# Preconception maternal exposure to *Nippostrongylus brasiliensis* transfers protection against *Nb* to her offspring

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## ***Abbreviations***

| | |
|---|---|
| IL-4Rα | Interleukin-4 receptor alpha |
| APC | Antigen presenting cells |
| B cells; T cells | B and T lymphocytes |
| B220 | B cell isoform of 220 kDa |
| CD19 | Cluster of Differentiation 19 |
| CD4+ | Cluster Designation 4 positive |
| CD8+ | Cluster Designation 8 positive |
| ELISA | Enzyme Linked Immunoabsorbent Assay |
| FACs | Flow cytometry, Fluorescently Activated Cell Sorter |
| FCS | Foetal Calf Serum |
| FM | Mature follicular B cell |
| FO | Follicular B cell |
| IFN-γ | Interferon gamma |
| IgG/IgE | Immuno-globulin G/E etc |
| IL-x (x = 1-...) | Interleukin-x |
| ILC | Innate Lympoid cell |
| IMDM | Iscove's Modified Dulbecco's Medium |
| JAK | Janus Tyrosine Kinase |
| KO | Knock out |
| MHC | Major Histocompatibility Complex |
| MZ | Marginal zone B cell |
| *N. brasiliensis, Nb* | *Nippostrongylus brasiliensis* |
| NaCl | Sodium chloride |
| NF | Newly formed B cell |
| Ova | Ovalbumin |
| p.i. | Post infection |
| PAMP | Pathogen-associated molecular patterns |
| PAS | Periodic Acid Schiffs |
| PBS | Phosphate Buffered Saline (solution) |
| PCp | Pre-conception |
| STAT | Signal transducer and activator of transcription |
| TCM | Central memory T cell |
| TCR | T cell receptor |
| Teff | Effector T cell |
| TH1/TH2 | T-helper (cell) type 1 or 2 |
| TLR | Toll Like Receptor |
| WT | Wild Type (Genetically unmodified) |

***Abstract***

In early life the immature immune system has a reduced ability to control infection. This susceptibility is offset by transfer of protective immune components from the mother. Helminth infections are widespread and can have a long lasting influence on host immunity. Children of mothers exposed to helminth infections may display T cell sensitization to endemic helminth infections and associations have been made between maternal helminth infection and altered immune responses to childhood diseases and vaccinations. This shows that helminth-modified maternal immunity may imprint on early offspring immune development *in-utero* or through breast milk in the form of transfer of, for example, antibodies, cytokines and lymphocytes.

Our study shows that, in mice, maternal infection with the helminth *Nippostrongylus brasiliensis* is not only associated with a passive transfer of antigen specific antibody (IgG1) but also inherently alters offspring immunity, increasing offspring cytokine production, alveolar macrophages, lung neutrophils and B cell population development and proliferation. Pups born to *N. brasiliensis* exposed mothers also had increased populations of lung and spleen CD4+ cells and higher sub-populations of central memory and effector CD4+ cells compared to pups born to naive mothers.

The modified immune populations in the offspring affected how they respond to disease. Our initial data suggests that increased numbers of activated cells in pups born to preconception *N. brasiliensis* infected mothers (PCp *Nb)* pups are able to demonstrate enhance TH2 protecitive immunity against *N. brasiliensis*. Therefore *N. brasiliensis* infected mothers transfer protective immunity against *N. brasiliensis* to

their offspring. Nursing alone could transfer the *N. brasiliensis* associated maternal protection; naïve pups nursed by a previously infected mother also showed enhanced control of *N. brasiliensis* infection when compared to pups nursed by naïve mothers. Together, these data indicate that pre-coneption maternal exposure to a helminth infection powerfully enhances offspring immune development and potential that confers protection against, in this case, *N. brasiliensis*.

# Chapter 1: Introduction

## 1.1 The Immune System

The immune system is a complex network of organs, cells and proteins whose primary role is to protect living organisms from a range of invasive pathogens and to limit the damage they cause [1]. This group of pathogens includes viruses, bacteria, fungi and parasites. 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, coordinating and responding in a manner that will deal with the pathogen effectively [2, 3]. Ideally this will result in complete and rapid clearance of the pathogen from the body of the host.

Leukocytes, the central focus of the immune system, originate primarily from hematopoetic stem cells in the bone marrow [4, 5]. They then develop into mature cells in various sites around the body, including the thymus and spleen before migrating to peripheral tissues, and are found in blood, lymph fluid, mucosal surfaces and secondary lymphoid organs such as the lymph nodes where they carry out protective activities [4]. Leukocytes act and interact by production of effector proteins such as cytokines and antibodies and secretion of cytotoxic molecules. Cytokines and chemokines, low molecular weight glycoproteins or polypeptides that act as chemical messengers, can stimulate and draw other immune cells to a site of infection, thus causing inflammation in that area [1, 6].

Control of immune responses is essential; cytokines may therefore also act in an anti-inflammatory manner by inhibiting cellular activation and modulating

homeostasis [7]. An immune system that responds poorly may allow a pathogen to thrive. But an over or incorrect response gives rise to immuno-pathology, and the resulting inflammatory response could be more dangerous than the original inciting stimulus. 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 [8]. All of these issues make the control of the inflammatory and anti-inflammatory systems crucial [7].

### 1.2 Innate vs. Adaptive

In order to provide efficient protection, a competent immune system includes several general approaches that recognise and destroy a broad spectrum of invasive organism, as well as some methods that give rise to defences that are specific to a particular species or sub-species of pathogen [9]. There are two basic components to the immune defence, the innate system which provides the immediate first-line non-specific response and the adaptive response which takes longer to respond to a primary infection but retains specific memory cells that respond quickly and effectively to a secondary challenge [9, 10].

The innate response is maintained by several cell types, including granulocytes, natural killer cells (NK), dendritic cells and phagocytes, such as macrophages and neutrophils. These cells can recognise highly conserved pathogen associated molecular patterns (PAMPs) including lipopolysacharides (LPS), DNA, double stranded RNA and flagellin, by germ-line encoded Pattern Recognition Receptors

(PRRs) and Toll Like Receptors (TLRs) [11]. These include 10 known TLRs, 3 RIG-I-like receptors (RLR), 3 C-type lectin receptors (CLR) and 2 nucleotide-binding oligomerization domain receptors (NOD-like receptors, NLRs) [12]. If there is an infection, cells encounter the foreign antigen and rapidly differentiate into short-lived effector cells [8].

B 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 the potential for a highly diverse range of antigen binding sites that are generated by DNA re-arrangement [13, 14]. About $10^6$ to $2\times10^6$ new T cells are generated each day [1]. 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 [10].

## 1.3 Innate Immunity

### 1.3.1 *Innate cells*

The basic role of innate cells is to detect pathogens, rapidly control infections and, if unable to effectively eliminate the pathogen, to induce the adaptive response [9]. Each cell type has a role to play in the overall construct of immunity. Phagocytic cells like macrophages and neutrophils detect and bind foreign organisms and cell debris in order to engulf and destroy them through fusion of the phagosome with acidic lysosomes. Granulocytes like basophils and eosinophils release enzymes (e.g. eosinophil protease) which can neutralise pathogens including helminths that

are too big to be phagocytosed. 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, 2].

Macrophages are present in virtually all tissues and are a functionally plastic and diverse population of cells. They primarily clear the interstitial environment of cellular debris and apoptotic cells. Macrophages also act as supervisors of the innate response and recruit other phagocytes to the site of infection using chemokines. Importantly, macrophages respond differently depending on the type of pathogen they encounter. Intracellular pathogens induce classically activated macrophages (CAMs) or M1 macrophages which induce a nitric oxide-mediated-killing inflammatory immune response. Alternatively activated macrophages (AAMs) or M2 macrophages defined by (for example) high Relmα and YM-1 expression respond to certain extracellular pathogens and have regulatory functions [15, 16]. They are also involved in allergic responses but may both promote allergic inflammation and have a suppressive role. Alveolar macrophages are a specialized type of macrophage found in the lung and play a critical role in pulmonary immunity [17, 18].

A recently discovered population of innate cells called Innate lymphoid cells (ILCs) belong to the lymphoid lineage but do not respond in an antigen-specific manner, as they lack a B or T cell receptor [19, 20]. They have different physiological functions, but are linked to assisting the helper T cell response. ILCs can be divided based on the cytokines that they can produce, and the transcription factors that

regulate their development and function. There are 3 groups; ILC1 (produce IFNγ), ILC2 (produce IL-4/IL-13) and ILC3 (IL-17) [21]. ILC cytokine secretion is an important link between the innate and adaptive responses and ILC2s (also known as nuocytes) have been shown to activate responses against helminths by secreting type 2 cytokines which polarize adaptive immunity towards helminth specific T cell responses [22, 23].

### 1.3.2 *Antigen and antigen presentation*

One of the most important roles for the collective innate response is to collaborate with the adaptive immune response. Antigen presentation is a common way of achieving this. Antigens take the form of a unique protein or other bio-molecular sequence which comes from the pathogen and is recognised as foreign [11]. This is especially likely to occur in phagocytic cells and dendritic cells which present peptide fragments from internalised and destroyed organisms to T cells on major histo-compatibility complex (MHC) proteins [24]. Along side this, costimulatory molecules expressed on Anigen Presenting Cells (APCs), such as CD80 and CD86, engage with CD28 receptors on T cells. Antigen presentation by innate cells alerts the adaptive response that clonal expansion of antigen-specific cells is required to control the infection and to instruct it on the nature of the pathogen and the type of adaptive response that is required [8]. Along with cytokine production by the innate cells, this helps to shape the adaptive immune response [24].

## 1.4 Adaptive Immunity

### 1.4.1 *Lymphocytes*

The protection provided by activated adaptive immune cells is very specific to a particular type or even to a sub-type of pathogen because the stimulating antigen is commonly unique to that pathogen [10]. 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. Most activated adaptive cells become highly active but short-lived effector cells while a small proportion of adaptive cells develop into relatively inactive longer-lived central memory cells [13]. Memory cells can remain dormant in the host for many years but if the pathogen is encountered again they rapidly respond by dividing to produce a new population of effector cells. Thus a re-infection is usually weak and brief [3].

### 1.4.2 *T cells*

The class of cells expressing CD3 is known as T cells and includes CD4+ helper cells and CD8+ cytotoxic cells. They are derived in the bone marrow and mature in the thymus [25]. Activation of CD4+ helper T cells requires TCR binding and stimulation by antigen on MHCII in a receptor complex. MHCII receptors are only found on professional APCs; these are dendritic cells, macrophages and B cells [5]. Following activation, helper T cells stimulate antibody production by B cells and innate effector mechanisms such as increased killing by phagocytes [26]. CD8+ cytotoxic cells are alerted to the presence of intracellular pathogens by antigen presentation on MHCI receptors, which are found on all nucleated cells. This causes

the CD8+ cell to destroy the infected cell by releasing cytotoxic granzymes which cause apoptosis of the target cell in order to contain the infection [27].

The antigen Thy-1 (CD90) was the first T cell marker to be identified and is present on all murine thymocytes, on mature T lymphocytes, and on neuronal cells. The function of Thy-1 has not yet been fully elucidated. It has been speculated that it could have possible roles in cell-cell and cell-matrix interactions, with implications in neurite outgrowth, nerve regeneration, apoptosis, metastasis, inflammation, and fibrosis. In mice, there are two alleles of CD90: Thy1.1 (Thy 1a, CD90.1) and Thy1.2 (Thy 1b, CD90.2). They differ by only one amino acid at position 108; an arginine in Thy-1.1 and a glutamine in Thy-1.2. Thy 1.2 is expressed by most strains of mice, whereas Thy1.1 is only expressed by a few like the AKR/J and PL mouse strains. Since these two proteins can be labeled individually without cross-reactivity, this occurrence is useful in studies which require separation of T cells into categories based on their origin, such as tracking T cells transferred from one mouse to another. This benefit is used later in our study.

### 1.4.3 *T helper 1 vs. T helper 2*

A significant development in the research into the role of CD4+ T cells shows that activated cells differentiate into separate, defined effector groups depending on the type of pathogen they are responding to and the resulting cytokine profile **(Figure 1A)** [26]. 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 nature of the antigen, 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 [28-30].

The two major classes of a T cell response are the cellular T helper cell 1 (TH1) response and the humoral T helper cell 2 (TH2) response and they are characterised by the cytokines they produce [31, 32]. Interleukin 12 (IL-12) is the predominant cytokine that induces TH1 differentiation via the STAT1/T-bet pathway. A TH1 response is defined by high levels of interferon gamma (IFN-γ), IL-2 and IL-12 production in a positive feedback loop and protects against most intracellular pathogens. Features of a TH1 response include IgG2a opsonizing and complement-fixing antibody production, classical macrophage activation and CD8+ cytotoxicity [33, 34].

TH2 responses are now known to be initiated by the secretion of IL-33 from epithelial cells which activate innate ILC2s via the T1/ST2 receptor. The ILC2s then produce the T cell polarizing cytokine Interleukin 4 (IL-4) which promotes a TH2 response via STAT6/GATA3 expression [34, 35]. TH2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 and are associated with helminths and allergens. IL-4 can be produced by conventional T and B cells as well as several innate sources, including ILC2s, basophils, γδ T-cells and eosinophils. This induces B cell class-switching to IgE and, in mice, IgG1. TH2 responses also include alternative macrophage activation, eosinophilic inflammation, mast cell proliferation, goblet cell hyperplasia and excessive mucous production at mucosal surfaces [26, 36]. An allergic reaction is an

exaggerated TH2 reaction to an otherwise harmless antigen in individuals who are predisposed to do so genetically [37, 38]. This can lead to anaphylactic shock and even death. The details of the TH2 response to helminths are discussed in depth later.

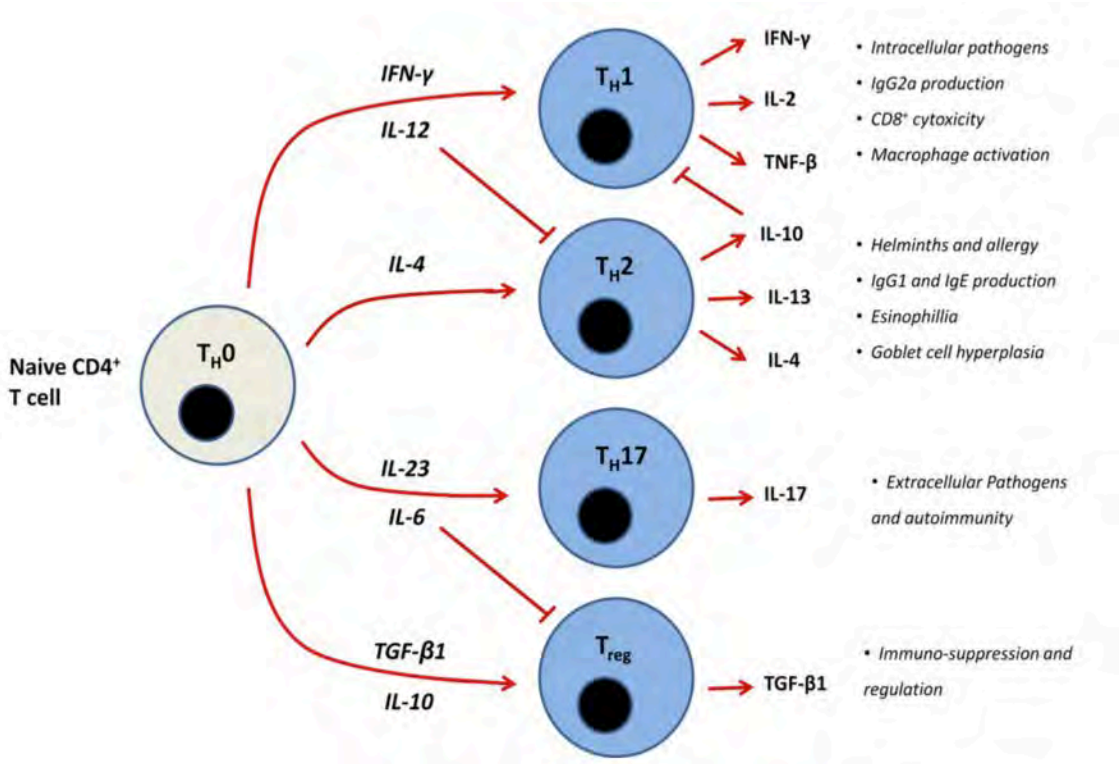



**Figure1A: Naïve CD4$^+$ T cells can polarise into different effector response classes.** This depends on the stimulating cytokine environment. Each type of response produces a different set of cytokines and effector mechanisms.

TH1 and TH2 effector responses counter regulate each other because of inhibitory intracellular effects i.e. the IL-12 and STAT1 produced by a TH1 response suppresses TH2 polarisation while IL-10 and GATA3 blocks TH1 cells from developing [31, 32].

### 1.4.4 *Other CD4+ T cell subsets*

The TH1/TH2 paradigm does not cover all helper T cell responses. Several other categories of effector T cells perform specific functions in co-ordinated action alongside TH1 or TH2 cells [39]. These subsets include TH17 cells, stimulated by IL-6 to produce IL-17 in immunity to specific extracellular comensal gut bacteria [40-42]. TGF-β1 drives the generation of CD4+CD25+FoxP3+ regulatory T cells that cause IL-10 mediated immuno-suppression to prevent dangerous inflammatory responses [43]. TGF-β also re-programs TH2 T helper cells to lose their characteristic IL-4 profile and switch to secretion of IL-9, making them TH9 cells [44, 45]. It is probable that there are even more T cell subsets than discussed here that are yet to be researched but it is unclear how many or what their role may be.

### 1.4.5 *B cells*

Similarly to T cells, B cells exist in distinct subsets; there are conventional B2 B cells found in the follicles of secondary lymphoid organs like the spleen, and B1 B cells found in the peritoneal and pleural cavities and the gut lamina propria [5, 46]. They can also be categorised as responsive to either T-dependent (TD) or T-independent (TI) antigens [47]. B2 B cells represent the largest proportion of adult B cells and are subdivided into mature follicular (FM) and marginal zone (MZ) B cells which respond to TD and TI antigens respectively. Before birth, B cells develop in the fetal liver from pluripotent hematopoietic cells but originate from the bone marrow after birth [2].

B2 cell progenitors from the bone marrow go through a process referred to as primary or central B cell development there [48, 49]. This generates immature,

surface-immunoglobulin (known as the B cell receptor or BCR) expressing B2 cells which migrate to the spleen where they mature through the transitional phases 1 and 2 (T1 and T2) in the splenic follicles **(Figure 1B)** [50, 51]; this process by which immature B2 cells are directed toward a specific mature B cell fate is known as secondary or peripheral development [52]. The immature T2 B2 cells can become mature FM or MZ cells. FM and MZ B cells respond to different types of antigen, require different activation signals and have different types of antibody responses [53-55]. MZ cells are only found in the spleen but naive follicular (FO) cells may re-circulate from there in the bloodstream to follicles in other secondary lymphoid organs such as lymph nodes [6, 56, 57]. Activation occurs when they encounter antigen which binds directly to the BCR, or when they interact with an activating T cell [58]. Activated FM B cells rapidly proliferate and go through somatic hyper-mutation (adapting to new foreign elements) and affinity maturation (becoming more antigen specific) in germinal centres (GC), developing into effector plasma cells or long-lived memory cells [54, 59, 60].

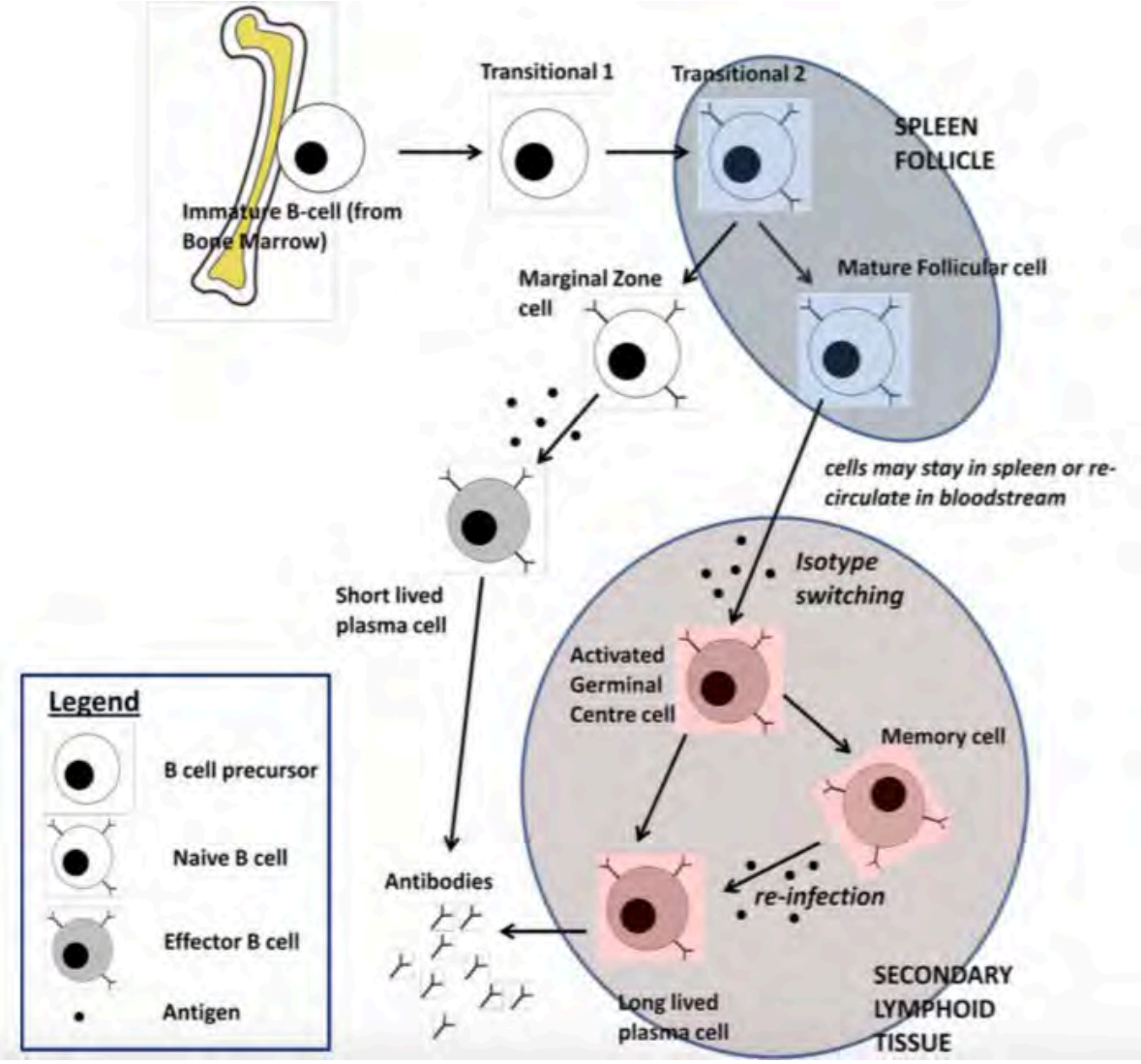



**Figure 1B: The progression of peripheral B cell development.** Naive B cells go through several stages of development and maturation before they encounter antigen or a CD4+ T cell which activates them turning them into germinal center/plasma cells.

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. This takes place in the bone marrow [49, 51, 61]. 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 [62]. Sometimes

IgD is also co-expressed with IgM on mature cells. Activated FM B cells often go through a process of istotype or class switching, whereby the cell progresses from producing low affinity IgM to producing high affinity IgG, IgA or IgE [63]. 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 [64, 65].

### 1.4.6 *Antibodies*

B lymphocytes stereotypically facilitate clearance of pathogens by producing and secreting antigen specific antibodies or immunoglobulins (Ig) [66, 67]. These are complex quaternary proteins that are made up of several polypeptide peptide chains bound together [68]. 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 [69]. 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, depending on the DNA rearrangement of the B cell [14, 70]. Secreted antibody specificity is identical to a B cells' BCR, meaning that antibodies bind the same antigen as their parent cell. 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 [63].

Antibodies function by binding to pathogens or their products via the antigen binding site [69]. 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 [67]. Other antibodies cause opsonisation of pathogens which enhances leukocyte recognition of antibody-bound pathogens. This marks the pathogen for ingestion and destruction by a phagocyte or for destruction by a process called antibody-dependent cellular cytotoxicity (ADCC) [71]. 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 [71]. 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 [72, 73]. Another mechanism used by antibodies is to activate complement proteins to attack an antibody-coated pathogen. Complement proteins can cause osmotic cell lysis or agglutination (binding together multiple units) of the pathogen, or opsonisation of the pathogen in a similar manner to antibody [74].

Antibodies of different isotype classes differ in their constant region and therefore their functional activity [63, 66]. IgM and IgD are the earliest secreted class of antibody. IgM forms pentamer polymers in the serum and strongly induces complement activation [75]. Polymerisation is important because this allows the antibody to bind to multiple antigens on the pathogen which increases binding affinity. The exact function of IgD is currently unclear but there is evidence that it has a similar role to IgM, and may enhance basophil responses [76, 77]. IgG is the most common antibody class in the plasma and has several subclasses (IgG1, IgG2a, IgG2b etc.). 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 [78, 79]. IgA can form dimers, and is able to cross epithelial surfaces; it is predominantly associated with mucosal sites [80]. IgE is involved in activating mast cells and basophils to produce hypersensitivity in allergic type reactions [81]. Antibodies have limited halflives and are constantly being turned-over [82].

### 1.4.7 *Spleen organisation*

The anatomical layout of spleen in highly organised. The immune cells are arranged in distinct locations in order to maximise the efficiency of cell development and cellular interaction with each other and with antigen to promote immune responses and cell migration is carefully controlled [57, 83-85]. The spleen mostly consists of red pulp, interspersed with discrete areas of lymphoid white pulp which is arranged around central arterioles [86, 87]. The red pulp contains splenic macrophages that phagocytose aged or dead red blood cells and opsonized blood-borne microbes. Lymphocytes in the spleen include CD4 and CD8 T cells, FM and MZ B cells [88, 89]. 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.

Lymphocytes such as naive mature T cells and immature newly-formed (NF)/transitional 1 (T1) B cells and antigen-loaded dendritic cells enter the white pulp via the central arteriole into the peri-arteriolar lymphoid sheath (PALS) [87]. The PALS, which is mostly made up of T cells with macrophages around its edges, is a site of T cell interaction with antigen-presenting dendritic cells (DCs) [89]. T cell

independent antigen-mediated selection, BAFF and transient BCR stimulation all play a role in driving T1 development into T2 cells [55, 90, 91]. As the T1 B cells develop into T2 cells they pass from the PALS into the follicle which mainly consists of B cells surrounded by a network of follicular DCs and follicular helper T cells. T2 cells can become either FM cells in the follicle or MZ cells in the Marginal Zone [88, 92]. The regulatory factors that control T2 differentiation include BCR signalling, cognate interactions, co-stimulation and antigen dependent selection [53, 90, 93]. Cells and antigen drain out of the white pulp in a trabecular vein. The organization of the white pulp of the spleen is similar to that of a lymph node, but while the spleen screens for blood borne antigens, lymph nodes detect localised antigens in draining lymph fluid [86, 94]. The development of B cells can be tracked based on the surface expression of various receptors (see methods).

### 1.4.8 *T-dependent vs. T-independent responses*

Nearly all protein antigens require cognate interaction between helper T cells and B cells, along with stimulation of the BCR in order to initiate antibody responses, and are called T cell dependent (TD) antigens [60]. Some non-protein bacterial antigens, such as capsular polysaccharides, are able to elicit an antibody response in nude mice, i.e. in the absence of T cell help, and are called T cell independent (TI) antigens [95]. Naive mature B cell antigen activation occurs in secondary lymphoid organs, the spleen being the most highly organized tissue for optimal B cell stimulation with antigen, interaction with T cells and splenic APCs [96].

The most abundant type of mature adult B cell, FM cells, are understood as conventional B cells; they are stimulated by antigen and T cells in the follicle to become germinal centre cells, they switch antibody isotype classes to produce high affinity antibodies, present antigen to T cells and generate plasma and memory cells [54, 59, 90, 97]. FM cells develop from the spleen but are found in all secondary lymphoid tissue such as the spleen and lymph nodes and at mucosal surfaces where they can screen large quantities of antigen [58, 98].

However in contrast to FM B cells, MZ and B-1 cells respond directly and rapidly to T-independent, blood-borne antigens and pathogens [95, 99]. Their germ-line encoded receptors are skewed towards common bacterial antigens and do not undergo somatic hypermuation. They can differentiate into extra-follicular plasma cells in one or two days and produce secreted circulating broad-specificity low-affinity IgM antibodies [100]. 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 [101]. They exhibit little or no *in vitro* proliferative response to BCR engagement. Furthermore MZ cells specifically respond to capsular polysaccaride antigens which crosslink many BCRs on the same B cell and induce a proliferative antibody response [102]. It must be noted though that MZ cells can activate T cells under some circumstances [103]. B1 B cells, comprised of B1a or B1b B cells, are differentiated from B2 B cells by their unique surface markers, anatomical location and their ability to self-renew [104, 105]. MZ and B1 B cells are the first to respond to infection, giving FO B cells time to form germinal centres and produce high affinity antibody producing cells [95, 106, 107] .

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 [62, 108].

### 1.4.9 *Other follicular B cell functions*

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 [109, 110]. B cells have been shown to regulate T cell mediated immune responses, even in mouse models which have B cells that do not secrete antibody competently; $IgM^{-/-}$ B cells can regulate immune responses via antibody independent mechanisms like activation of T cells [111, 112]. These other effector functions of B cells include cytokine production, antigen presentation to CD4+ T cells and expression of co-stimulatory molecules **(Figure 1C)** [113, 114]. 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 [60, 109].

B cells can internalise antigen selectively via the BCR; it is then processed in the endoplasmic reticulum and presented on MHCII receptors [109]. Co-stimulatory molecules such as CD80 act together with the antigen presenting MHCII to trigger the T cell to respond. 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 [115]. The co-operative interactions between B and T lymphocytes result in a positive feedback loop which causes both cells to become activated [116, 117]. 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 [118]. 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 [119].

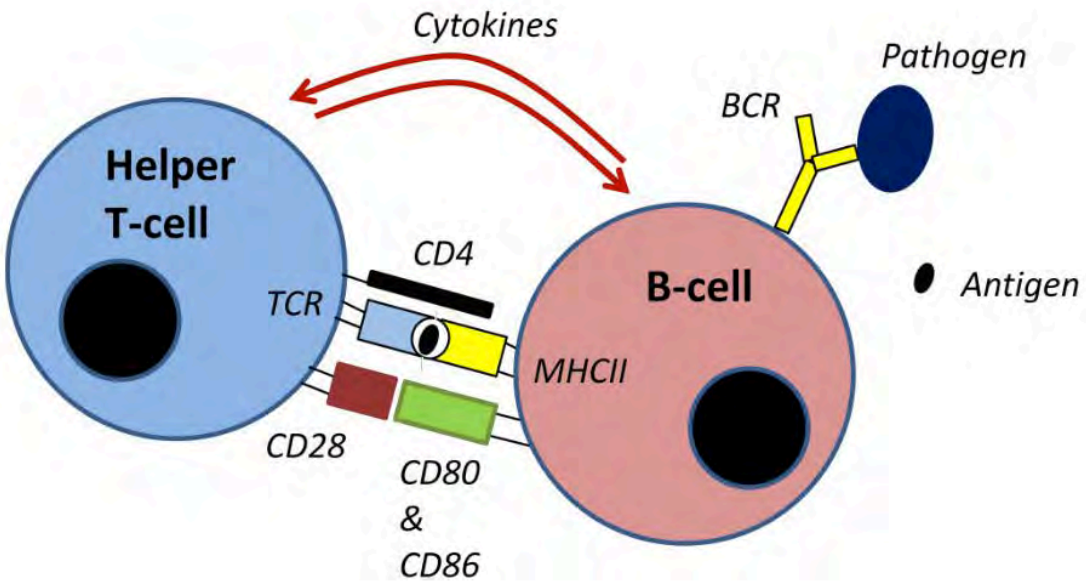



**Figure 1C: The interaction between B-cells and T-cells.** B cells present antigen to T cells on MHCII. The T cells form a cell surface receptor complex by binding to the MHCII with their TCR and CD4. Co-stimulation is provided by CD28 interaction with CD80/86, and cytokine production.

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 [120]. 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 Bregs suppress T cell mediated inflammation and play a vital protective role in several autoimmune diseases [121]. These cytokine-producing effector B cells can modulate T cell responses and can induce strong TH1 or TH2 immunity [109].

### 1.4.10 *B effector 1 vs. B effector 2*

It has been shown that activated effector B-cells (Be), like helper T cells, can also be sub-divided into discrete subsets, producing Be-1 [120], Be-2 [119, 122] or B-regulatory [121, 123] 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. Naive B-cells primed by interactions with TH1 cells and stimulated by IFN-γ and IL-12 become Be-1 cells that produce the TH1 cytokines IFN-γ, TNF-α and IL-10 [120, 124]. B cells that are primed by IL-4 and interact with TH2 cells differentiate into Be-2 cells and produce IL-4, IL-13, IL-2 and IL-6 [110, 122]. Functionally Be-1 cells contribute significantly to immunity to bacterial infections, such as Salmonella [125]. Be-2 cells have been demonstrated to be important for immunity to helminth infections, including the humoral and cellular response to *Heligmosomoides polygyrus* [119, 126].

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 TH1 and Be-2 into TH2 [35, 114, 117]. 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 TH2 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 [119]. 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.

### 1.4.11 *Immune memory*

Memory immune cells are a sub-type of antigen-experienced lymphocytes that are formed from activated germinal centers following primary infection and are important in generating a robust and accelerated immune response in the case of re-infection (a secondary immune response) [2, 96, 127]. Memory cells can rapidly reproduce to mount a stronger, faster immune response than during the primary invasion. They have higher sensitivity to antigenic stimulation, are less dependent on costimulation, and upregulate inflamatory markers to a greater extent, thus providing more effective stimulatory feedback to other cells in the immune system [128]. In this way we gain life-long active immunity to infections.

Positively selected GC B cells are antigen specific with high affinity. A proportion of these cells move to the red pulp and differentiate into long lived memory B cell and short lived effector plasma B cells [77, 98, 129]. While memory B and plasma cells both secrete large amounts of antibody, memory cells can still proliferate whereas plasma cells have stopped proliferating [130]. Short lived plasma cells disappear from the spleen within two weeks of infection [131]. The survival of long lived memory B cells is ensured by the survival niche in which they reside. When re-activated by antigen, memory B cells proliferate to produce a new population of short-lived plasma cells [132, 133]. Memory and plasma cells may migrate to the inflamed tissue, the bone marrow or the red pulp of the spleen. All three tissues provide plasma cell survival niches [134]. However, once the infection and tissue inflammation has been resolved, these cells die by apoptosis [135]. Most memory B cells end up in the bone marrow where they become immobile and can survive for years and account for long-term antibody production and humoral memory [136, 137].

The response of T cells can be tracked based on the surface expression of various receptors [138]. Activated T cells divide into effector or central memory T cell populations which have different functions, although these two broad subsets are similar in expression of many costimulatory molecules, adhesion, and chemokine receptors [139]. Central memory T cells (TCM) cells express CD44 and CD62L and they secrete high levels of IL-2, although not much IFNγ or IL-4, but after stimulation and proliferation they efficiently differentiate to effector cells able to produce effector cytokines [140, 141]. TCM cells have a capacity for self-renewal due to high levels of

STAT5 [142] and have been shown to confer superior protection against certain viruses [27], bacteria [27] and cancer [143] in several different models compared with Teff cells. Effector T cells (Teff) cells also express CD44 but not CD62L and can produce effector cytokines like IFNγ and IL-4 [144]. Compared with TCM, Teff cells are characterized by a more rapid effector function. Memory T cells can reside in secondary lymphoid organs or various peripheral tissues without recirculating and rapidly respond *in situ* [145, 146]. TCM and Teff show differential patterns of distribution. Teff cells are enriched in lung, liver, and gut, whereas lymph nodes contain greater proportions of TCM cells [128]. Memory CD4 T cells have the potential to impact a broad range of both innate and adaptive immune effector functions during a secondary response through both cell-to-cell contact and cytokine and chemokine production, including recruitment of B cells [58], CD8 cells [141] and DC or macrophage activation [140].

### 1.4.12 *The Innate capability 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 [14, 147]. However a class of cells known as innate-like lymphocytes, including γδ T cells, B-1 B cells and NK T cells, shows less stringent antigen specific activation requirements [148, 149]. The receptors found on these subsets are encoded by a few common genes and little re-arrangement 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 [14, 150]. 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 in a manner similar to innate immune cells, a quality not possible in lymphocyte populations which require clonal expansion to launch an effective and highly specific response [99, 151].

Even classical mature adaptive cells can sometimes exhibit innate-like functions when they respond to antigen in a TCR or BCR independent manner [152, 153]. B cells have been shown to express TLRs; currently 10 mammalian TLR's have been defined. These non-specific receptors allow the cells to recognise and respond to some types of antigens rapidly by cytokine and antibody production [154, 155]. For example MZ B-cells are shown to respond to LPS rapidly via TLR signalling and innate response activator (IRA); these B cells have been shown to protect against microbial sepsis in a TLR dependant manner [156, 157]. FM B cells and MZ B cells have different cytokine profiles in response to TLR ligands [158]. T2 cell development toward FM B cells has been shown to be enhanced upon simultaneous BCR and TLR activation [159].

## 1.5 Experimental Infection Models

### 1.5.1 *Helminths*

Parasitic nematode infections are an extremely important and widespread global public health issue and are especially common in tropical areas and developing countries in Africa, Asia and South America [160]. They are common to both humans and domestic livestock. Infections with *Ascaris lumbricoides, Trichuris trichuria* and

the hookworms *Ancylstoma duodenale* and *Necator americanus* occur in an estimated third of the world human population [161].

The silent but devastating effects of infection cause morbidity in health and economic damage. For example infection by hookworms or roundworms causes anaemia, malnutrition, poor immunity towards some bacterial co-infections, widespread child developmental and growth problems, and can affect maternal *in-utero* fetal imprinting [162-165]. Strong associations have been made with maternal helminth infection and reduced childhood vaccine efficacy in offspring, discussed later in the chapter. Effects on cognitive development as a result of repeated childhood infections are widely reported [166, 167]. Exacerbation of chronic lung pathologies associated with parasitic larval migrations in the host may also represent a further major health implication in endemic areas [168]. 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 [162, 169]. Infection is chronic and may last 1-5 years for *Necator americanus* and about 6 months for *Ancylostoma duodenale* [170]. Although treatable with drugs like albendazole and praziquantel [171], parasitic nematode re-infections occur as humans do not develop protective immunity and to date no vaccination strategies exist against these helminths, although this is being actively investigated [172-178]. In order to understand how the public health burden caused by these infections will

be best controlled and to accelerate and optimize the development of effective vaccines, it is important to understand how the various components of the immune system respond to the infection.

### 1.5.2 *Nippostrongylus brasiliensis*

*Nippostrongylus brasiliensis* is a gastro-intestinal parasite of rodents, natural to rats, but it has been adapted for use in mice [179]. *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 [170]. It is easy to work with, the life cycle is straightforward and simple to maintain and it is not fatally harmful to rodents [180]. 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 [179].

### 1.5.3 *The life cycle of N. 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 1D)** [160, 169]. The larvae hatch at the L1 stage, which is free living and motile [179, 180]. Further development carries them through to the free-living infectious L3 larval phase. The L3 larvae can infect the host by oral ingestion or more likely they infiltrate the skin of the potential

host [162]. To mimic this in the laboratory, the L3 larvae are injected subcutaneously into the rodent's abdomen. The circulatory system carries the larvae to the lungs [181], where they get trapped in the capillaries, undergo maturation and moulting to L4 stage, breach the capillaries and enter the pulmonary alveoli. Then they get coughed up into the oesophagus and are ingested when the animal swallows and penetrate of the mucosal epithelium of the upper intestinal system. In this way they migrate back to the gastro-intestinal system 4-5 day's post infection (p.i.). They bind to the intestinal wall and there they develop and mature into adults (L5) which mate to produce eggs about day 6 post infection. 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 [182, 183]. 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 [184-186].

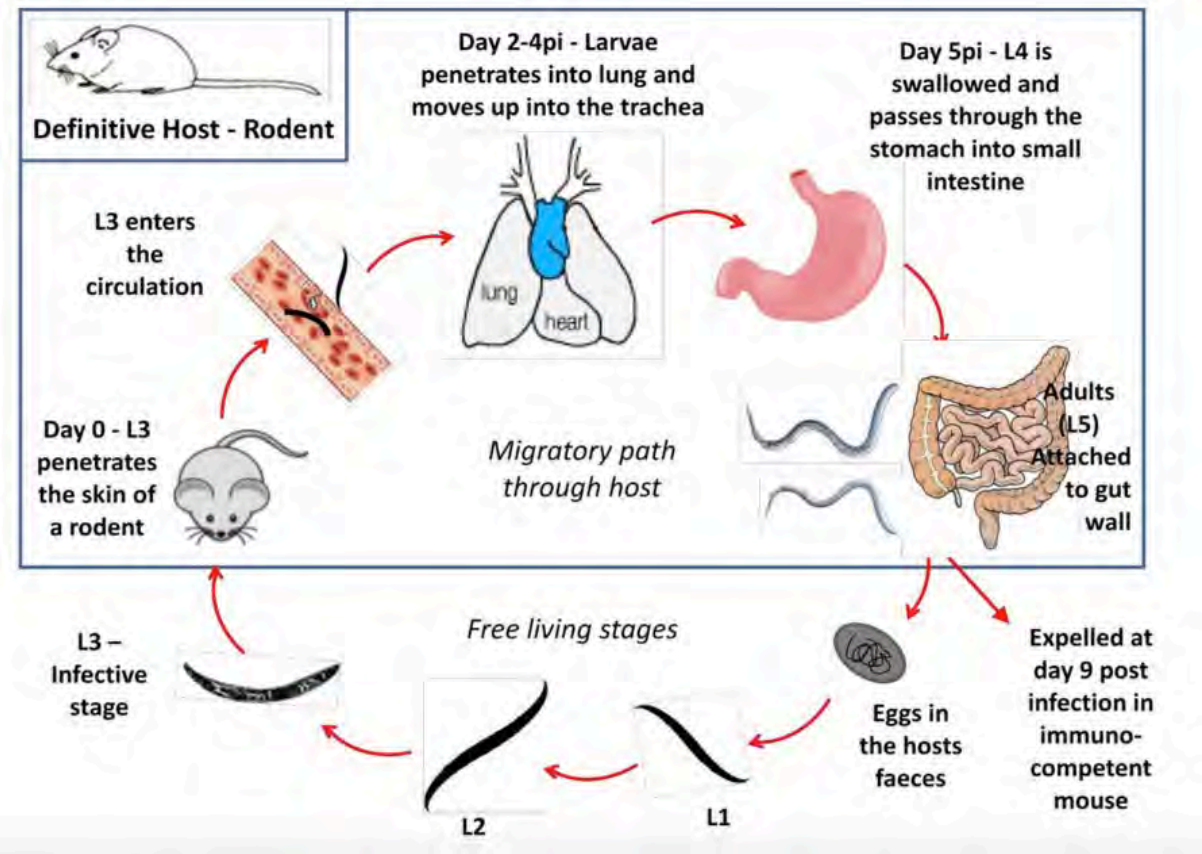



**Figure 1D: The life cycle of a primary *Nippostrongylus brasiliensis* infection.** There are six developmental stages in the cycle, the egg, the four larval stages (L1-L4) and the adult stage (L5). There is no intermediate host. The eggs produced by the adults in the gut leave the infected rodent in the faeces. The larval stages L1-L3 are free-living in the soil and motile. The L3 larva is the infective stage.

### 1.5.4 *Primary immune response to N. brasiliensis (part 1)*

Use of experimental parasitic nematode models has established that TH2 immune responses drive host resolution of primary helminth infections and these responses correlate with those found in humans [187-189]. An anti-helmith TH2 response includes eosinophillia and basophillia (eosinophil and basophil recruitment into tissues), goblet cell hyperplasia (differentiation and growth) and mucous secretion, CD4+ T cell dependant generation of IgE and secretion of cytokines IL-4, IL-5, IL-9 and IL-13 as well as isotype switching of B cells to produce IgG1 [147, 170, 179,

187]. However, while this immune response may be rapid and robust as a consequence of helminth infection, it can be ineffective at eliminating certain parasite models, as is the case for *Shistosoma mansoni* where the TH2 response is merely able to control the infection [190].

IL-4 has been shown to be important in driving TH2 mediated protection to helmiths [191], but despite its importance IL-4 is not essential to protective responses against *N. brasiliensis* [188]. It is primarily IL-13 that drives the TH2 response to *N. brasiliensis*, although it is aided by IL-4 and other TH2 cytokines, and it has a wide range of TH2 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 [192, 193]. Underlying effective expulsion of murine model parasites, such as *N. brasiliensis*, *Heligmosomoides polygyrus* and *Trichuris muris*, is host expression of IL-4 receptor alpha (IL-4Rα) [187].

### 1.5.5 *The importance of IL-4Rα*

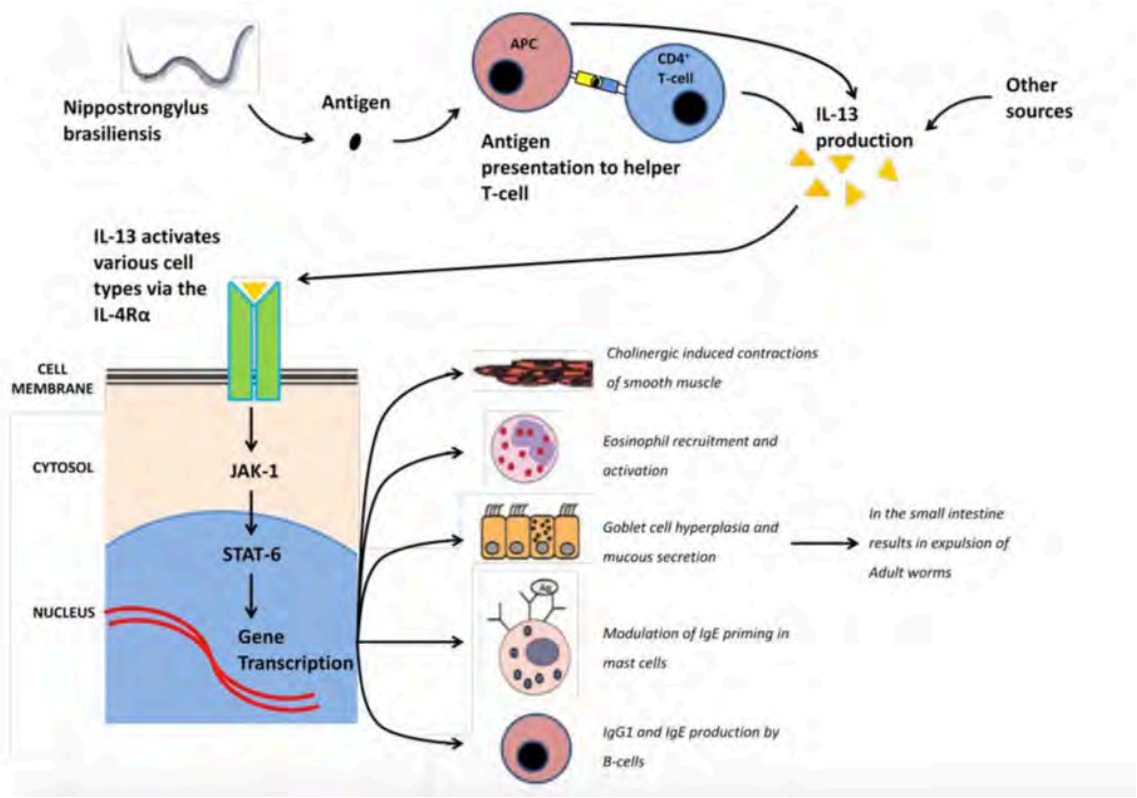
IL-4Rα is found on many types of cells, including haematopoietc, muscle, epithelial and endothelial, fibroblast and neurons [194]. IL-4Rα is an essential component of the cell surface heterodimeric receptors required for IL-4 and IL-13 signalling which ultimately drives host immune polarisation to TH2 [185, 193, 195, 196]. Use of IL-4Rα$^{-/-}$ mice has clearly demonstrated an absolute requirement for IL-4Rα expression in resolving primary nematode infections [109, 186]. It has been shown that IL-4/IL-13 signalling through the IL-4R activates the Signal Transducer and Activator of Transcription 6 (STAT6) and Janus Kinase 1 (JAK1) intracellular pathway. The

JAK1/STAT6 pathway promotes gene transcription that results in parasite clearance [188, 194]. 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-haematopoetic cells including smooth muscle cells [185] and epithelial cells [197, 198]. It has been found that in modified chimeric mice which only express IL-4R normally on non-haematopoetic/bone marrow derived cells, expulsion of worms is unaffected [199]. In mice that selectively display IL-4R on immune/haematopoetic cells, the worms are not expelled [199].

### 1.5.6 *Primary immune response to N. brasiliensis (part 2)*

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” [179]. The tissue that appears to be most involved is the small intestine. IL-4 and IL-13 induce several STAT 6 dependant changes there [200], 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 [179, 186, 188, 199, 201]. IL-4R 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 [185]. Mice that had smooth muscle cell specific IL-4R deletions showed a delay in goblet cell differentiation and reduced mucous production and therefore do not eliminate a *N. brasiliensis* infection as quickly as controls [185, 202]. 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 **(Figure 1E)** [185, 199].




**<u>Figure 1E:</u> 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-4Rα initiates a range of STAT6 dependent TH2 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 inflamation/pathology and airway hyper responsiveness and are also potential sites of parasite killing [203]. 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 [181]. There is strong activation of alternative alveolar macrophages [168, 182, 204], structural damage, inflammation and other cellular and molecular changes that persist for days after the larvae have moved on. 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 [168, 205]. 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 [181]. There is also an increase in eosinophil numbers and goblet cell differentiation in the lung and bronchioles and helminth induced epigenetic modification of phagocytic cells [206, 207]. 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” [168].

### 1.5.7 *Secondary immune response to N. brasiliensis*

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 [168, 182, 208], CD4+ T cells [209, 210], parasite specific type 2 antibody responses and B cell cytokine production **(Figure 1F)** [109, 119, 211]. Studies with *N.brasiliensis* show that the lung is the key site of host secondary responses and rapid resolution of re-infection [203, 212]. Furthermore, roles for eosinophils [212, 213], basophils [214], neutrophils [215], antigen specific antibody [216-218], antibody independent B cell

responses [109], and CD4+ T cells [209, 219, 220] in coordinating this immunity have also been demonstrated .

Even though IL-4Rα expression on hematopoietic cells is apparently not essential to primary parasite clearance it does impact on the magnitude of the hosts secondary TH2 response to *N. brasiliensis*. For example, disruption of IL-4Rα expression on CD4+ T cells results in a significantly reduced TH2 response to *N. brasiliensis* infection [205]. It has been suggested that B cells do not play a significant role in immunity to *N. brasiliensis* infection and re-infection [211]; here Liu et. al. demonstrate that, in the absence of B cells and antibody, sufficient IL-13 to mediate protective immunity is provided by other sources. However, even though the immune system can compensate for the absence of B cells, they can contribute to an immune response due to their multi-functional role in the immune system. In a study on *H. polygyrus*, 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 re-activate TH2 memory cells [119]. Studies in our lab have demonstrated that B cells are essential in the development of effective immune memory to *N.brasiliensis*. B cell production of IL-13, signalling through IL-4Rα and antigen presentation all play a role [109, 216]. Together with the fact that T cells are essential to generate protective memory against a nematode re-infection [209], this implies an important role for adaptive immunity in the immune response to the parasite.

In this study, we used *N.brasiliensis* infection as a model for maternal helminth infection to assess the effects of this on immunity in offspring. We investigated whether the effects of the strong TH2 response to a primary infection on immune cells and cytokine profiles, and the development of immune memory in the mother may be transferred to offspring. We hypothesized that any observed altered immune components in offspring may be acquired from the mother *in-utero* and via breastmilk.

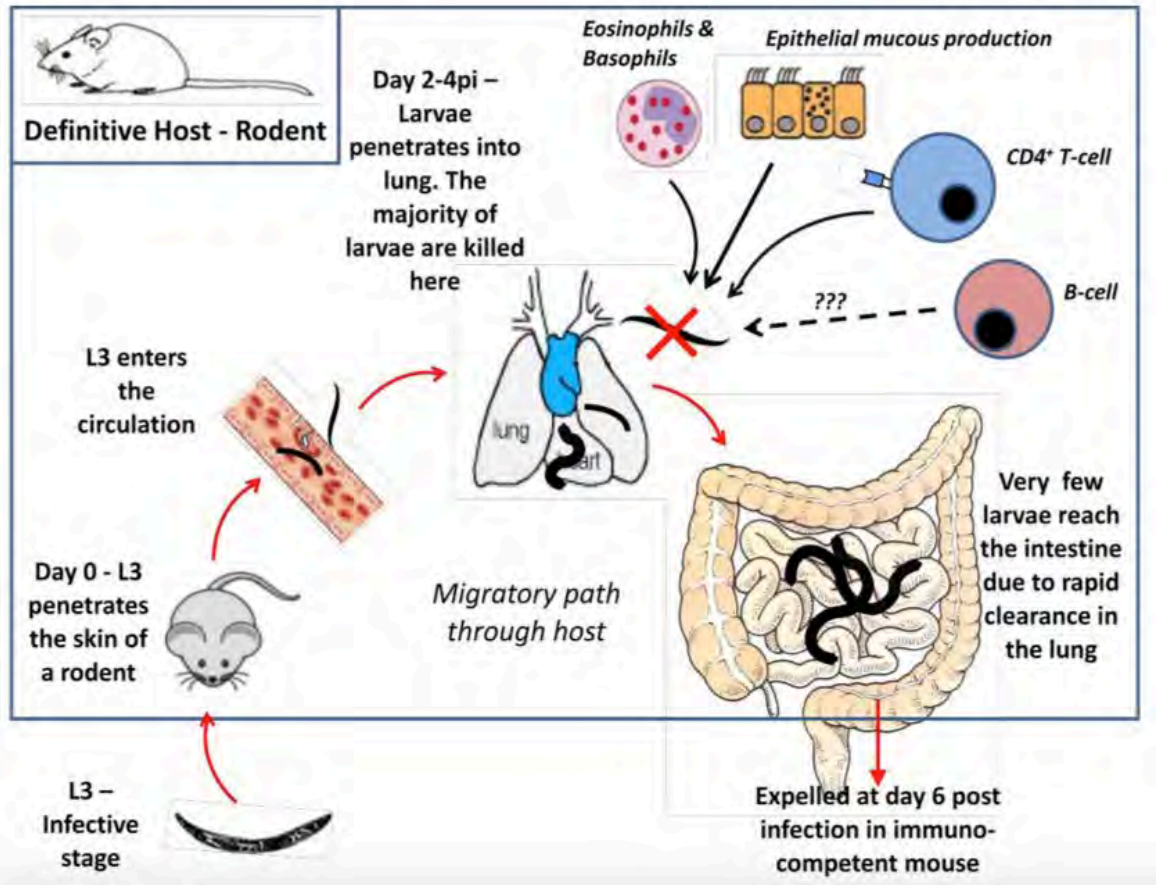



**Figure 1F: The life cycle of a memory *Nippostrongylus brasiliensis* infection.** The lung is the main site of parasite clearance in immuno-competent mouse. This has been shown to be associated with lung eosinophil and T cell functions.

## 1.6 Maternal and Neonatal Immunity

In early life the immature immune system has a reduced ability to control infection due to lack of immune memory, and newborns are highly susceptible to pathogens. Infectious disease is a major cause of human infant mortality in developing countries and hospitalization in industrialized countries. Each year more than 2 million children aged between 1 and 6 months die due to respiratory infections or diarrheal disease and early life infections like influenza, respiratory syncytial virus (RSV) and bacterial infections account for a significant proportion of infant hospitalizations [221-224].

This susceptibility is offset by transfer of protective immune components from the mother. A mothers infectious history may influence how effective these components are at conferring protection in the offspring [225]. Preconception maternal health can have profound effects on the health of her offspring [226]. Maternal (and even grand-maternal) lifestyle choices alter children's risk of a range of important diseases. For example epidemiology studies of pre-conception maternal smoking have found strong associations with increased risk of early childhood allergy [227, 228]. Additionally, maternal pre-conception exposure to potential pathogens can also be an important source of neonatal protection against potentially life threatening infections such as non-typhoidal salmonella (NTS) [229]. The mechanisms and contributing factors affecting the transfer of protective immune components from mother to infant are a key area of investigation.

### 1.6.1 *The use of rodent vs. human infant models*

While the neonatal period in humans is defined as the first 28 days after birth, the murine neonatal period has not been precisely defined. Initial studies suggested varying time points (≤24 h, ≤48 h and ≤7 days), yielding confusing results [230]. Highly variable results were obtained from mouse immunisations within the first 3-4 days of life, which suggests individual variation in appearance of DCs and T cells in peripheral lymphoid organs during the first week of life [231, 232]. Even neonatal priming at ≤96 h after birth resulted in weak secondary antibody responses. Together these initial experiments suggested that murine neonatal immune maturation was delayed compared to humans [230]. However, primary antibody responses elicited in mice aged 1-3 weeks of age compared well to those of human neonates and infants, increasing stepwise to an adult-like primary antibody response at age ≥4-5 weeks. Thus a neonatal period of 1-2 weeks was established with similar responses in BALB/c and C57BL/6 mice [230, 233, 234]. But full development of the spleen architecture is usually delayed until about 3-4 weeks after birth in rodents and 2 years in humans [235, 236].

The degree of development of soluble and cellular components of neonatal immune systems varies widely between mammalian species [237]. So the human neonatal immune system has been studied using umbilical cord blood mononuclear cells (CBMCs) as stand-ins for research on the newborn, where murine and other mammalian models are not sufficient for maternal studies. While the use of human CBMCs has been informative and readily available, the modulating effects of the

corticosteroids and maternal cytokines released during parturition may give false impressions of the cell populations circulating in the days after parturition [237].

### 1.6.2 *Pregnancy induced changes to a mothers immune system*

For a pregnancy to be successful, the maternal endometrium must accept infiltration and invasion of semi-allergenic foeto-paternal RNA and DNA. During viral infections, this type of invasion normally induces a strong TH1 response. But pro-inflammatory TH1 cytokines, like IFNγ and TNFα, promote expression of the pro-apoptotic transmembrane protein, Fas, and would therefore increase the sensitivity of the trophoblast cells to apoptosis [238]. In order for the maternal immune system to not reject and destroy the fetus by a TH1 cytotoxic response it must accept the fetus as an allograft, and so suppression of the maternal inflammatory response is necessary at the maternal-foetal interface [238-241].

This is achieved by creating an environment which represses the production of TH1 mediators. Instead the placenta produces regulatory TH2 cytokines, like IL-6 and IL-10, which block TH1 cytokines and activation of NK cells and macrophages, and promote the implantation and maintenance of the trophoblast [242-246]. Foeto-placental tissue is known to spontaneously secrete TH2 cytokines, and studies in mice have shown that if this tissue is restimulated, the TH2:TH1 cytokine ratio is greater that that of re-stimulated maternal spleen cells [247]. Also it has been shown that *in-utero*, $CD4^{+}CD25^{hi}Foxp3^{+}$ regulatory T cells dominate the foetal circulation [248]. Thus a normal pregnancy requires a TH2 biased environment to avoid loss of the trophoblast [249, 250].

This regulatory immune imbalance that protects the foetus *in-utero* is passed onto the foetus itself and extends into the neonatal period [241]. It is known that the capacity of the neonatal immune system to generate a TH1 response is compromised because it is naturally biased towards a TH2 response [251, 252]. This indicates that certain susceptibilities of infants are not because the neonatal immune system is under developed or immature at birth but, rather, due to it being specifically adapted for survival *in-utero* [253, 254]. After birth the neonate encounters a new world of pathogens, many of which are viruses and bacteria. This bias against a TH1 response together with an antigen inexperienced immune system means neonates are highly susceptible to viral and bacterial infections [223, 255, 256].

### 1.6.3 *Transfer of maternal immunity to offspring*

As mentioned the increased susceptibility to infectious disease during the neonatal development period is reduced by transfer of a moderate range of immunity from the mother, either across the placenta or via breast feeding **(Figure 1G)**. This can be in the form of a various assortment of potential immune mediators, including antibodies, cytokines, antigens, immune cells or commensal bacteria [257].

The passive transfer of maternal antibodies (MatAb) is the factor most commonly associated with offspring protection [258]. This is because antibodies perfectly fit the role of transfer of passive protection from one organism to another since they are highly stable for long periods and target pathogens very antigen specifically without

being overly immunogenic themselves. Also they are produced at measurable levels by the mother throughout her life post-infection without detrimental inflammatory side-effects, as would be the case for persistent production of cytokines. Neonates are highly dependent upon MatAb for protection against a wide range of pathogens; on the other hand however MatAb can suppress an infant's own primary antibody response to infection and vaccination [230]. The transfer of cytokines or antigen is less likely than antibodies, except in cases where the mother has an active infection during pregnancy or breastfeeding. Most of these components exist only for a short time in the offspring since they are meant to have a role limited to protecting the neonate only during its most vulnerable period [259].

There is also the possibility that the transfer of maternal immunity may play a more active role in shaping the offspring's own immune system. Mouse studies have shown that passive MatAb influence development of the B cell repertoire of offspring [260]. Furthermore maternal stem cells and mature T cells have been found in the breastmilk which are transferred to the offspring and assimilate into the offsprings' bone marrow and immune structure [261-264]. In addition, transfer of maternal commensal bacteria during birth through vaginal delivery has been shown to be critical for establishment of the offspring microbiome, which affects the offsprings response to allergy and asthma [265, 266]. This raises the potential for long term maternal-mediated immune effects, operating long after the transfer of maternal immunity has ceased.

### 1.6.4 *Breastfeeding vs. trans-placental*

Evolving research in the field of mother-child health is constantly finding links between maternal immunity and offspring immune development and responses. It is becoming increasingly apparent that the development of the fetal immune system and the immune responses to homologous and possibly even heterologous antigens in later life is already determined *in-utero* [267, 268]. The period of nursing is equally important, and breastmilk has been shown to play a decisive role in offspring immune tolerance and disease resolution, as well as general health [260, 269].

The uterine cavity has long been regarded as a sterile environment and infringement of this usually results in a pathological condition [270]. The upper genital tract contains many immune detection and effector systems that maintain this sterility, while the amniotic fluid contains several protective acute phase proteins. The vaginal tract, which is colonised with microorganisms, is separated from the sterile intra-uterine compartment by the cervical plug, which contains multiple antimicrobial proteins and peptides [271]. Together this protects the foetus from life threatening infections. Thus the dominant route that affects offspring exposure to immune factors *in-utero* is via the placenta and umbilical cord [268, 272]. Naturally transport of proteins, cells and other mediators across this obstacle is limited for the protection of the infant. Substances that are able to pass from maternal blood to fetal blood must traverse the histological barrier, which consists of two cell layers: the multinucleated syncytiotrophoblasts (STBs) and endothelial cells of the fetal capillaries [273].

IgG is the only antibody class that significantly crosses the placenta in humans and rodents. This crossing is mediated by neonatal Fc receptors (FcRn or Fcγ) expressed on syncytiotrophoblast cells [274]. There is evidence that IgG transfer depends on maternal levels of total and specific IgG antibodies as well as the IgG subclass type, and the newborn IgG antibody levels usually correlate with maternal ones. Preferential transport occurs for IgG1, followed by IgG4, IgG3, and IgG2, for which the FcRn receptors have reduced affinity [275]. In cases of maternal human immuno-virus (HIV) infection or placental injury-causing diseases, like malaria, a great decrease in antibody transfer has been reported [276-279].

Additional immune support is given by the mother through breast milk, which contains functional nutrients, lactoferrin and IgA antibodies. Even maternal stem cells [263, 280, 281] and functional memory T cells [261, 282, 283] have been found in the breastmilk [262, 284]. Therefore a breastfed infant is better protected against numerous common infections than one not breastfed. Breastfeeding may also actively stimulate the infant's immune system by anti-idiotypic antibodies [285] and uptake of milk lymphocytes meaning a breastfed child could be better protected against certain infections long-term [286]. Vaccine responses are also often enhanced in breastfed infants. Breastfeeding decreases the risk of respiratory tract infections and diarrhea [287]. Other benefits include a lower risk of skin disease and allergies [288, 289], celiac disease [290], type 2 diabetes [291], and leukemia [292]. Breastfeeding may also improve cognitive development [293] and decrease the risk of obesity in adulthood [294, 295]. The mother's own immunity against tetanus,

diphtheria, whooping cough and influenza can protect the baby from these diseases in the early years of its life [260].

Studies performed in recent years have shown that exposure of a newborn to both pre-natal and post-natal environmental allergens influences asthma and allergy development [296, 297]. Pregnant mice that are exposed to ovalbumin (OVA) have been shown to transfer the antigen to their offspring through breastmilk, which protects them from anti-OVA hypersensitivity [298, 299]. Even pre-conception maternal exposure can lead to allergy prevention. Offspring of mice orally exposed to OVA prior to pregnancy were protected, whereas those of naïve mothers showed symptoms of a stronger allergy response, expressing higher levels of IgE and TH2 cytokines [300]. Use of radiolabelled 125i-OVA assays shows that it moves via the digestive tract of mother to the mammary gland where it is transferred to her infant [301]. OVA mediated protection in the offspring was shown to be long-lasting and is still present 8 months later.

OVA tolerised mothers showed increased anti-OVA IgG1 immunoglobulin's in both their serum and breast milk, correlating to higher levels in offspring [302]. But pups breastfed by OVA exposed B cell deficient μMT mothers had similar protection, low lung IL-13 secretion and reduced OVA-specific serum IgE compared with the pups breastfed by exposed wild type mothers, indicating that transfer of antibodies is not essential to tolerance induction [303, 304]. Since IL-10 was not detected in breastmilk, breastfeeding-dependent protection could rely on TGF-ß signalling mediated by regulatory T cells (Treg) [301]. Mice immunized with a monoclonal Ab to

TGF-ß before exposure to airborne OVA do not induce protection and experimental data has shown the presence of Tregs in the breast milk of allergen exposed mothers.

Alternative evidence, however, suggests the maternal transfer of immunity is not always protective against allergy and may even be considered as a risk factor for asthma in the infant. Studies of mothers that were tolerised by OVA show protection but when exposed to different allergens such as beta-lactoglobulin (BLG), offspring hypersenitivity can result [305]. These studies of murine models show that breastfeeding plays a role in increasing the risk of asthma development in the offspring [306-310]. Furthermore maternal exposure to tobacco smoke and air pollutants such as exhaust particles, has been shown to cause offspring epigenetic modification of histones or chromatin. Exposure to these pollutants during pregnancy affects foetal programming resulting in enhanced production of IgE and a subsequent allergic sensitivity [311]. It is recently reported by Clark et al. that there is a correlation between high maternal exposure to pollutants in air and risk of development of asthma symptoms in children [312]. Therefore while tolerance and protection against asthma and allergic diseases can be induced and transferred from the mother to the offspring, breastfeeding may be a risk factor in some circumstances and sensitize the progeny to some allergens [313]. But the strong evidence supporting its advantageous role in protection of infants means the benefits outweigh the risks of breastfeeding.

Some pre-conception infections can be transmitted vertically *in-utero* resulting in adverse health outcomes in children. This has been reported for *Listeria monocytogenes*, HIV, rubella, *Toxoplasma gondii* and CMV [314]. Screening for pre-conception exposure to infections such as cytomegalovirus (CMV) is considered to be an effective approach for reducing potential adverse events during and after pregnancy [315]. Three viruses (CMV, HIV, and HTLV-I)(Human T-cell lymphotropic virus) frequently cause infection or disease as a result of breast-milk transmission [316]. Interestingly much of the research on maternal HIV transmission via breastfeeding indicates this is possible due to the presence of infected T cells in the milk [282]. Other maternal viral and bacterial infections are rarely transmitted to their infants through breast milk [317]. In the context of the current study vertical transmission of gastro-intestinal nematodes outside of the usual fecal-oral route appears to be unusual [318]; however trans-mammary transmission of *Strongylosides stercoralis* has been reported in dogs and as such should not be ruled out as a potential route of infection in humans [319].

### 1.6.5 *Maternal antibodies*

Neonates and infants display a limited ability to generate primary antibody responses. Vaccination of infants is thus only partially successful but crucial in protecting the highly susceptible newborn. Maternal antibodies (MatAb) account for some protection for the first 3 to 12 months after birth [320], and vaccination of pregnant women in developing countries is a good way to transfer protective IgG to their babies. Maternally derived antibody immunity is generally accepted to be passive and temporary owing to the half-life of the transferred maternal components

[275]. This means the transferred proteins from the mother will decline and be removed from the body once breast-feeding is stopped. The half-life of murine IgG1 is 6-8 days [82]. With weaning and the decay of MatAb, there may be a window period where the infant is left unprotected before its adaptive response is fully matured.

While MatAb prevents some offspring B cell priming during vaccination due to a common antigen epitope specificity, MatAb do not influence T cell priming since TCRs recognize different epitopes on the vaccine antigen [321]. This has been shown in mouse and clinical studies where MatAb-inhibition of a primary antibody response did not inhibit secondary priming; a T-dependent secondary response after booster immunization suggests that this is the case in human infants [322].

### 1.6.6 *Neonatal immune development*

The neonatal immune system is qualitatively quite complete at birth, but it is different to that of a mature system in humoral and cell-mediated immune responses [235]. Certain cytokines are only produced at low levels, due to the TH2 bias already discussed, and many immune cell types are present in low numbers due to lack of antigen encounter and pre-existing immunological memory. Therefore the concentrations of soluble mediators, absolute numbers and ratios of immune cells and their activation states differ from those of an adult [237]. But while neonates may respond weakly to vaccination and infection due to an antigen inexperienced adaptive response, adult-like B and T cell responses have been demonstrated under

specific conditions and stimulations, displaying the plastic nature of the neonatal immune system [323].

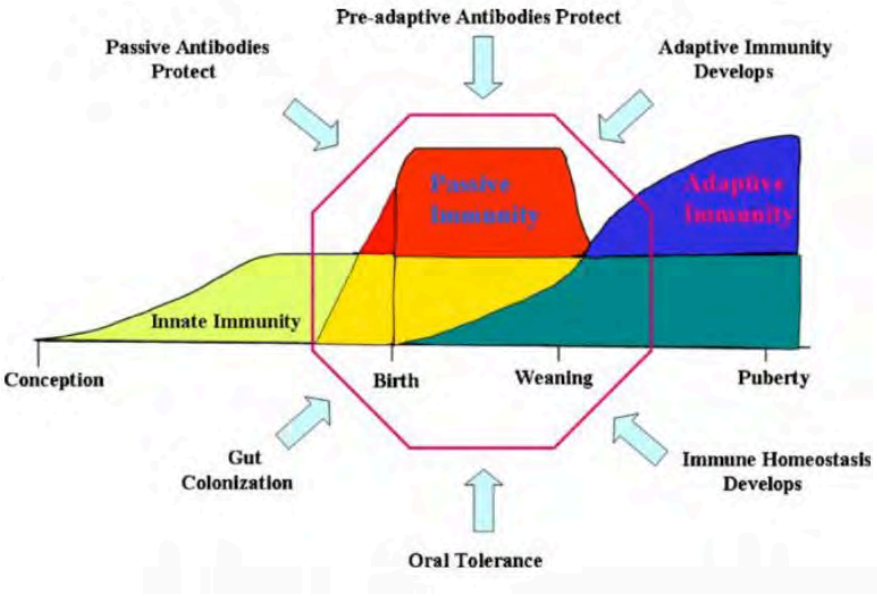



**Figure 1G: Passive protection of neonates and young infants by maternal antibodies before the adaptive response develops.** Maternal antibodies are transferred *in-utero* and via breastmilk and protect neonates from early infections. Breastmilk antibodies and transferred antigen in the form of maternal antigen and food antigen help to colonize the neonate's gut and induce oral tolerance. Before the adaptive response is fully matured and once maternal antibodies wane, the infant enters a window period where it is left unprotected [324].

Monocytes/macrophages, DCs, neutrophils and lymphocyte populations are all very limited in number at birth, and the immune system of new-born mice has been shown to be only a few percent of that of an adult [235]. And while these cells are reported to be functionally adequate, they have limitations in chemotactic responsiveness. Infant blood monocytes produce less IFN-α, IFN-γ, and IL-12p70 than cells obtained from adults, but production of these cytokines rapidly increases between birth and infancy [325, 326]. The major impetus for the expansion of the

lymphoid population is the exposure to the microbial flora that colonise the gut from birth onwards, shown by studies of germ-free animals [327]. But it takes time for the neonate to build up and develop its immune system and expand its range of antigen specificities.

Infant T cells show a great capacity to produce IL-10 and to differentiate to IL-17-producing cells (TH17 cells) in response to TLR stimulation [328], but individual infant cells are less able to produce multiple cytokines simultaneously than adult cells. The predominance of a Th17-like pattern combined with considerable IL-10 production is related to the *in-utero* TH2 bias and contributes to diminished TH1 responses, resulting in greater susceptibility to intracellular infections and diminished vaccine responses during infancy [252]. Neonatal CD4+ T cell responses, but not CD8+ T cell responses, develop slowly in infants after primary infection and present an intrinsic immaturity with a diminished capacity to generate memory cells and reduced production of IFN-γ [329, 330].

### 1.6.7 *Neonatal B cells*

Human infants have higher relative counts of total B cells than adults, increasing in the first weeks of life and remaining high for the first year of life [331]. There are numerous phenotypic and functional differences between neonatal and adult mouse splenic B cells, although fewer when comparing neonatal and adult peripheral B cells. Immature transitional ($IgM^{hi}IgD_{lo/-}$) B cells dominate in the neonatal mouse spleen and fail to up-regulate co-stimulatory molecules and MHCII, essential for effective interaction with T cells, upon ligation of their BCR [235, 332]. Neonatal

splenic B cells have limited expression of CD40, CD80, CD86 and limited interaction with ligands such as B cell activating factor (BAFF) and/or proliferation-inducing ligand (APRIL), all of which are plasma cell supporting factors [333, 334].

There is no trans-placental transfer of complement system elements, and neonates have relatively low levels of some components [335, 336]. Neonatal splenic marginal zone B cells are deficient in mice and humans [337] and have low expression of CD21 (complement receptor 2), which explains their sometimes inadequate response to TI bacterial polysaccharides [333]. In mice, MZ cells are found after 1-2 weeks of age and in humans they appear after 2 years of life and coincide with the ability to mount a response to T independent antigens [338]. Human neonatal spleens contain fewer marginal zone macrophages and these cells differ in their capacity to produce cytokines required for B cell activation [337]. Neonates also have impaired GC formation. In humans, GCs only appear at about 4 months after birth [339]. With the appearance of GCs in mice at 3 weeks of age, they also acquire the ability to induce adult-like antibody responses [340]. This delayed GC reactivity is due to delayed maturation of the essential follicular dendritic cell (FDC) network, resulting from FDC precursors failing to respond to B cell lymphotoxin-α signalling. This has been shown in mice, even with potent adjuvants that induce adult-like B and T cell interaction [334, 340].

Regarding neonatal antibody responses, the most important neonatal difference is that a B cell response favors memory induction rather than high affinity antibody production through plasma cell development [341, 342]. Several studies have shown

a delayed onset, lower peak levels and a shorter duration of antibody production compared to adults [343, 344]. There are also differences in the distribution of IgG isotypes, with infants showing lower IgG2 than adults, and lower affinity and reduced heterogeneity. The preferential differentiation into memory B cells is indicated by the inability to elicit a specific primary antibody response with vaccination at birth [345, 346]. The impaired ability to generate GC reactions and long-lived plasmablasts has implications for vaccination with TD antigens and is important in vaccination schemes. Functional neonatal B-1 progenitor cells are also present in the embryo [347, 348].

### 1.6.8 *Neonatal antigen presentation*

Neonatal APC-T cell interactions are sub-optimal and could contribute to the TH2 biased neonatal immune response. Dendritic cells are the most important APCs for stimulating naive T cells; they produce various factors, such as IL-12, which polarize CD4+ T cells to a TH1 type response [349]. But absolute numbers of neonatal mouse DCs are reduced by several logs compared to adults. This may contribute to decreased antibody production since once activated, T cells are meant to provide help to B cells for antibody production. The splenic T cell to DC ratio reaches adult proportions by 1 week after birth yet seems to be functionally immature in some settings [236]. This immaturity has been attributed to low expression of costimulatory molecules and IL-12. But as with neonatal T and B cells, under certain circumstances neonatal DCs are capable of mature response. In humans, cord blood mononuclear cell (CBMC)-derived DCs express limited IL-12 and IL-15 [350], which seems to persist for up to 1 year after birth [230]. Optimal APC function is thus a key

element for overcoming the TH2 biased neonatal immune response and eventually eliciting an adult-like TH1 response.

### 1.6.9 *Neonatal vaccination*

Many infant diseases could be prevented by available vaccines. However, only three vaccines are currently given at birth: oral polio vaccine (OPV), hepatitis B virus (HBV) vaccine and bacille-Calmette Guerin (BCG) and, with the exception of BCG, they require several booster immunizations [223]. Young infants (<3 months) are incompletely protected by vaccine schedules, with some vaccines initiated too late, and no influenza vaccine licensed for use prior to the age of 6 months while there is no licensed RSV vaccine [223].

So far, strategies to protect neonates and young infants against overwhelming infections have included indirect protection via maternal vaccination, cocoon strategies where people in contact with the neonate are vaccinated to prevent spread of disease, or direct neonatal immunisation. Concerns regarding neonatal immune tolerance to vaccine antigens and unpredictable adverse effects arising from an immune system that does not respond like that of an adult, have hindered the development of neonatal vaccination. However, recent advances have revealed much about this unique period of immune response and show various potential strategies for the development of neonatal vaccines.

MatAb are known to interfere with primary antibody responses to immunizations in infants [351, 352]. By epitope masking, antigen specific MatAb bind to vaccine

antigens in an epitope-specific manner and prevent infant B cells from accessing immunodominant vaccine epitopes for recognition by their BCRs and activation of a functional primary antibody response [322]. MatAb mediated inhibition is titre dependent and has the capacity to be circumvented by increasing the dose of vaccine antigen. This epitope-specificity of MatAb-mediated inhibition has not been shown or reported in human infants, only in mice [321]. Another proposed mechanism of MatAb-mediated inhibition is the mopping up of vaccine antigen by the uptake of MatAb:antigen immune complexes by neonatal APCs [322]. In addition, responses to some vaccines, such as vaccines for hepatitis B virus and oral poliovirus vaccine, result in less TH1 activity and a bias toward TH2 function [353].

### 1.6.10 *Helminth infections in pregnancy and neonates*

Helminth infections are highly prevalent in sub-Saharan Africa (SSA) where over 40% of women of childbearing age are infected with helminthes [169]. A considerable parasitic infection rate during pregnancy has been reported [354]. In general, poor maternal health as a result of insufficient nutrition or anemia in particular is a major contributor to increased maternal and infant mortality and low birth weight [355, 356]. Helminth infections can cause and/or exacerbate both malnutrition and anemia and are therefore likely to contribute to problems during and after pregnancy as low-birth weight itself is associated with an increased risk to develop diseases later in life [357, 358]. But as mentioned previously, congenital helminthic infection in humans is rarely described [318].

However a mother's infectious history and immunity can affect her offspring in other ways. Studies have demonstrated that maternal helminth infection can influence susceptibility to a homologous infection during childhood without previous feto-maternal transmission of the infectious agent itself during pregnancy [359]. Whether this is caused by chronic maternal immune responses (cells or cytokines) or transmission of helminth derived antigen/proteins, and furthermore which developmental stage of the offspring's immune system is affected by such factors and what clinical implications these results have regarding vaccination strategies, needs to be investigated [360]. Children of mothers exposed to helminth infections may display T cell [361, 362] or B cell [363] sensitization to endemic helminth infections.

Associations have been made between maternal helminth infection and impaired immune responses to childhood diseases and vaccinations [364]. For example, generation of protective antibody to *Haemophilus influenzae type B* post vaccination is inversely proportional to the number of maternal helminth infections during pregnancy [365]. Altered neonatal immunity to malaria [366] and other protozoan infections [367], eczema [289], tuberculosis and BCG vaccination [368-370] as well as other microbial infections [371] has been associated with helminth infection.

While a maternal *N. brasiliensis* model has not yet been described, mouse studies have shown transfer of maternal antibody can confer high levels of protection against *H. polygyrus* infection [320]. How this affects offspring ability to launch antibody mediated protective responses to other infections is unclear. But maternal transfer of

regulatory immune components can have a range of effects on children's subsequent immunity. Children of filarial infected mothers have been shown to demonstrate reduced ability to control a subsequent filarial infection [372] possibly due to expanded regulatory T cell populations in the offspring impairing responsiveness to antigenic challenge [248]. Potentially related to this is the demonstration that anti-helmintic treatment during pregnancy can increase the likelihood of atopic disease in children [289]. However, transfer of regulatory immune components may not be the only cause of this protection from allergy; for example Schistosome induced maternal IFNγ has been demonstrated to protect against experimental offspring allergy [373].

### 1.7 Objectives

As discussed above, the effects of preconception maternal infection with the helminth *Nippostrongylus brasiliensis* is not well defined. However this has been studied in other helminth infections. Our aim is to investigate any effects maternal *N. brasiliensis* infection has on offspring disease susceptibility. Related to this, we aim to elucidate the effects of preconception maternal *N. brasiliensis* infection on innate and adaptive immunity in offspring and we will define the development of the B and T cell immune populations.

Helminth infections induce a highly polarized TH2 response. We hypothesize that a memory-type TH2 response may be transferred to offspring *in-utero* and through breastmilk in the form of lymphocytes, antibodies and cytokines, imprinting on early offspring immune development and possibly extending into later life.

- **Aim1: How does maternal helminth infection affect immune development in her offspring?**
- **Aim2: How does maternal helminth infection influence offspring immune responses to *N. brasiliensis* infection?**
- **Aim3: What components of the mothers immune system play a role in the previously seen transfer of immunity?**

Our study showed that, in mice, maternal infection with the helminth *N. brasiliensis* impacts on immune development of offspring by increasing T cell populations and B cell proliferation. In offspring born to *N. brasiliensis* exposed mothers control of *N. brasiliensis* was heightened. This *N. brasiliensis* associated maternal transfer of protection could be transferred by nursing alone. These data indicate that maternal exposure to a helminth infection may offer some protection against infection.

# Chapter 2: Materials and Methods

## 2.1 Animal Work

### 2.1.1 *Mice used, animal unit and ethics*

In this study the following BALB/c background mice were used: Wild type BALB/c control, IL-4Rα$^{-/-}$ [described as *IL4ra*$^{tm1Fbb}$*/IL4ra*$^{tm1Fbb}$], IL-13$^{-/-}$, IL-4$^{-/-}$ and µMT (B cell deficient) mice. BALB/c background T cell-specific IL-4Rα deficient *ilck1*$^{Cre}$IL-4Rα$^{-/lox}$ and B cell-specific IL-4Rα deficient *MB*1$^{Cre}$IL-4Rα$^{-/loxp}$ [described as *IL4ra*$^{tm1Fbb}$*/IL4ra*$^{tm2Fbb}$*Tg (Cd79a*$^{tm1(cre)Reth}$*)*] or their hemizygous IL-4Rα$^{-/loxp}$ littermate controls were also used.

All studies were carried out in accordance with protocol 012/054 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 used between 2–15 weeks of age with appropriate littermate controls of the same generation. Mice were killed by halothane inhalation.

### 2.1.2 *Targeting IL-4Rα*

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-4Rα from that cell type. The gene for IL-4Rα 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 [374]. 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-4Rα gene of that cell. If the promoter is only active in certain cell types then the Cre induced IL-4Rα deletion is specific to that type of cell.

To identify IL-4Rα-mediated effects on B cell function during *N. brasiliensis* re-infection, B cell specific IL-4Rα knock outs were generated. B cell-specific IL-4Rα-deficient BALB/c mice were established by intercrossing floxed IL-4Rα mice with mice expressing Cre- recombinase (Cre) under the control of the *MB1* promoter ($MB1^{Cre}$ mice) [375]; The *MB1* gene encodes the Ig-α signalling subunit of the B cell antigen receptor. Transgene-bearing hemizygous mice ($MB1^{Cre}$IL-4Rα$^{-/lox}$) were identified by PCR genotyping and efficient B cell-specific depletion was demonstrated on a genomic, protein and functional level [376].

T cell-specific IL-4Rα-deficient BALB/c mice were established by intercrossing floxed IL-4Rα mice with mice expressing Cre under the control of the *lck* promoter ($lck^{cre}$ mice). Lck is a tyrosine kinase, which phosphorylates tyrosine residues of certain proteins involved in T cell intracellular signaling pathways [377]. Transgene-bearing hemizygous mice ($ilck1^{Cre}$IL-4Rα$^{-/lox}$) were identified by PCR genotyping. Efficient T cell-specific depletion was demonstrated on a genomic, protein and functional level [378].

### 2.1.3 *Mating and litter-swaps*

Female mice aged between 7 and 8 weeks were mated with male mice at one male per cage of two females over two weeks after which the male was removed. Females gave birth 21 days after fertilisation and birth of pups was monitored daily. A maximum of two mothers and 8 pups per mother were housed per cage. In the long-term experiments pups were weaned at 3 weeks of age and separated from their mothers according to sex.

### 2.1.4 *Experiment end point*

At specific time points mice were killed, peripheral blood was taken by cardiac puncture and spleens, lymph nodes or lungs were removed by incision under sterile conditions. Blood was collected in serum separation tubes and spun at 8,000rpm (7155xg) for 20min to separate plasma from red blood cells. The left lobe of the lung was snap frozen in liquid nitrogen and subsequently stored at -80$^{o}$C until cytokine analysis. Blood and lung samples were stored at -80°C. Organs were weighed whole before use for either flow cytometry or in vitro splenocyte restimulation for cytokine assessment by ELISA. Milk was isolated from the stomach of the pups using the method established by Verhasselt et al. [301]. This was used for cytokine and antibody assessment by ELISA.

## 2.2 Working with *Nippostrongylus brasiliensis (Nb)*

### 2.2.1 *Maintenance of Nb*

The *Nippostrongylus brasiliensis* stock was maintained by the division of Immunology (Faculty of Heath Science, UCT), by passage through Wistar rats.

Every two weeks, a naive group of rats were infected subcutaneously with 5000 L3 larvae per rat in 500µl of 0.9% NaCl. Rat faecal pellets were collected on day 6, 7 and 8 post infection and liquefied in 5µg/ml fungizone in $H_2O$, 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 were harvested at the L3 stage for future infections.

### 2.2.2 *Infection with Nb*

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 adult mouse was injected sub-cutaneously with 500 x *N. brasiliensis* L3 larvae in 200 µl of 0.9%NaCl. Pups were infected with a half dose of 250 x *N. brasiliensis* L3 larvae in 100 µl of 0.9%NaCl. Mice were killed at the relevant timepoint p.i., day 5 in most cases.

Most mice used (except IL-4R $\alpha^{-/-}$) for infection experiments would naturally clear *Nb* at approximately 9 days p.i. but to ensure that all worms were expelled potential mothers were treated with 10 mg/ml Ivermectin (Virbamec LA, South Africa)(correct dose is approximately 0.004mg ivermectin per 20g mouse per administration; this dose equates 400ul of 10 mg/ml drinking water per mouse per administration) in their drinking water from day 7 to 14 post primary infection to clear the pathogen.

### 2.2.3 *Generating Nb somatic antigen*

L3 larvae were washed from the edge of the filter paper into $H_2O$. Pen./Strep. (Penicillin + Streptomycin) at 50µg/ml was added to the $H_2O$ 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 distilled $H_20$. The larvae are then concentrated into 2 ml of distilled $H_2O$ and dipped into liquid nitrogen to snap freeze them and disrupt cellular walls and membrane in order to release the cells' contents. Following this they are completely homogenized before the whole solution is centrifuged at 8,000rpm (7155xg) 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). Antigen was used to determine the antigen specific antibody levels or to stimulate cells to measure cytokine production by intracellular facs staining.

### 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, lung 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 $1x10^7$ cells/ml in complete media with 10% Foetal Calf Serum (FCS) added (see Appendix B). The cells were then ready for re-stimulation or FACS analysis.

To prepare lung homogenates, 400µl of MACs buffer containing protease inhibitor (Sigma) was added to each previously snap-frozen lung and homogenized with a homogenizer (Polytron). The homogenates were centrifuged at 14000 rpm for 5 mins and the protein concentrations of the supernatants were determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Thereafter, protein concentrations for all samples were equalized to 5mg/ml and levels of IL-4 and IL-13 were determined with enzyme-linked immunosorbent assay (ELISA) *(Section2.6.2)*.

## 2.4 In vitro re-stimulation of cells

Single cell suspensions of splenocytes in complete media were plated at $1x10^6$ cells per well in 96 well plates (Nunc, Maxisorp) and restimulated with homemade anti-CD3 (Monoclonal hamster IgG Clone #145-2C11; protein G purified from hybridoma culture supernatant; lyophilized from a 0.2 µm filtered solution in PBS with Trehalose and Mannitol) [379-382] at 10µg/ml in a total volume of 200µl per well in complete

media. Cells restimulated with anti-CD3 were incubated for 72 hours. Cells were then pelleted (1,200rpm for 5min at 4°C) and the supernatants frozen at -20°C for cytokine analysis by ELISA.

## 2.5 Flow Cytometry

### 2.5.1 *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 $1x10^6$ cells per FACS sample was used. The samples were stained with 25µl antibody master mix (FACS buffer containing 2% Rat serum, 2% anti-FcRγII/III and the required antibodies (see Appendix A) diluted to the correct concentration) for 30min on ice, before the antibody was washed off in FACS buffer (see Appendix B). The cells were re-suspended in FACS buffer and analysed on a 4 laser BECTON DICKINSON FACS LSR FORTESSA. Antibodies were from BD Pharmingen (San Diego, CA). The data was collected by DIVA© BD (San Diego, CA). FACS data was analysed by FlowJo© Treestar (Ashland, OR). 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.

### 2.5.2 *Intracellular FACS*

Intracellular cytokine staining was performed on cells re-suspended in complete media at $1x10^7$/ml and stimulated with 10µg/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 (as in *2.5.1*), then fixed and permeabilised with Cytofix/Cytoperm Plus (BD Pharmingen). Intracellular staining was performed by staining cells with appropriate antibody (see Appendix A), mostly IL-13 PeCy7 (Clone: ebio13a)(eBioscience; San Diego, CA) or appropriate IgG1 isotype control.

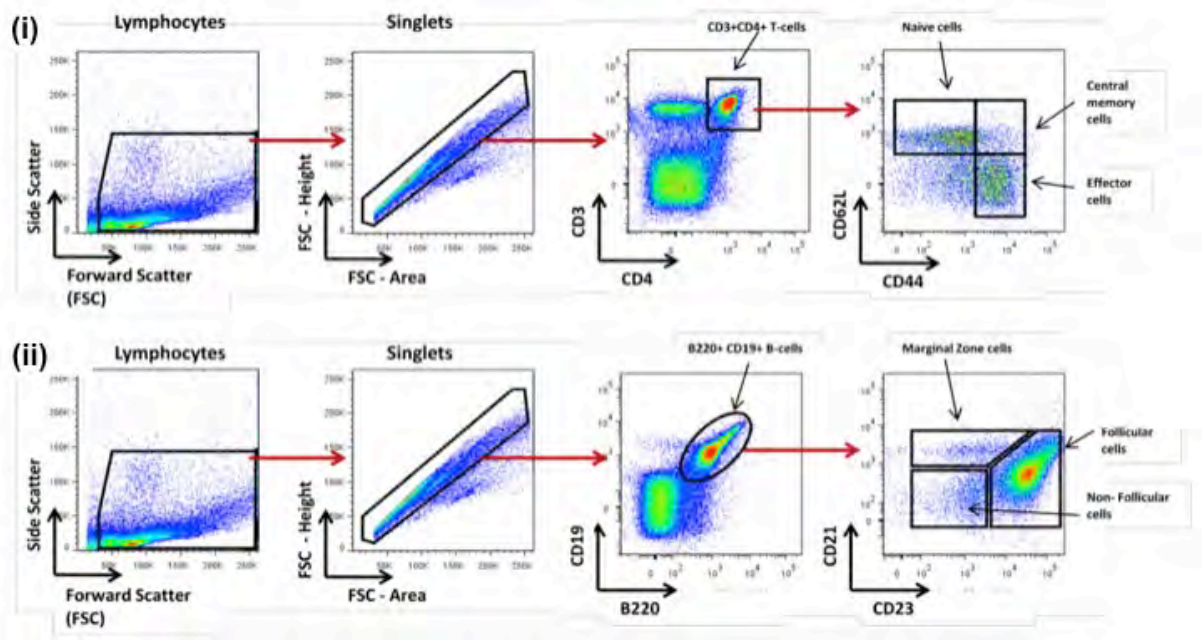



**Figure2A: Flow cytometry gating strategies for T cells and B cells**. Raw data is gated for lymphocytes based on size (Forward Scatter, FSC) and granularity (Side Scatter, SSC), then helper ($CD3^{+}CD4^{+}$) T cells, and the activation status of helper T cells is determined as naive ($CD44_{lo}CD62L^{hi}$), effector ($CD44^{hi}CD62L_{lo}$), central memory ($CD44^{hi}CD62L^{hi}$) helper T cells **(i)**. Lymphocytes are separated into $B220^{+}CD19^{+}$ B cells, whose maturation status is assessed in terms of newly-formed (NF) ($CD21^{-}CD23^{-}$), follicular (FO) ($CD23^{hi}CD21_{int\text{-}lo}$) and marginal zone (MZ) ($CD21^{hi}CD23_{lo}$) B cells, and MHCII activated B cells **(ii)**.

### 2.5.3 *Antibodies and gating strategies*

All flow cytometry antibodies (from BD Pharmingen except IL-13) used in this study are listed in Appendix. T and B cell populations and sub-populations were determined using gating strategies shown (Fig 2A-E). Unstained cells and

CompBeads (BD Pharmingen) were used to set parameters on the Facs Fortessa machine.

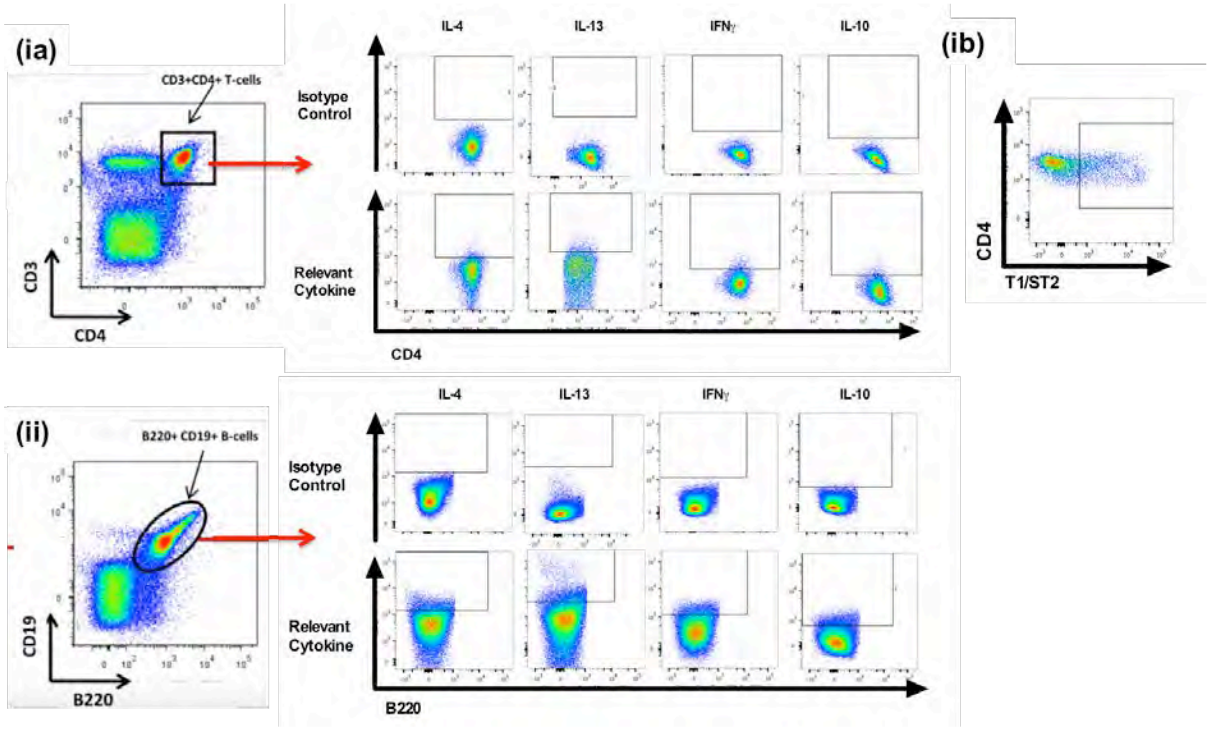



**Figure2B: Flow cytometry gating strategies for cytokines and T1/ST2**. Raw data is gated for lymphocytes based on size (FSC) and granularity (SSC), then helper T cells ($CD3^+CD4^+$) **(i)** or $B220^+CD19^+$ B cells **(ii)**. These are then analysed for expression of cytokines (intracellular) **(ia & ii)** or T1/ST2 **(ib).**

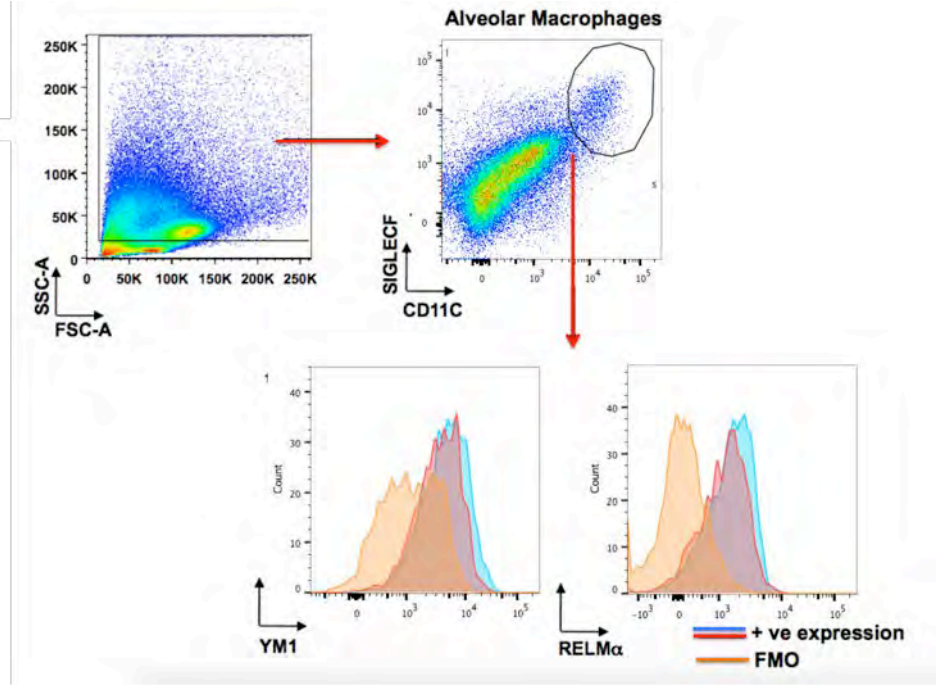



**Figure2C: Flow cytometry gating strategy for alveolar macrophages**. Raw data is gated for cells based on size (FSC) and granularity (SSC), then alveolar macrophages (SiglecF$^{+}$CD11c$^{+}$). Alternative activation was measured by YM-1 and Relmα expression.

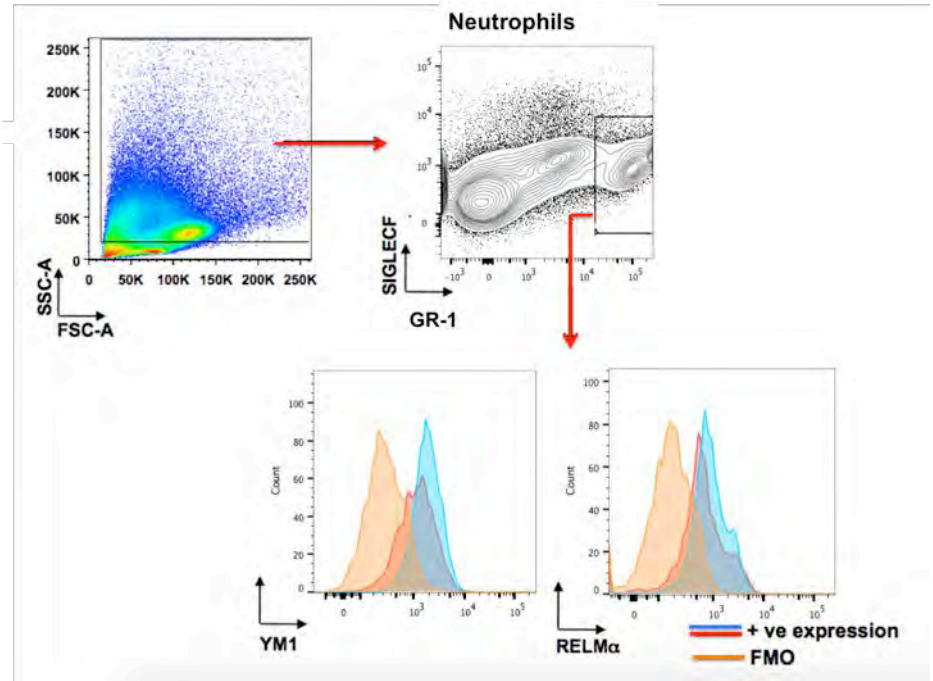



**Figure2D: Flow cytometry gating strategy for neutrophils**. Raw data is gated for cells based on size (FSC) and granularity (SSC), then neutrophils (SiglecF$^{-}$GR1$^{+}$). Alternative activation was measured by YM-1 and Relmα expression.

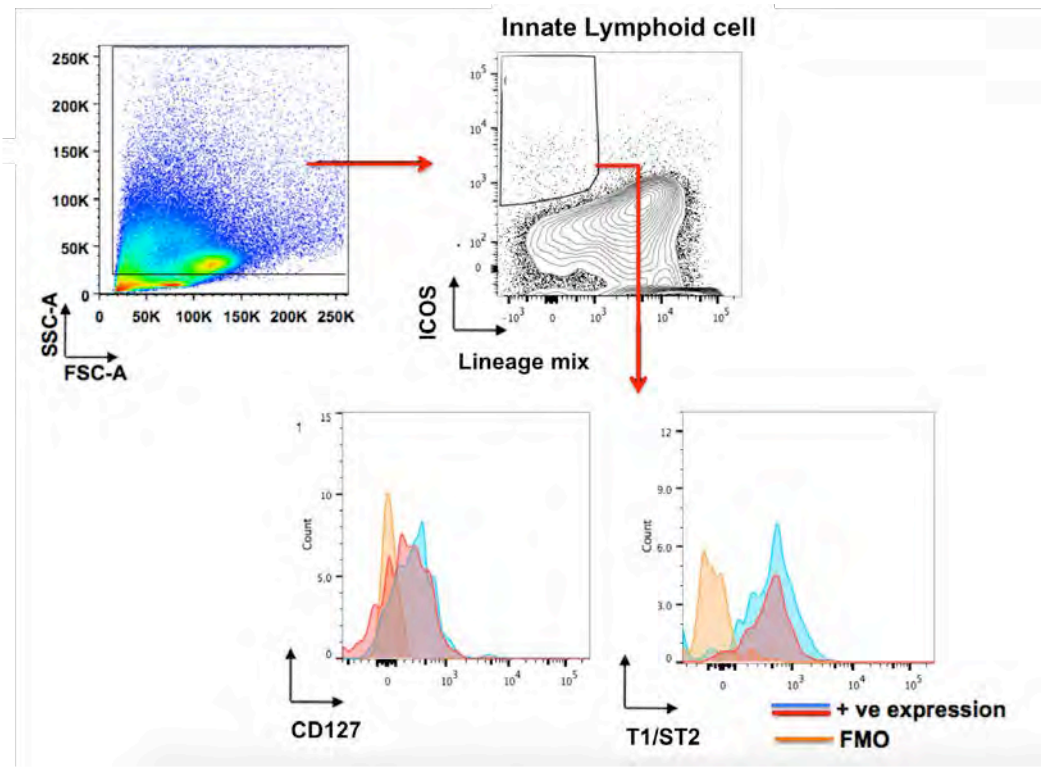



**Figure2E: Flow cytometry gating strategies for ILCs**. Raw data is gated for cells based on size (FSC) and granularity (SSC), then ILCs (Lineage$^-$ICOS$^+$). Activation was measured by CD127 and T1/ST2 expression

## 2.6 Enzyme-linked Immunosorbent assay (ELISA)

### 2.6.1 *Antibody ELISA*

Relative antigen specific serum antibody levels were determined by ELISA. Briefly, a 96 well flat bottomed plate (Nunc, Maxisorp) was coated with 10µg/ml of *Nb* somatic antigen in carbonate coating buffer (see Appendix B) overnight at 4°C before blocking buffer was added for 1hr at 37°C, after which the plate was washed in washing buffer (see Appendix B). The blood serum from *2.1.4* was added to the wells, starting with a 1/3 dilution and this was serially diluted at 1/3 down the plate using diluting buffer (see Appendix B). The plate was incubated overnight at 4°C and washed the next day, before the AP conjugated secondary antibody for IgG1 or

IgG2a was added and the plate was again incubated overnight at 4°C. After a washing step, 4-P-Nitrophenol-Phosphate (Sigma-Aldrich) in substrate buffer (see Appendix B)(1mg/ml) added to develop the reaction. Plates were incubated with the substrate at 37°C until the desired colour intensity was observed, after which the absorbance was read at λ405nm against a reference measurement of λ490nm using the Softmax Pro software on a VersaMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA). Relative antigen specific antibody levels were plotted as dilution graphs. All antibody ELISA sandwich antibodies were from BD Pharmingen.

### 2.6.2 *Cytokine ELISA*

Cytokine levels were quantified from supernatants taken from antigen-restimulated splenocytes *(Section2.4)* or lung homogenates *(Section2.3)*. For all solution and antibody details for cytokine ELISAs, refer to Appendix A/B. Briefly, IL-4, IL-10, IFNγ and IL-13 levels were quantified by coating flat-bottomed 96 well plates (Nunc, Maxisorp) with coating antibody in 1 x PBS overnight at 4°C. After washing and blocking steps were performed as for antibody ELISAs, the samples were diluted to a starting concentration of 1/3 in dilution buffer, loaded into wells and further diluted 1/3 two times to a final dilution of 1/27. Recombinant protein standards were used to generate a standard curve, and diluted serially at 1/2 from 100ng/ml across 11 wells. Plates were then incubated overnight at 4°C. After washing three times, a biotinlyated secondary antibody was added in dilution buffer and again incubated at 4°C over night. After washing three times, streptavidin-linked horseradish peroxidase (HRP) was added at a dilution of 1/5000 in a volume of 50µl per well and incubated

for 1 hour at 37°C. After washing, the plates were developed with 50µl TMB Microwell Peroxidase Substrate System (KPL), and once the titration of the standard was clearly visible, the reaction was stopped with 25µl 1M $H_3PO_4$ and the signal was read at λ450nm against a reference measurement of λ540nm on a VersaMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA). Cytokine concentrations from samples were determined from the standard curve.

### 2.7 Statistics

The results below are expressed as either individual mice/datapoints or group means ± standard deviation (SD). P values and significances were determined using either the one-tailed 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 (*:$p<0.05$, **:$p<0.01$, ***:$p<0.001$). [380]

# Chapter 3: Results 1

## Can preconception maternal exposure to *N. brasiliensis* transfer protective immunity to *N. brasiliensis* to offspring?

*Introduction*

In endemic areas a number of studies have shown that maternal exposure to a range helminth infections does alter children's immunity and these changes may have important ramifications for natural and vaccine mediated immunity to both helminths and other diseases [237, 289].

In the study presented here we developed and applied a model of pre-conception (PCp) exposure to a model helminth infection (*Nippostrongylus brasiliensis: Nb*). We then used this model to establish how PCp *Nb* exposure influenced offspring immunity to a subsequent *Nb* infection.

*Results*

**Pre-conception maternal exposure to *Nb* transfers *Nb* protective immunity to offspring.**

Female BALB/c mice were infected with 500 L3 *Nb* and 7 days post infection (p.i.) the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later mice were mated. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i. **(Figure 1.1A).**

Pups born to mothers who had a preconception (PCp) *Nb* infection had reduced intestinal numbers of adult *Nb* when compared to pups born to un-infected mothers **(Figure 1.1B).** As an important component of maternal transfer of immunity can be transfer of antibody to the offspring [320] we established if PCp *Nb* infection influenced pup immunoglobulin levels. Pups born to PCp *Nb* mothers had elevated levels of anti-*Nb* IgG1 and IgG2 **(Figure 1.1C)**. PCp *Nb* infection therefore resulted in increased anti-*Nb* antibody responses and these associated with increased protection against infection. Maternal helminth infections have also been associated with increased type 2 cytokine responses in offspring [369], and such an influence could also contribute to enhanced offspring immunity to *Nb*. Offspring born to PCp *Nb* infected mothers showed significantly elevated levels of IL-4, IL-13 and IL-10 following anti-CD3 re-stimulation of total splenocytes **(Figure 1.1D)**; IFN-γ was not changed. Offspring born to PCp *Nb* infected mothers also had significantly more splenocytes than pups born to un-infected mothers **(Figure 1.1E)**.

These data demonstrate that a PCp *Nb* infection confers a significant level of protective immunity to offspring when compared to offspring born to un-infected mothers. Protection related to increased antigen specific antibodies and systemic ability to secrete type 2 cytokines. We next tested how these responses related to immune cell populations known to confer protection against *Nb*.

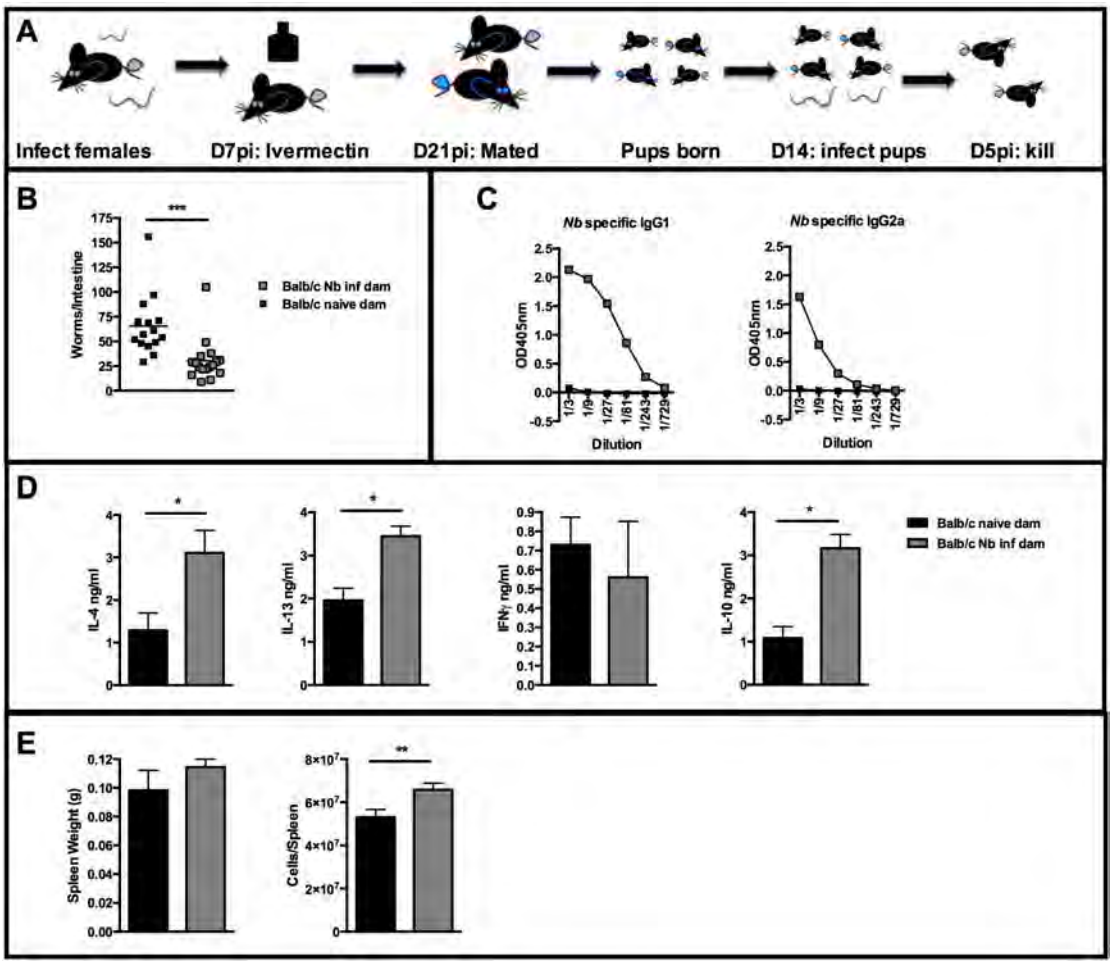



**Figure 1.1: Preconception maternal infection with *Nb* confers immune protection against *Nb* infection to offspring.** Female BALB/c mice were infected with 500 L3 *Nb* and 7 days post infection (p.i.) the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later mice were mated. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i. **(A).** Intestinal worm burden was analysed at D5p.i. **(B).** Serum antigen specific IgG1 and IgG2a levels at D5p.i. **(C).** IL-4, IL-13, IFN-γ and IL-10 secretion by total splenocytes restimulated with αCD3 **(D).** Spleen weight and cellularity per

mouse **(E).** Data is representative (except for worm counts which are pooled)(Mean +/- SD) of 3 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

**Increased offspring splenic B cell populations and expression of MHCII associate with PCp-maternal-exposure-linked protective immunity to *Nb*.**

B cells make important contributions to protective immunity to *Nb* infection [109]. We analyzed how PCp *Nb* influenced systemic B cell development and immune responses in offspring.

Increased splenic $B220^{+}CD19^{+}$ B cell proportions and numbers were found in pups born to PCp *Nb* infected mice compared to those born to naïve mice **(Figure 1.2A)**. No difference in the proportions of follicular (FO; $B220^{+}CD19^{+}CD23^{+}$) or newly formed (NF; $B220^{+}CD19^{+}CD23^{-}CD21^{-}$) B cells between naïve or PCp *Nb* mice were found. But pups born to PCp *Nb* infected mice did have increased proportions and numbers of marginal zone (MZ; $B220^{+}CD19^{+}CD23^{-}CD21^{+}$) B cells **(Figure 1.2B)**. B cell antigen presentation has been shown to be an important component of protection in a range of diseases. We have previously shown that expression of MHCII on B cells associated with host protective immunity to *Nb* [109]. In the current study we found that all PCp *Nb* B cell sub-populations showed increased MHCII expression when compared to naïve mice **(Figure 1.2C)**. B cell cytokine production has also been shown to mediate protection against a number of helminth species [110, 135]. We found splenic $CD19^{+}$ B cell populations showed increased numbers (but not proportions) of IL-4, IL-13 and IFN-γ producing cells in PCp *Nb* infected mice in response to PMA/Ionomycin stimulation **(Figure 1.2D)**.

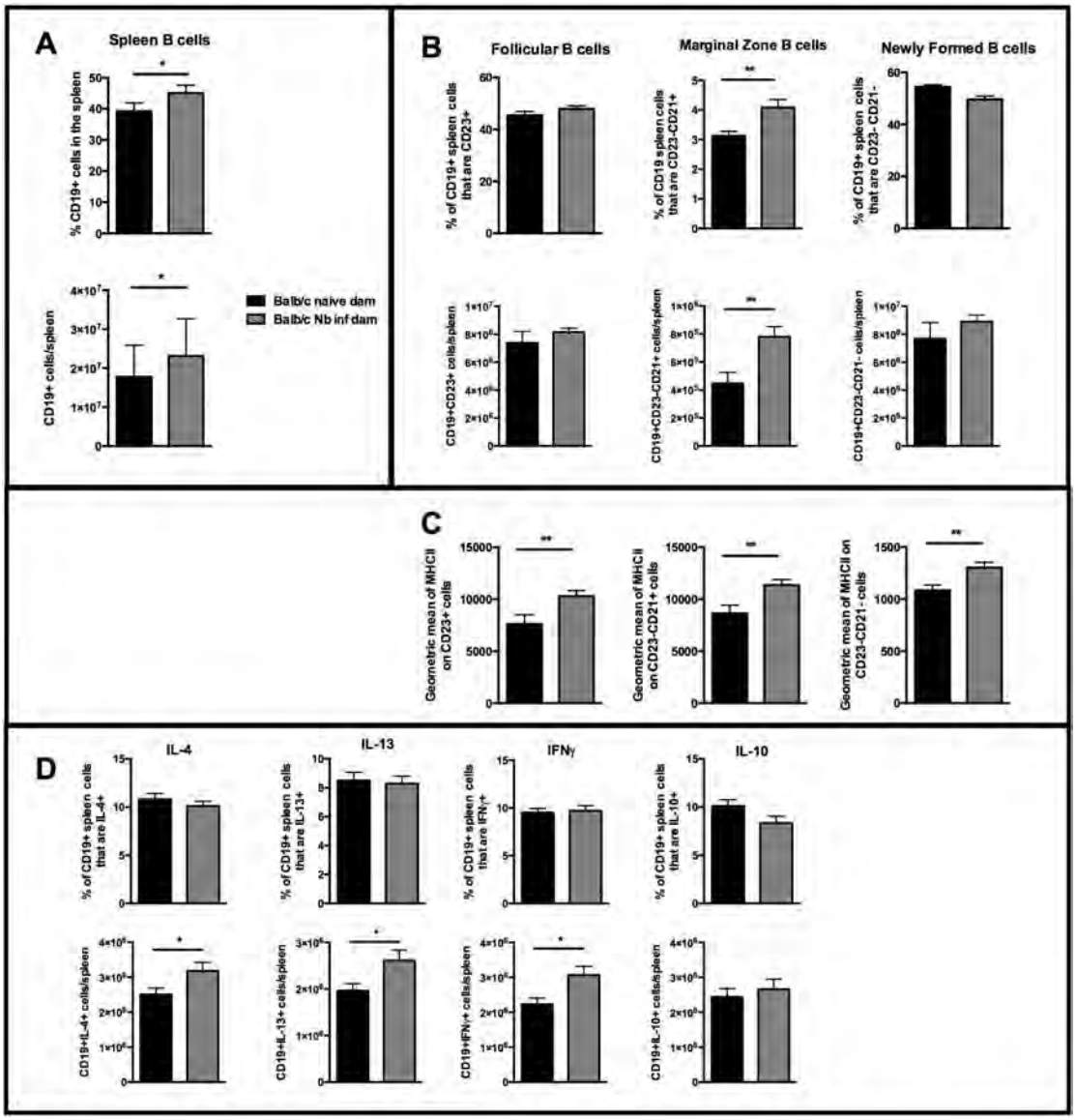



**Figure 1.2: Preconception maternal infection with *Nb* associates with increased splenic B cell populations and activation.** Spleen $CD19^+B220^+$ B cell populations were analysed by FACS at D5p.i. *(Methods Figure2A)* **(A).** Spleen B cells were stratified into FO ($B220^+CD19^+CD23^+$), MZ ($B220^+CD19^+CD23^-CD21^+$) and NF ($B220^+CD19^+CD23^-CD21^-$) populations **(B).** MHCII expression on FO, MZ and NF cells was analysed by FACS **(C).** Spleen $CD19^+B220^+$ B cell populations that produce IL-4, IL-13, IFN-γ and IL-10 were analysed by FACS at D5p.i. *(Methods Figure2B)* **(D).** Data is representative (Mean +/- SD) of 3 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

Taken together this data shows that PCp *Nb* infection increased certain B cell populations in the spleen when compared to pups born to naïve mothers. And although B cells had similar proportions of cytokine production, pups born to *Nb* infected mothers do have increased numbers of cytokine producing B cells. Importantly we found that MHCII expression by these B cells was increased suggesting that protection could be mediated via a similar mechanism as that suggested in our previous work [109].

**Activation of offspring splenic CD4 T cell populations associated with PCp-maternal-exposure-linked protective immunity to *Nb*.**

CD4 T cells are a key source of protective immunity to an *Nb* infection [35, 219]. We analyzed how PCp *Nb* influenced the development of potentially protective systemic CD4 T cell responses in offspring.

Splenic $CD3^+CD4^+$ T cell populations showed equivalent proportions in both groups but mice born to PCp *Nb* infected mice had increased numbers of helper T cells **(Figure 1.3A).** Analysis of T cell activation showed that $CD3^+CD4^+CD44^+$ activated T cells also showed equivalent proportions between groups but increased numbers in PCp *Nb* infected mice compared to naïve pups **(Figure 1.3B).** Stratification of activated T cells into effector (Teff; $CD3^+CD4^+CD44^+CD62L_{lo}$) and central memory (TCM; $CD3^+CD4^+$ $CD44^+CD62L^{hi}$) T cells showed a similar trend; numbers of these sub-populations were increased in PCp *Nb* infected mice **(Figure 1.3C)**. Additionally relative expression of the IL-33 receptor (T1/ST2) on CD4 T cells was also equivalent between both naive and PCp *Nb* infected mice but again the PCp *Nb* had

higher numbers of expressing cells **(Figure 1.3D)**. This shows increased TH2 induction in PCp *Nb* mice. Analysis of splenic $CD3^+CD4^+$ T cell cytokine production showed increased proportions and and numbers of IL-4, IL-13 and IFN-γ producing cells in PCp *Nb* infected mice following ex vivo PMA/ionomycin stimulation **(Figure 1.3E)**.

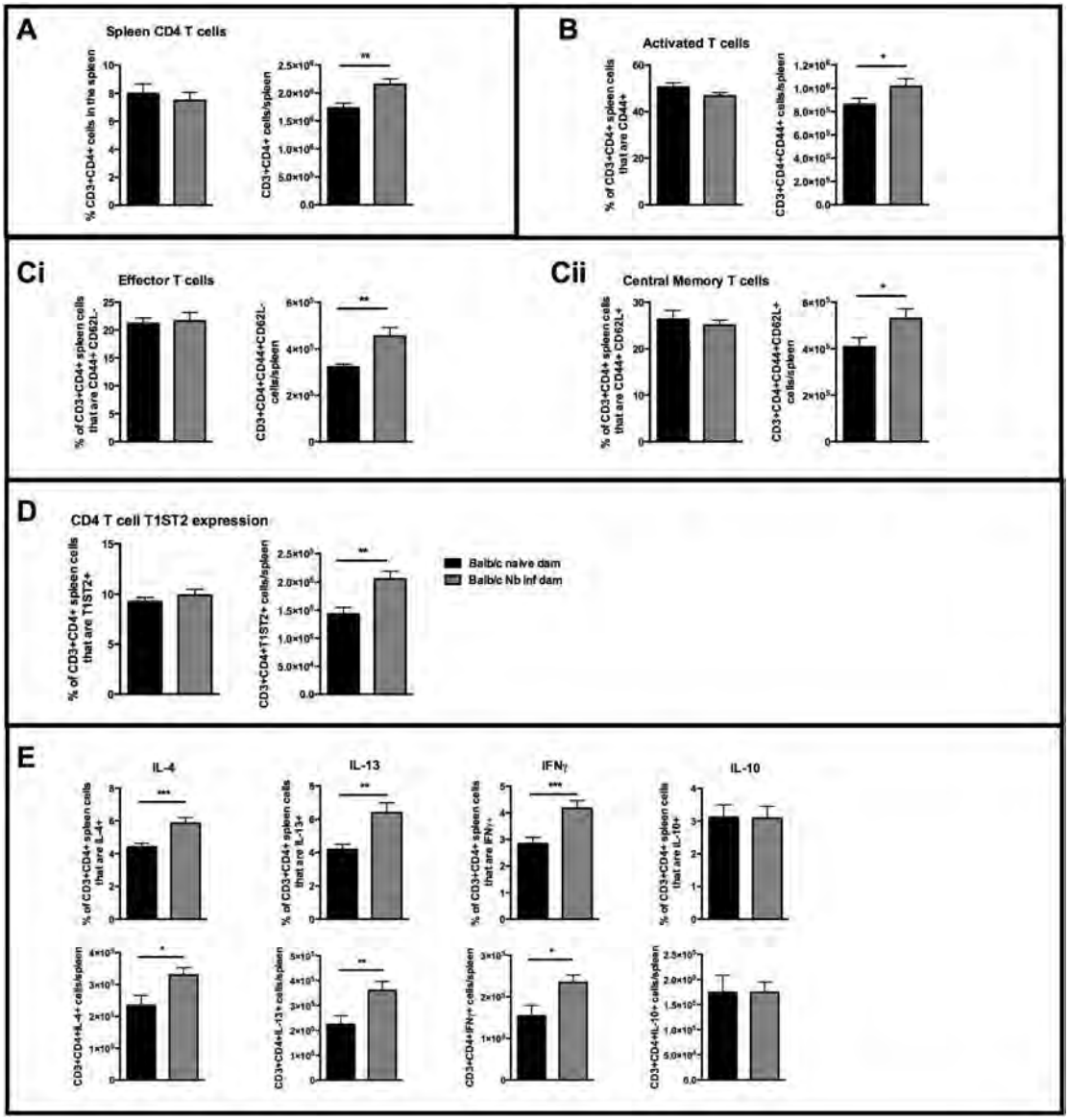



**Figure 1.3: Preconception maternal infection with *Nb* associates with increased splenic T cell populations and activation.** Spleen $CD3^+CD4^+$ T cell populations were analysed by FACS at D5p.i. *(Methods Figure2A)* **(A).** Spleen T cells were stratified into

activated ($CD3^+CD4^+CD44^+$)**(B)**, Teff ($CD3^+CD4^+CD44^+CD62L_{lo}$)**(Cii)** and TCM ($CD3^+CD4^+CD44^+CD62L^{hi}$)**(Ciii)** populations**.** T1/ST2 expression on $CD3^+CD4^+$ T cells was analysed by FACS **(D).** Spleen $CD3^+CD4^+$ T cell populations that produce IL-4, IL-13, IFN-γ and IL-10 were analysed by FACS at D5p.i. *(Methods Figure2B)* **(D).** Data is representative (Mean +/- SD) of 3 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

This analysis shows that PCp *Nb* infection does not alter relative memory or effector phenotype activation of CD4 T cells but PCp *Nb* do have greater numbers of activated helper T cells in the spleen when compared to pups born to naïve mothers and these cells may contribute to the protective immune response via cytokine production.

**Increased lung immune responses in offspring associate with PCp-maternal-exposure-linked protective immunity to *Nb*.**

We have established that systemic protection-associated-markers of immunity to *Nb* are increased in pups born to PCp *Nb* infected mothers when compared to those born to naïve mothers. But the reduced parasite numbers we see at day 5p.i. are indicative of an early immune reaction in a site before the larvae reach the intestine, and previous work by us and others indicates that priming in the lung is important for inducing an early reduction in parasite burdens [203, 219]. So we next sought to establish if increased immune responses were also seen at an essential local site of control, the lung.

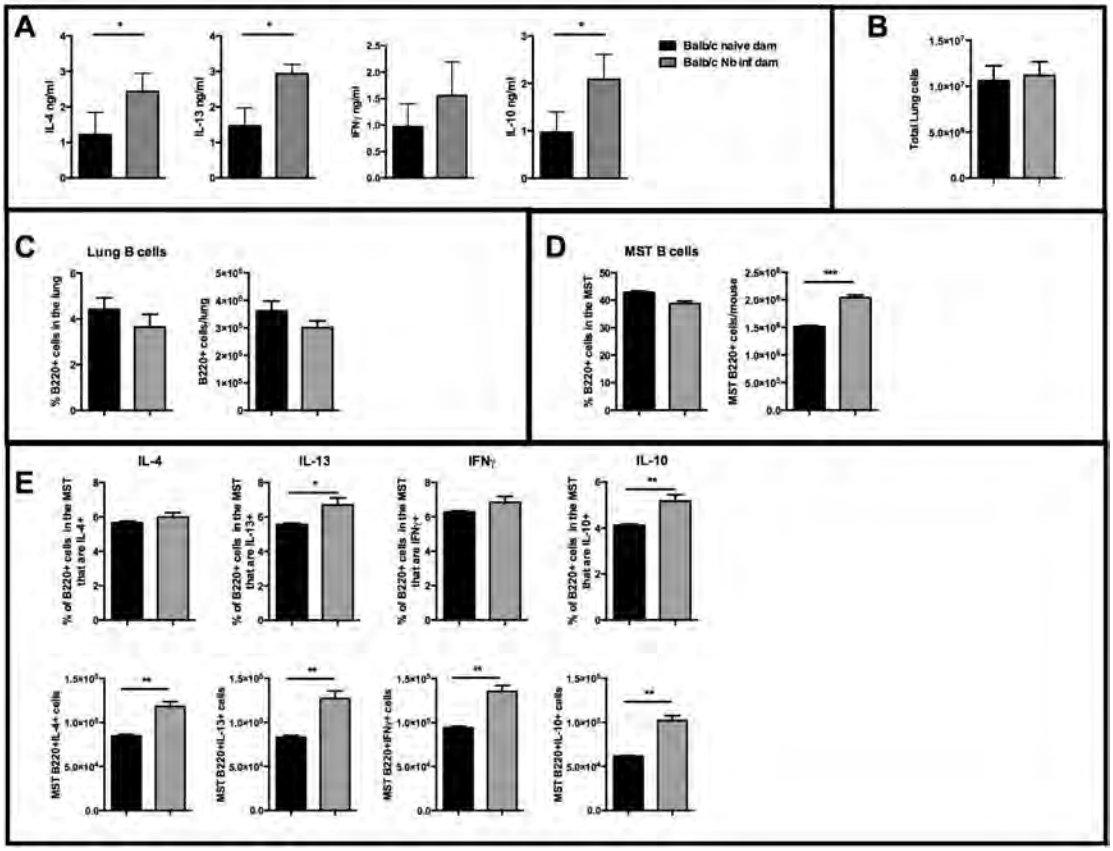



**Figure 1.4: Preconception maternal infection with *Nb* alters lung immune protection against *Nb* in offspring.** Lung IL-4, IL-13, IFN-γ and IL-10 levels found in tissue homogenates at D5p.i. **(A).** Lung cellularity per mouse **(B).** Lung $CD4^{-}B220^{+}$ B cell populations were analysed by FACS *(Methods Figure2A)* **(C).** MST $CD4^{-}B220^{+}$ B cell populations were analysed by FACS **(D).** MST $B220^{+}$ B cell populations that produce IL-4, IL-13, IFN-γ and IL-10 were analysed by FACS *(Methods Figure2B)* **(E).** Data is representative (Mean +/- SD) of 3 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

Offspring born to PCp *Nb* infected mothers showed significantly elevated levels of IL-4, IL-13 and IL-10 in lung tissue homogenates **(Figure 1.4A)**. Lung cellularity was similar between groups **(Figure 4B),** but analysis of immune cell populations both in the lung and the mediastinal lymph nodes (MST) revealed important differences. Previous analysis of B cell populations in the lung draining lymph node has shown MST B cell cytokine production to be associated with control of *Nb* infection [109]. In

this study we found increased numbers of B cells in the MST of PCp *Nb* mice **(Figure 1.4D)**. Pups born to PCp *Nb* mothers had increased numbers and proportions of IL-13 and IL-10 positive MST B cells and while only numbers of IL-4 and IFN-γ positive cells were increased **(Figure 1.4E)**.

Lung associated T cells are also important mediators of immunity to *Nb* infections [203, 219]. Analysis of lung $CD3^+CD4^+$ T cells showed proportions and numbers were increased in pups born to PCp *Nb* infected mice **(Figure 1.5A).** $CD3^+CD4^+CD44^+$ activated T cells showed equivalent proportions between groups, but PCp *Nb* infected mice also showed increased numbers **(Figure 1.5Bi).** Stratification of activated T cells into Teff and TCM T cells showed an increased proportion of TCMs in pups born to naïve mice while Teff proportions and numbers were increased in pups born to PCp *Nb* infected mice **(Figure 1.5Bii and iii)**. As in the spleen expression of the IL-33 receptor (T1/ST2) was equivalent between both naive and PCp *Nb* infected mice although PCp *Nb* mice had more total T1/ST2 expressing cells **(Figure 1.5C)**. Moreover, $CD3^+CD4^+$ T cells in the MST from PCp *Nb* infected mice were increased **(Figure 1.5D)** and produced more IL-4, IL-13, IL-10 and IFNγ in response to PMA/ionomycin stimulation **(Figure 1.5E)**.

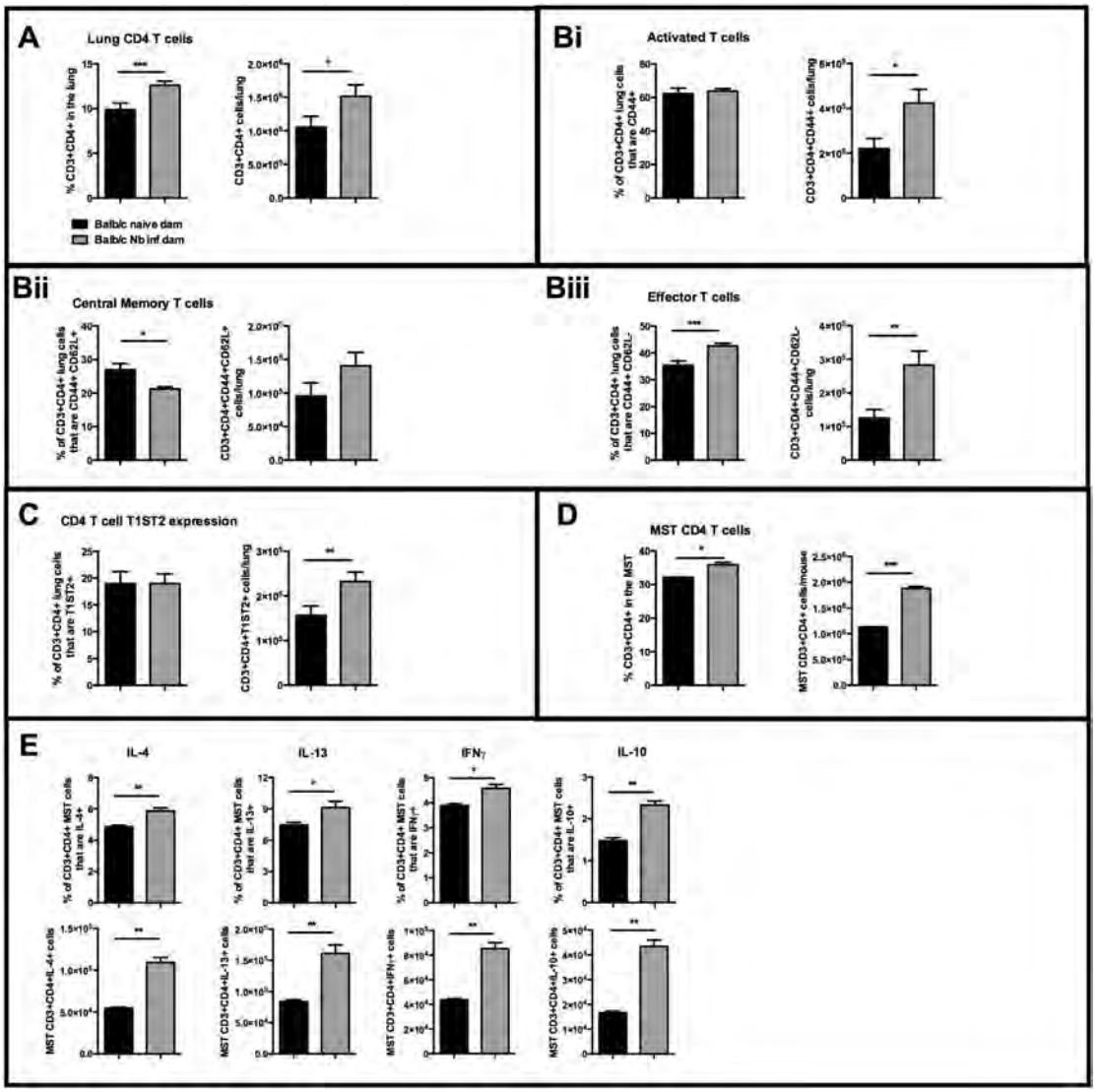



**Figure 1.5: Preconception maternal infection with *Nb* associates with increased Lung and MST T cell populations and activation.** Lung $CD3^+CD4^+$ T cell populations were analysed by FACS at D5p.i. *(Methods Figure2A)* **(A).** Spleen T cells were stratified into activated **(Bi)**, TCM **(Bii)** and Teff **(Biii)** populations**.** T1/ST2 expression on $CD3^+CD4^+$ T cells was analysed by FACS **(C).** MST $CD3^+CD4^+$ T cell populations were analysed by FACS **(D).** MST $CD3^+CD4^+$ T cell populations that produce IL-4, IL-13, IFN-γ and IL-10 were analysed by FACS at D5p.i. *(Methods Figure2B)* **(E).** Data is representative (Mean +/- SD) of 3 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

This analysis shows that PCp *Nb* infection increased offspring lung tissue cytokines and effector T cell populations, as well as IL-13 producing MST B and T cells. These

altered conditions and adaptive cell populations in the lung may well be driven by changes to innate effector lung populations. Therefore next we analysed if a PCp *Nb* infection influenced the phenotype of innate lung cells, specifically ILCs, macrophages and neutrophils.

**Effect of preconception maternal *Nb* infection on innate immunity in offspring**

Lung ILCs are a recently described cell population that is essential for mucosal immunity to *Nb* by initiating and sustaining protective type 2 immunity [22]. Here we show increased ILCs (lineage$^-$ ICOS$^+$) in the lungs of pups born to PCp *Nb* infected mothers compared to naïve mice **(Figure 1.6Ai)** but expression of markers of ILC2 function was not altered in these cells (CD127/IL-7R and T1ST2/IL-33R expression)**(Figure 1.6Aii)**.

Alveolar macrophages are also an essential effector cell in lung Type 2 immunity [182]. We found alveolar macrophages were increased in pups born to PCp *Nb* infected mothers and also had increased expression of the type 2 markers Relmα and YM-1 **(Figure 1.6B)**, indicating a greater commitment to a Type 2 phenotype in pups born to PCp *Nb* infected mothers compared to naïve mice.

Finally we looked at lung neutrophil populations, which have recently been shown to also be able to develop into a Type 1 or Type 2 phenotype [215]. We found total lung neutrophils were increased in pups born to PCp *Nb* infected mothers and also have increased expression of YM-1 **(Figure 1.6C).**

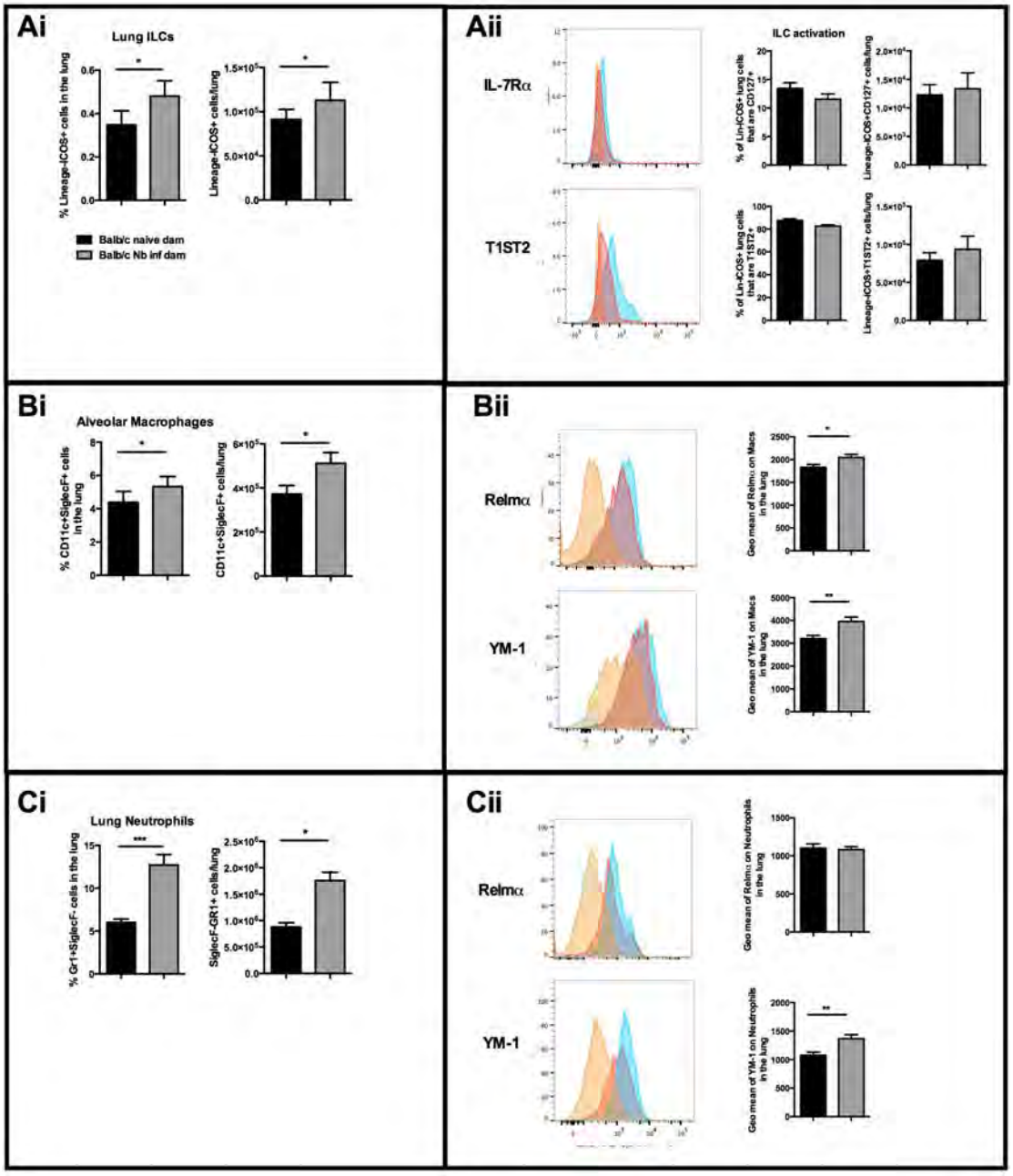



**Figure 1.6: Preconception maternal infection with *Nb* associates with altered Lung innate cell populations and activation.** Lung ILC populations (lineage$^-$ICOS$^+$) were analysed by FACS at D5p.i. *(Methods Figure2E)* **(Ai).** Expression of CD127 (IL-7R) and T1/ST2 (IL-33R) on ILCs was examined **(Aii)** Alveolar Macrophage populations (SiglecF$^+$CD11c$^+$) were quantified *(Methods Figure2C)* **(Bi).** Relmα and YM1 expression on macs was analysed by FACS **(Bii).** Lung neutrophil populations (SiglecF$^-$GR1$^+$)*(Methods Figure2D)* **(Ci).** Relmα and YM1 expression on neutrophils was looked at by FACS **(Cii).** Data is representative (Mean +/- SD) of 2 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

Our analysis shows that there is an increase in both lymphocyte and innate lung and lung-associated effector populations and activation related to maternal PCp *Nb* infection. Thus we have established that protection associated markers of localised immunity in the lung to *Nb* are increased in pups born to PCp *Nb* infected mothers when compared to those born to naïve mothers.

**PCp-maternal-exposure-linked protective immunity to *Nb* in offspring persists until adulthood.**

Maternally derived immunity is generally accepted to be passive and temporary owing to the half-life of the transferred maternal components (eg. antibodies) [225]. This means the transferred immune components from the mother will decline and be removed from the body once breast-feeding is stopped. The half-life of IgG1 in mice is 6-8 days [82]. Therefore if the protection shown in Figure 1.1 is dependent on passive maternal antibody we would expect it to be lost in adult mice. We analysed mice at 7 and 12 weeks old to investigate the persistence of the protection.

Female BALB/c mice were infected with 500 L3 *Nb* and 7 days post infection (p.i.) the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later mice were mated. The offspring were infected when 49 or 86 days old with 500xL3 *Nb* and killed 5 days p.i. **(Figure 1.7A**). Pups born to mothers who had a preconception (PCp) *Nb* infection had reduced intestinal numbers of adult *Nb* at both 7 and 12 weeks old when compared to pups born to un-infected mothers **(Figure 1.7B).** Pups born to PCp *Nb* mothers still had elevated levels of anti-*Nb* IgG1 at 7

weeks old. **(Figure 1.7C)**. PCp *Nb* infection therefore results in persistently increased anti-*Nb* antibody responses that associated with protection against infection indicating that the offspring's anti-*Nb* response has an active component to it and leads to questions about what the immune components transferred from the mother could be.

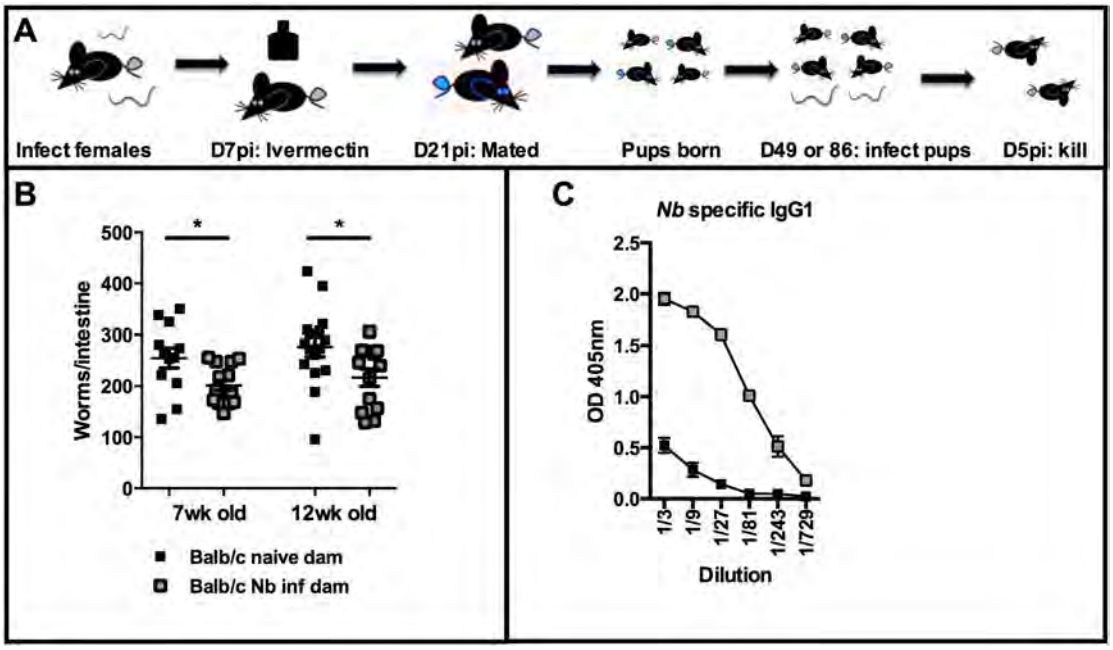



**Figure 1.7: Preconception maternal infection with *Nb* confers persistant immune protection against *Nb* infection to offspring.** Female BALB/c mice were infected with 500 L3 *Nb* and 7 days post infection (p.i.) the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later mice were mated. The offspring were infected when 49 or 86 days old with 500xL3 *Nb* and killed 5 days p.i. **(A).** Intestinal worm burden was analysed at D5p.i. **(B).** Serum antigen specific IgG1 and IgG2a levels at 7 weeks old **(C).** Data is representative (except for worm counts which are pooled)(Mean +/- SD) of 2 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

*Discussion*

So far our study has showed that, in mice, maternal infection with the helminth *N. brasiliensis* impacts on immune development of offspring by enhancing T cell and B cell population development and proliferation. Pups born to *N. brasiliensis* exposed mothers had increased CD4 helper cells in the spleen, lung and MST, as well as higher sub-populations of central memory and effector CD4 cells compared to pups born to naive mothers. Many splenic developing and mature B cell populations are also increased in pups born to previously infected mothers; analysis of the spleen showed an increase in a number of total B cell populations and, notably, MZ cells. Our initial data suggests that these early B and T cells are able to respond to *N. brasiliensis* infection, by increased activation and IL-13 production. There is also increased lung innate cell sensitivity to anti-*N. brasiliensis* immune responses in pups born to previously infected mothers, with alveolar macrophages and neutrophils demonstrating greater type 2 and total cell populations

These changes promote a level of immunity in the offspring to *N. brasiliensis* in pups born to previously infected mothers, associated with anti-*Nb* antibody levels and increased type 2 cytokine responses. The maternal components that contribute to this protection were analysed in subsequent chapters. But it is interesting to note that the maternally derived protection was maintained long after the pups had reached maturity suggesting that the offspring's anti-*Nb* response has an active component to it, which is able to modify the offspring immune system long term

# Chapter 4: Results 2

## Does preconception maternal exposure to *N. brasiliensis* transfer protective immunity to offspring *in-utero* or via breastfeeding?

*Introduction*

Immunity can be transferred from the mother to offspring either *in-utero* via the placenta or through the mother's milk in breastfeeding. The placenta is important in the development of immune tolerance and regulation of fetal immune development [267]. Immune transfer can be mediated by components of the maternal immune system such as cytokines, transfer of antibodies and even some transfer of immune cells such as helper T cells [267].

Placental transfer of maternal IgG antibodies to the fetus is an important mechanism that provides protection to the infant while his/her humoral response is inefficient. IgG is the only antibody class that significantly crosses the human placenta [268]. Transfer of IgG across the placenta is mediated by FcRn expressed on syncytiotrophoblast cells [268]. Maternal infection can lead important changes in the nature of IgG transfer. For example, HIV infection or malaria, lead to decreased placental antibody transfer [225, 226]. Other immunogenic components transferred from the mother to child during pregnancy, OVA antigen for example, can affect defense against various diseases including allergic responses in the offspring [359]. Additional offspring immune support is also derived from breast milk, which contains nutrients, cytokines and antibodies that provide important early life protection while the newborns' immune system develops [260].

During breastfeeding, approximately 0.25–0.5 grams per day of immunoglobulin's, including secretory IgA antibodies, pass to the baby via milk [269]. It is estimated that more than a million babies could be saved globally per year through breastfeeding. Breastfeeding decreases the risk of respiratory tract infections and diarrhea. Other benefits include a lower risk of asthma and allergies [288], celiac disease [290], type 2 diabetes [291], and leukemia [292]. Breastfeeding may also improve cognitive development [293] and decrease the risk of obesity in adulthood [294]. The mother's own immunity against tetanus, diphtheria, whooping cough and influenza can protect the baby from these diseases in the early years of its life [260].

Studies have demonstrated that maternal helminth infection can influence susceptibility to a homologous infection during childhood without previous feto-maternal transmission of the infectious agent itself during pregnancy [359]. Children of mothers exposed to helminth infections may display T cell sensitization to endemic helminth infections [369]. Associations have been made between maternal helminth infection and impaired immune responses to childhood diseases and vaccinations [289]. Whether this is caused by chronic maternal immune responses (cells or cytokines) or transmission of helminth derived antigen/proteins, and furthermore which developmental stage of the offspring's immune system is affected by such factors and what clinical implications these results have regarding vaccination strategies, needs to be investigated.

*Results*

**Pre-conception maternal exposure to *Nb* did not transfer *Nb* protective immunity to offspring *in-utero*.**

Female BALB/c mice were infected with 500 L3 *Nb* and 7 days p.i. the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later mice were mated. 3 days after the offspring were born, we transferred pups born to PCp *Nb* mothers to be breastfed by lactating naïve mothers to examine the importance of *in-utero* transfer of immunity. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i. **(Figure 2.1A).**

Pups born to mothers who had a preconception (PCp) *Nb* infection but breastfed by naïve mothers had similar intestinal burdens of adult *Nb* to pups born to and breastfed by un-infected mothers **(Figure 2.1B).** Pups born to PCp *Nb* mothers had marginally elevated levels of anti-*Nb* IgG1 **(Figure 2.1C)**. In addition, offspring breastfed by PCp *Nb* infected mothers had similar sized spleens to pups breastfed by to un-infected mothers **(Figure 2.1D)**.

As in the first results chapter we examined responses related to the immune cells most likely to drive them, CD4 T cells and B cells. Splenic $B220^{+}CD19^{+}$ B cell populations showed similar proportions and numbers in pups born to PCp *Nb* infected mice compared to those born to naïve mice **(Figure 2.2A)**. Further analysis showed no differences in the proportions of FO, MZ or NF B cells between naïve or PCp *Nb* mice **(Figure 2.2B)**. MHCII expression was also equivalent between groups **(Figure 2.2C)**. This indicates that maternal-*Nb*-linked transfer of immunity *in-utero*

may not induce B cell populations in the spleen that have an enhanced ability to control an *Nb* infection

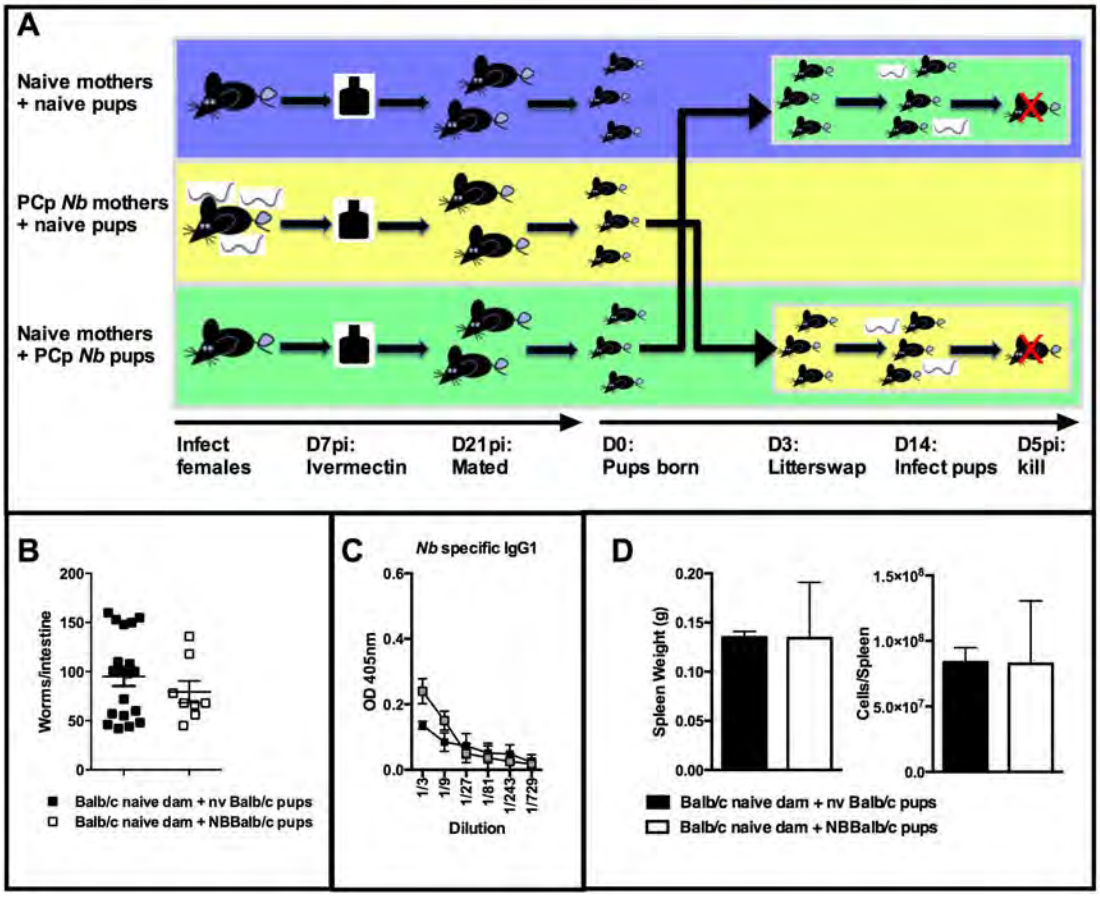



**Figure 2.1: Preconception maternal infection with *Nb* does not confer immune protection against *Nb* infection to offspring *in-utero*.** Female BALB/c mice were infected with 500 L3 *Nb* and 7 days post infection (p.i.) the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later mice were mated. 3 days after the offspring were born, we transferred some pups born to PCp *Nb* mothers to be breastfed by lactating naïve mothers. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i. **(A).** Intestinal worm burden was analysed at D5p.i. **(B).** Serum antigen specific IgG1 levels at D5p.i. **(C).** Spleen weight and cellularity per mouse **(D).** Data is representative (except for worm counts which are pooled)(Mean +/- SD) of 2 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

Analysis of splenic $CD3^+CD4^+$ T cell populations also showed equivalent proportions and numbers in mice born to both naive and PCp *Nb* infected mice **(Figure 2.3A).** $CD3^+CD4^+CD44^+$ activated T cells, Teff and TCM demonstrated equivalent proportions and numbers of these sub-populations between both naive and PCp *Nb* infected mice **(Figure 2.3B)**. Additionally expression of the IL-33 receptor (T1ST2) was equivalent between both naive and PCp *Nb* infected mice **(Figure 2.3C)**. This analysis shows that PCp *Nb* infection does not alter memory or effector phenotype activation of splenic CD4 T cells in the spleen *in-utero*.

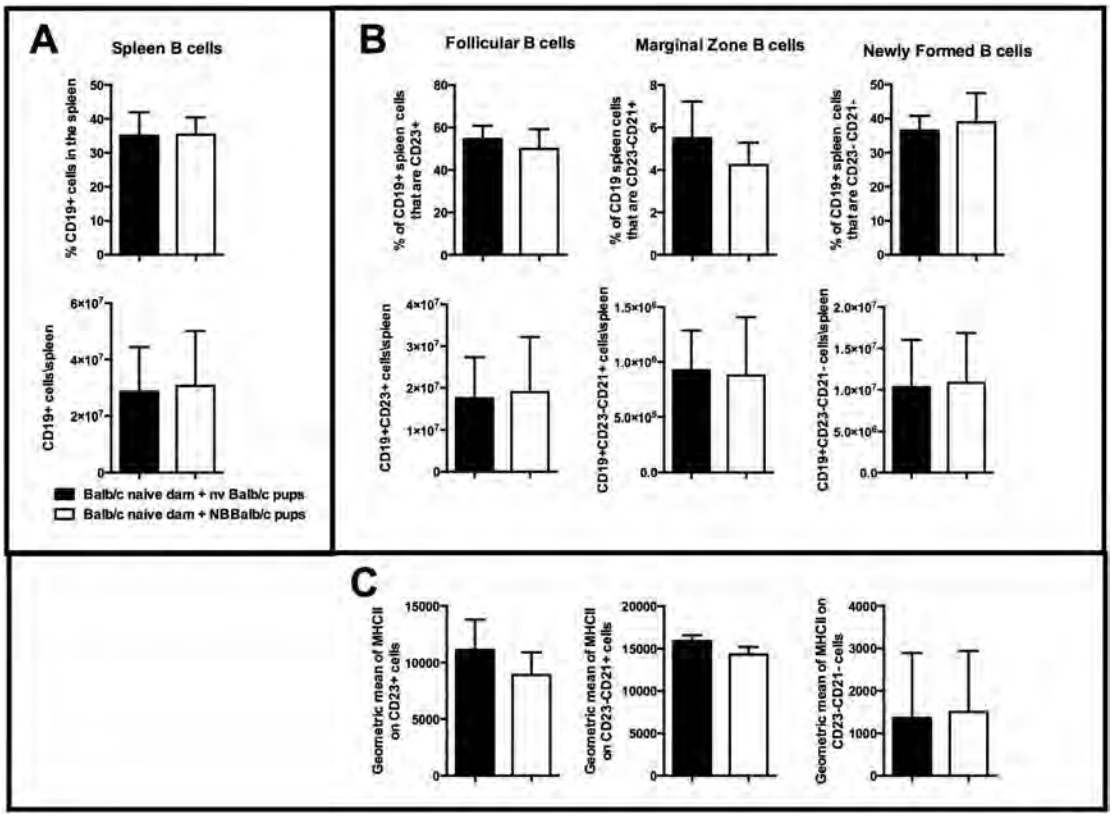



**Figure 2.2: Preconception maternal infection with *Nb* does not alter splenic B cell populations or activation *in-utero*.** Spleen $CD19^+B220^+$ B cell populations were analysed by FACS at D5p.i. *(Methods Figure2A)* **(A).** Spleen B cells were stratified into FO ($B220^+CD19^+CD23^+$), MZ ($B220^+CD19^+CD23^-CD21^+$) and NF ($B220^+CD19^+CD23^-CD21^-$) populations **(B).** MHCII expression on FO, MZ and NF cells was analysed by FACS **(C).**

Data is representative (Mean +/- SD) of 2 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

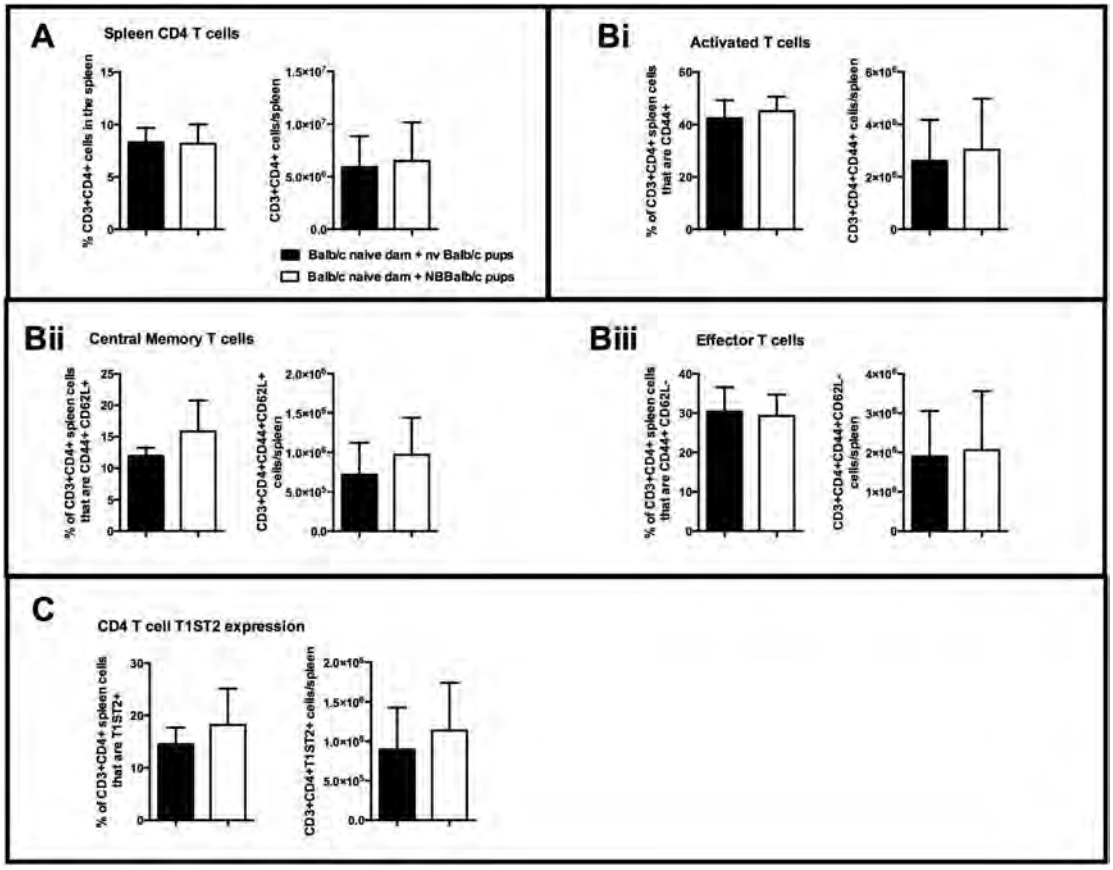



**Figure 2.3: Preconception maternal infection with *Nb* does not alter spleen T cell populations or activation *in-utero*.** Spleen $CD3^+CD4^+$ T cell populations were analysed by FACS at D5p.i. *(Methods Figure2A)* **(A).** Spleen T cells were stratified into activated ($CD3^+CD4^+CD44^+$)**(Bii)**, TCM ($CD3^+CD4^+CD44^+CD62L^{hi}$)**(Biv)** and Teff ($CD3^+CD4^+CD44^+CD62L_{lo}$)**(Bv)** populations**.** T1/ST2 expression on $CD3^+CD4^+$ T cells was analysed by FACS **(C).** Data is representative (Mean +/- SD) of 2 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

Lung $CD3^+CD4^+$ T cell proportions and numbers were increased in pups born to PCp *Nb* infected mice **(Figure 2.4B).** $CD3^+CD4^+CD44^+$ activated T cells showed equivalent proportions between groups but had increased numbers in pups born to PCp *Nb* infected mice **(Figure 2.4Ci)** and stratification of activated T cells into Teff

and TCM T cells showed an increased proportion of TCMs in pups born to and breastfed by naïve mice while Teff proportions were increased in pups born to PCp *Nb* mice and fed by naïve mothers **(Figure 2.4Cii and Ciii)**. Expression of the IL-33 receptor (T1/ST2) was equivalent between both naive and PCp *Nb* infected mice **(Figure 2.4D)**. Alveolar macrophage proportions and numbers were increased in pups born to PCp *Nb* infected mothers compared to those born to naïve mice **(Figure 2.4E)**.

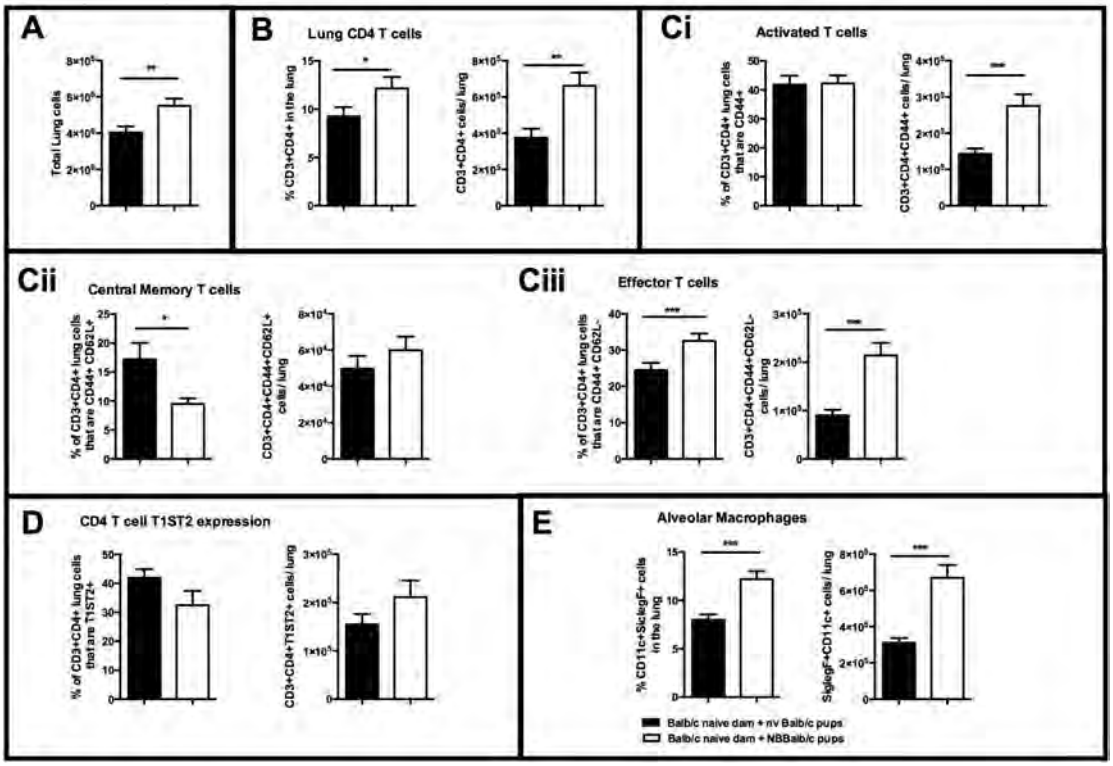



**Figure 2.4: Preconception maternal infection with *Nb* affects Lung T cell populations and activation *in-utero*.** Lung $CD3^+CD4^+$ T cell populations were analysed by FACS at D5p.i. *(Methods Figure2A)* **(B).** Spleen T cells were stratified into activated **(Ci)**, TCM **(Cii)** and Teff **(Ciii)** populations**.** T1/ST2 expression on $CD3^+CD4^+$ T cells was analysed by FACS **(D). A**lveolar macrophages ($SiglecF^+CD11c^+$) were analysed by FACS **(E).** Data is representative (Mean +/- SD) of 2 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

This data shows that pups that were born to PCp *Nb* mothers but were breastfed by naïve mothers had similar spleen structure to pups born to and breastfed by naïve mothers. PCp *Nb* mice did however have increased lung and alveolar macrophage populations but this did not appear to contribute to enhanced parasite clearance. Taken together this demonstrates that a PCp *Nb* infection does not confer protective immunity to offspring *in-utero*. We excluded the pups born to PCp *Nb* mice but breastfed by naïve mothers from further analysis because they do not shown protection despite elevation of certain lung immune responses.

**Pre-conception maternal exposure to *Nb* transfers *Nb* protective immunity to offspring via breastfeeding.**

Female BALB/c mice were infected with 500 L3 *Nb* and 7 days post infection (p.i.) the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later mice were mated. 3 days after the offspring were born, pups born to naïve mothers were transferred to lactating PCp *Nb* mothers to examine the role of breastfeeding in transfer of immunity. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i. **(Figure 2.5A).**

Pups breastfed by mothers who had a preconception (PCp) *Nb* infection had reduced intestinal numbers of adult *Nb* when compared to pups breastfed by un-infected mothers **(Figure 2.5B).** As seen in chapter 1, pups breastfed by PCp *Nb* mothers had elevated levels of anti-*Nb* IgG1 **(Figure 2.5C)**. Offspring born to PCp *Nb* infected mothers showed significantly elevated levels of IL-13 as demonstrated by anti-CD3 re-stimulation of total splenocytes **(Figure 2.5D)**. PCp *Nb* infection

therefore mediates increased anti-*Nb* antibody and cytokine responses via breastfeeding that associated with protection against infection. But offspring breastfed by PCp *Nb* infected mothers had similar sized spleens to pups breastfed by to un-infected mothers **(Figure 2.5E)**.

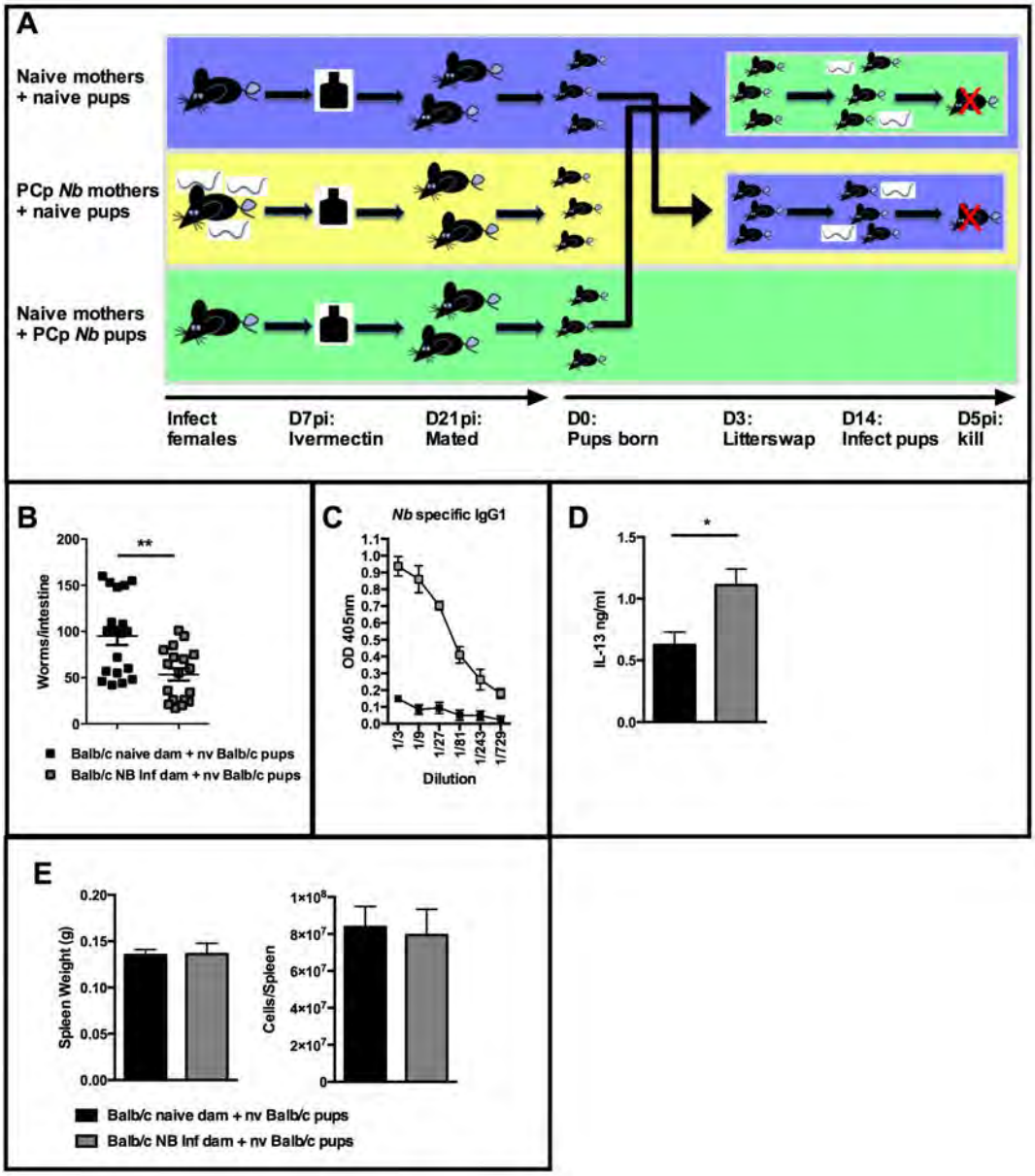



**Figure 2.5: Preconception maternal infection with *Nb* confers immune protection against *Nb* infection to offspring via breastfeeding.** Female BALB/c mice were infected with 500 L3 *Nb* and 7 days post infection (p.i.) the infection was cleared by a 5 day oral

treatment with ivermectin; 14 days later mice were mated. 3 days after the offspring were born, we transferred some pups born to naïve mothers to be breastfed by lactating PCp *Nb* mothers. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i. **(A).** Intestinal worm burden was analysed at D5p.i. **(B).** Serum antigen specific IgG1 levels at D5p.i. **(C).** IL-13 secretion by total splenocytes restimulated with αCD3 **(D).** Spleen weight and cellularity per mouse **(E).** Data is representative (except for worm counts which are pooled)(Mean +/- SD) of 3 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

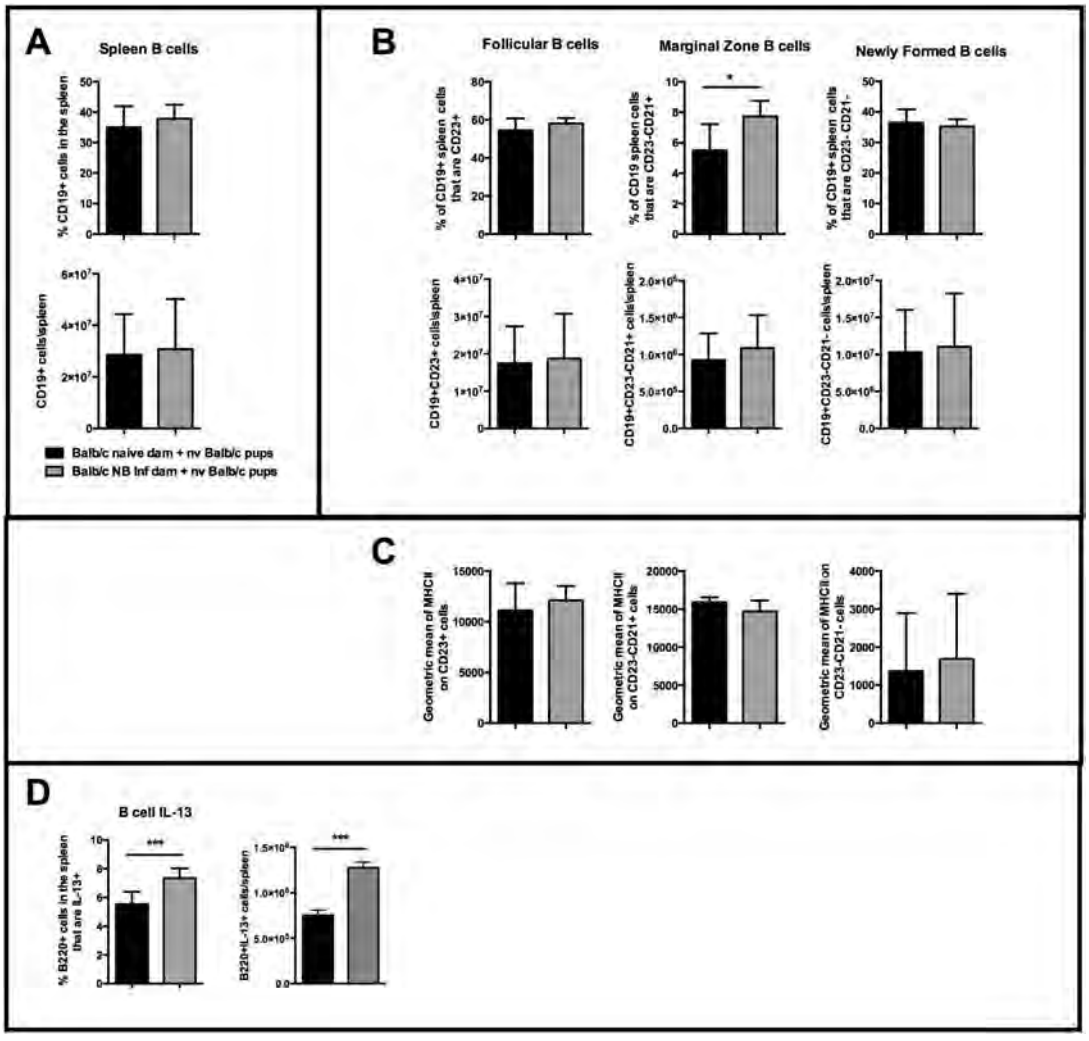



**Figure 2.6: Preconception maternal infection with *Nb* alters splenic B cell populations and activation by breastfeeding.** Spleen $CD19^{+}B220^{+}$ B cell populations were analysed by FACS at D5p.i. *(Methods Figure2A)* **(A).** B cells were stratified into FO, MZ and NF populations **(B).** MHCII expression on FO, MZ and NF cells was analysed by FACS **(C).** $CD19^{+}B220^{+}$ B cell populations that produce IL-13 were analysed by FACS at D5p.i. **(D).**

Data is representative (Mean +/- SD) of 3 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

Splenic $CD19^+$ B cell populations showed similar proportions and numbers in pups breastfed by PCp *Nb* infected mice compared to those fed by naïve mice **(Figure 2.6A)**. Further analysis showed no difference in the proportions of follicular or newly formed B cells between naïve or PCp *Nb* mice, but pups breastfed by PCp *Nb* infected mice did have increased proportions of marginal zone B cells as seen in chapter 1, compared to naïve pups fed by naïve mothers **(Figure 2.6B)**. MHCII expression was not altered **(Figure 2.6C)**. Splenic $B220^+$ B cell populations showed higher IL-13 production in PCp *Nb* infected mice in response to PMA/ionomycin stimulation **(Figure 2.6D)**. This shows that maternal-*Nb*-linked transfer of immunity via breastfeeding can alter spleen B cell populations when compared to pups breastfed by naïve mothers by increasing MZ cell populations and IL-13 production.

Furthermore splenic $CD3^+CD4^+$ T cell populations showed equivalent proportions and numbers in mice breastfed by either naïve or PCp *Nb* infected mice **(Figure 2.7A).** $CD3^+CD4^+CD44^+$ activated T cells, Teff and TCM also showed equivalent proportions and numbers of these sub-populations between both naive and PCp *Nb* infected groups **(Figure 2.7B)**. Additionally expression of the IL-33 receptor (T1/ST2) was also equivalent between groups **(Figure 2.7C)**. Splenic $CD3^+CD4^+$ T cells from PCp *Nb* infected mice had equivalent proportions of IL-13 producing cells in response to PMA/ionomycin stimulation compared to naïve mice but had increased numbers overall **(Figure 2.7D).** This analysis shows that PCp *Nb* infection does not alter certain phenotypes of CD4 T cells in the spleen via breastfeeding but activation

can cause higher IL-13 production when compared to pups breastfed by naïve mothers.

These data demonstrate that a PCp *Nb* infection confers a significant level of protective immunity to offspring via breastfeeding when compared to offspring breastfed by uninfected mothers. Because pups that were born to PCp *Nb* mothers but were breastfed by naïve mothers were not protected, the data suggests that breastfeeding is essential to this protection. Protection was associated with higher levels of antigen-specific antibody and protective cytokines and increased proportions of marginal zone cells.

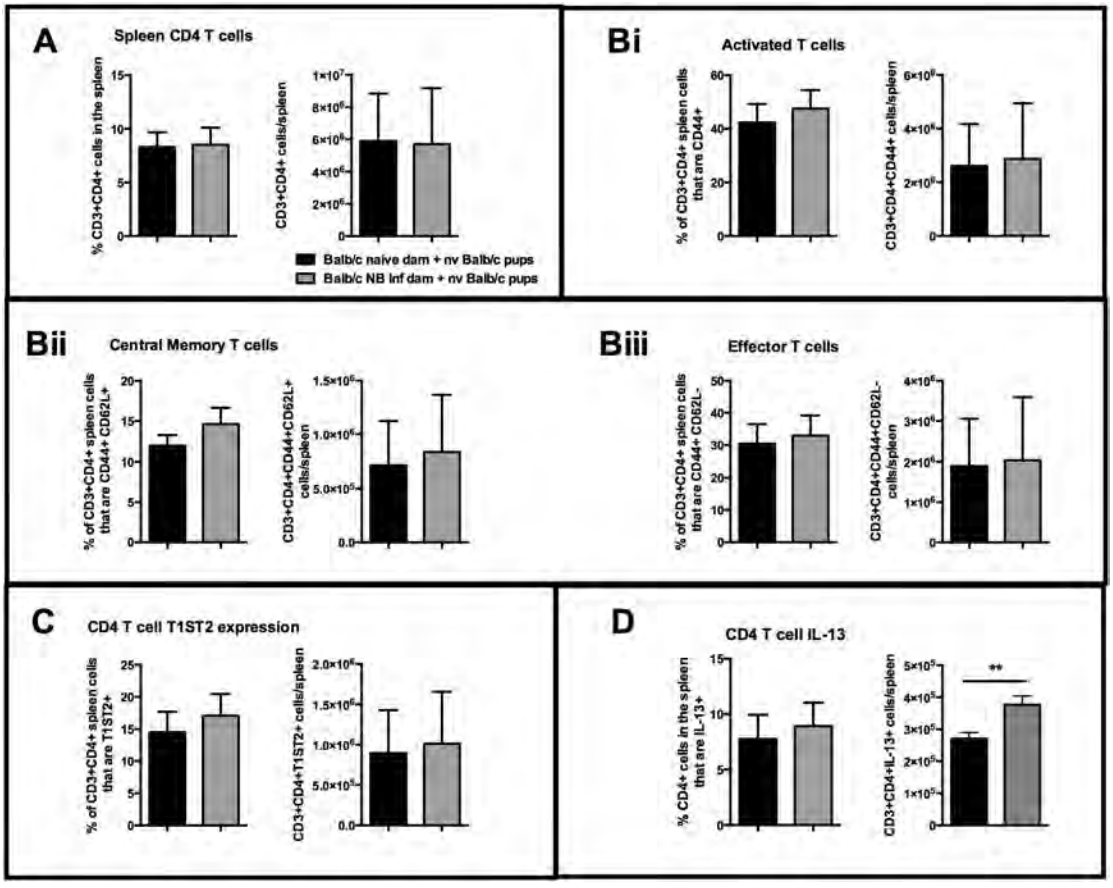


**Figure 2.7: Preconception maternal infection with *Nb* does not change most splenic T cell populations through breastfeeding.** Spleen $CD3^+CD4^+$ T cell populations were analysed by FACS at D5p.i. *(Methods Figure2A)* **(A).** Spleen T cells were stratified into activated **(Bi)**, TCM **(Bii)** and Teff **(Biii)** populations**.** T1/ST2 expression on $CD3^+CD4^+$ T cells was analysed by FACS **(C).** Spleen $CD3^+CD4^+$ T cell populations that produce IL-13 were analysed by FACS at D5p.i. **(D).** Data is representative (Mean +/- SD) of 3 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

## Increased lung immune responses in offspring associate with PCp-maternal-exposure-linked protective immunity to *Nb*.

We have established that certain protection-associated markers of systemic immunity to *Nb* are increased in pups breastfed by PCp *Nb* infected mothers when compared to those fed by naïve mothers. As previously we again looked in the lung for altered populations of localized effector cells.

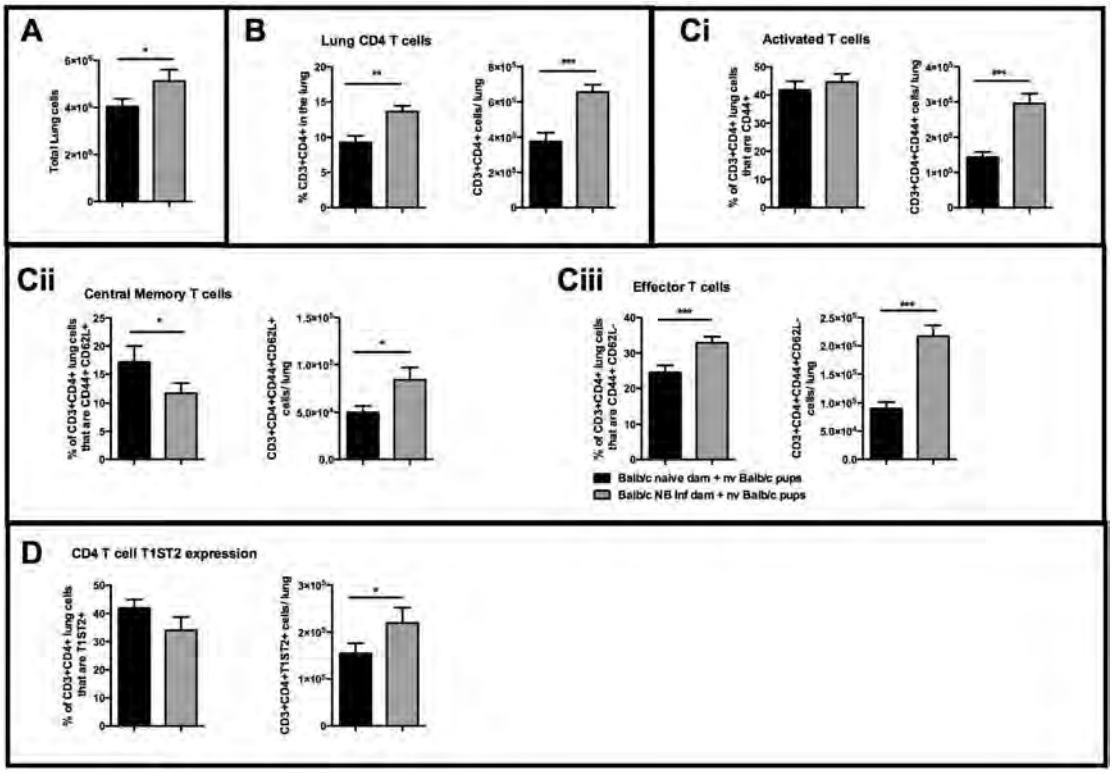


**Figure 2.8: Preconception maternal infection with *Nb* associates with increased lung T cell populations and activation through breastfeeding.** Lung $CD3^+CD4^+$ T cell populations were analysed by FACS at D5p.i. *(Methods Figure2A)* **(B).** Lung T cells were stratified into activated **(Ci)**, TCM **(Cii)** and Teff **(Ciii)** populations**.** T1/ST2 expression on $CD3^+CD4^+$ T cells was analysed by FACS **(D).** Data is representative (Mean +/- SD) of 3 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

Lung $CD3^+CD4^+$ T cell proportions and numbers were increased in pups' breastfed by PCp *Nb* infected mice **(Figure 2.8B).** $CD3^+CD4^+CD44^+$ activation showed equivalent T cell proportions between groups but had increased numbers in pups fed by PCp *Nb* infected mice **(Figure 2.8Ci)** and stratification of activated T cells into Teff and TCM T cells showed an increased proportion of TCMs in pups born to and breastfed by naïve mice while TCM cell numbers and Teff proportions and numbers were increased in pups fed by PCp *Nb* mothers **(Figure 2.8Cii and Ciii)**. Expression of the IL-33 receptor (T1/ST2) was equivalent between both naive and PCp *Nb* infected mice but there were increased numbers in pups fed by PCp *Nb* infected mice **(Figure 2.8D)**.

Offspring breast fed by PCp *Nb* infected mothers showed significantly elevated levels of IL-13 as demonstrated by ELISA on lung tissue homogenates **(Figure 2.9A).** Populations of $CD3^+CD4^+$ T cells in the MST from PCp *Nb* infected mice were expanded **(Figure 2.9Bi)** and more likely to produce IL-13 in response to PMA/ionomycin re-stimulation **(Figure 2.9Bii)**. Moreover MST $CD19^+$ B cells were decreased in the MST of pups fed by PCp *Nb* infected mice compared to those fed by naïve mice **(Figure 2.9Ci)** but produce elevated levels of IL-13 **(Figure 2.9Cii)**.

This analysis shows that PCp *Nb* infection increased offspring lung tissue cytokines and effector T cell populations, as well as IL-13 producing MST B and T cells.

Finally we analysed the innate cell populations of the lung. We found equivalent proportions but increased numbers of ILCs (lineage- $ICOS^{+}$) in the lungs of pups' breastfed by PCp *Nb* infected mothers compared to those fed by naïve mice **(Figure 2.10Ai)**; ILCs expressed equivalent relative levels of type 2 activation markers (CD127/IL-7R and T1ST2/IL-33R expression) but total numbers of ILCs expressing these markers were increased in offspring of PCp *Nb* infected mothers **(Figure 2.10Aii)**. Alveolar macrophages were also increased in PCp *Nb* infected mice and expressed higher levels of Relmα and YM-1 **(Figure 2.10B)**, indicating a greater commitment to an M2 phenotype in pups breastfed by PCp *Nb* infected mothers compared to naïve mice. Furthermore we found total lung neutrophils were increased in pups breastfed by PCp *Nb* infected mothers although with decreased relative expression of YM-1 **(Figure 2.10C)**.

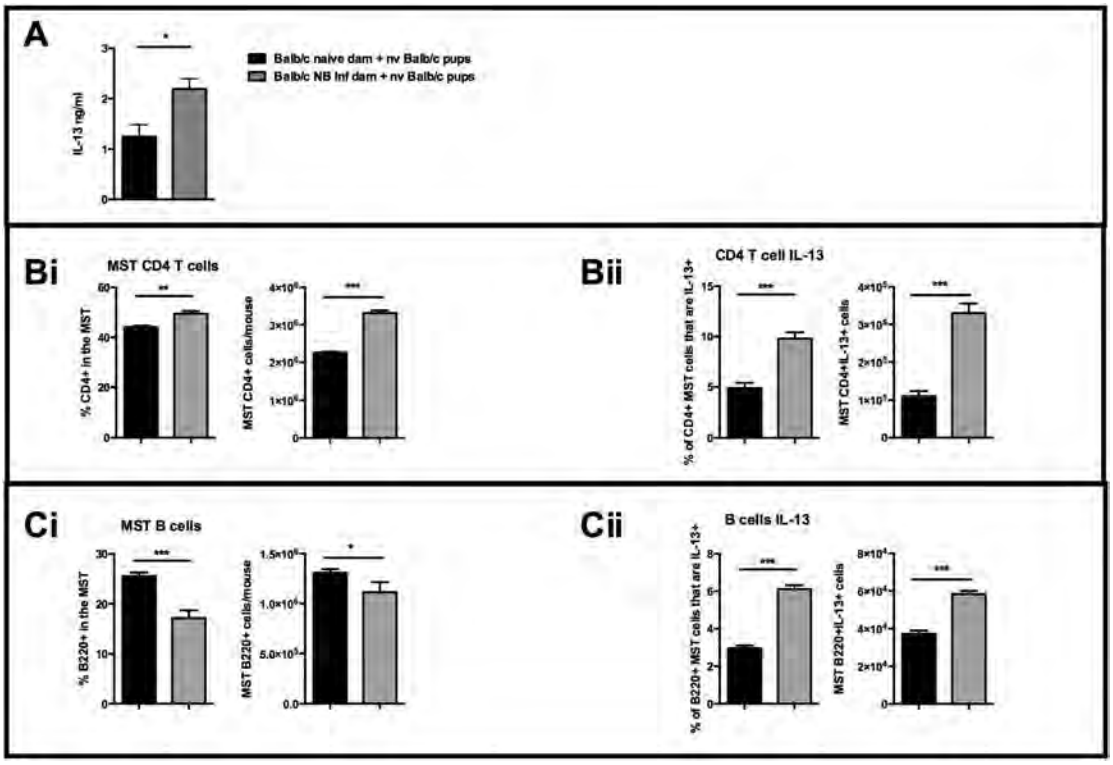



**Figure 2.9: Preconception maternal infection with *Nb* associates with increased lung and MST IL-13 production through breastfeeding.** Lung IL-13 levels found in tissue homogenates at D5p.i **(A).** MST $B220^{-}CD4^{+}$ T cell populations were analysed by FACS *(Methods Figure2A)* **(Bi).** MST $B220^{-}CD4^{+}$ T cell populations that produce IL-13 were analysed by FACS at D5p.i. **(Bii).** MST $CD4^{-}B220^{+}$ B cell populations were analysed by FACS **(Ci).** MST $B220^{+}$ B cell populations that produce IL-13 in response to PMA/iono were analysed by FACS **(Cii).** Data is representative (Mean +/- SD) of 2 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

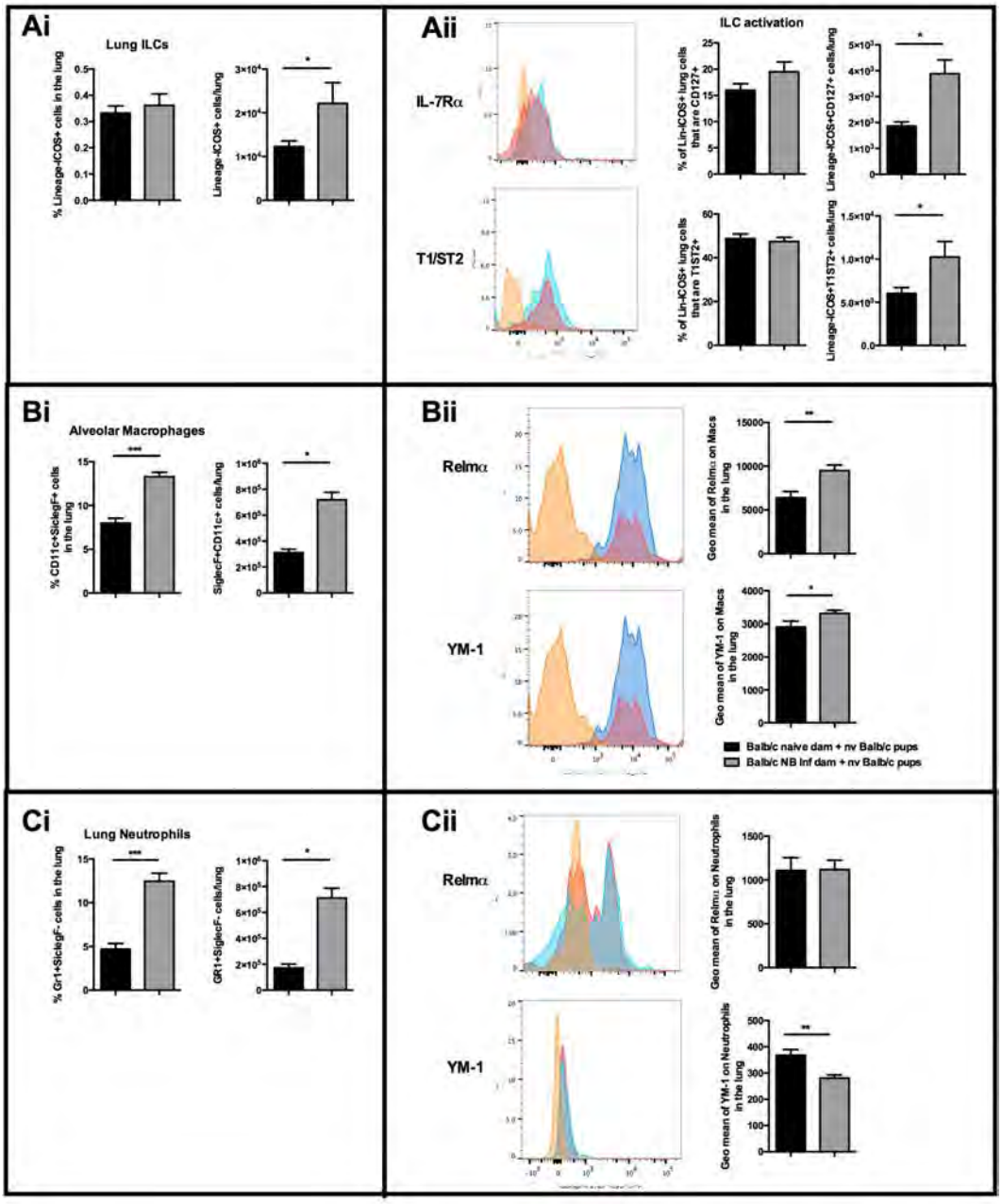



**Figure 2.10: Preconception maternal infection with *Nb* associates with altered lung innate cell populations and activation through breastfeeding.** Lung ILC populations (lineage$^-$ICOS$^+$) were analysed by FACS at D5p.i. *(Methods Figure2E)* **(Ai).** Expression of CD127 (IL-7R) and T1/ST2 (IL-33R) on ILCs was examined **(Aii)** Alveolar Macrophage populations (SiglecF$^+$CD11c$^+$) were quantified *(Methods Figure2C)* **(Bi).** Relmα and YM1 expression on macs was looked at by FACS **(Bii).** Lung neutrophil populations (SiglecF$^-$ GR1$^+$) were analysed *(Methods Figure2D)* **(Ci).** Relmα and YM1 expression on neutrophils was looked at by FACS **(Cii).** Data is representative (Mean +/- SD) of 2 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

Our analysis shows that effective maternally derived protection against *Nb* can be transferred by breastfeeding from mothers exposed to PCp *Nb* infection. This is associated with increased lung adaptive and innate effector cell populations and heightened activation of spleen MZ populations, protective TH2 cytokines and antibodies. *In-utero* transfer of immunity also resulted in increased lung effector populations but did not associate with protection in pups born to PCp *Nb* mice but breastfed by naïve mothers.

*Discussion*

Here we have shown that, in mice, PCp *Nb* maternal infection impacts on immune development of select populations both *in-utero* and via breastfeeding. However while maternally-derived protection to *Nb* was associated with breastfeeding, pups born to PCp *Nb* dams but fed by naïve foster mothers did not show protection to *Nb.* Therefore the data suggests that breastfeeding is essential to this protection. And as seen in results chapter 1, protection was associated with higher levels of antigen-specific antibody and protective cytokines and increased proportions of marginal zone cells.

Pups only exposed to PCp *Nb* maternal infection *in-utero* demonstrated similarly enhanced lung immune cell development compared to pups only exposed to PCp *Nb* maternal infection during breastfeeding, when both are compared to unexposed pups. Both exposed groups had higher lung T cell and alveolar macrophage populations. Very few spleen populations exhibited alteration by exposure either *in-utero* or by nursing independent of one another.

# Chapter 5: Results 3

## Can maternally transferred protective immunity to offspring override immune-deficient related disease susceptibility?

*Introduction:*

IL-4Rα is an essential component of the cell surface heterodimeric receptors required for IL-4 and IL-13 signalling which drives host immune polarisation to type 2/TH2 [188]. Use of IL-4Rα$^{-/-}$ mice has clearly demonstrated an absolute requirement for IL-4Rα expression in resolving nematode infections [194]. Studies by others have shown that mothers can transfer immune components via breast feeding that can override genetic immune-deficiency [383]. Letterio et al. show that maternal sources of transforming growth factor-beta 1 (TGF-β 1) can contribute to the normal appearance and survival of TGF-β 1 deficient newborn mice [383].

In this study we examined if maternal transfer of immunity could rescue genetic susceptibility to an *N. brasiliensis* infection. Specifically we investigated whether the effects of the strong type 2 maternal response to a primary *N. brasiliensis* infection on immune cells and cytokine profiles, and the development of immune memory in the mother was transferred to offspring and whether this would be sufficient to protect IL-4Rα$^{-/-}$ offspring. We hypothesized that any altered immune components in offspring are acquired from the mother *in-utero* and via breastmilk.

*Results:*

**Pre-conception maternal exposure to *Nb* is sufficient to protect IL-4Rα deficient pups via breastfeeding.**

Female BALB/c mice were infected with 500 L3 *Nb* and 7 days post infection (p.i.) the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later these PCp BALB/c, naïve BALB/c or naive IL-4Rα$^{-/-}$ mice were mated with appropriate males. 3 days after the offspring were born, the pups born to naïve IL-4Rα$^{-/-}$ mothers were transferred to naïve or PCp *Nb* BALB/c lactating mothers. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i. **(Figure 3.1A).**

Pups breastfed by mothers who had a preconception (PCp) *Nb* infection showed significantly reduced intestinal numbers of adult *Nb* when compared to pups breastfed by un-infected mothers **(Figure 3.1B).** Moreover protected pups breastfed by PCp *Nb* mothers had elevated levels of IL-4 and IL-13 but reduced IL-10 in response to CD3 stimulation of total splenocytes despite a IL-4Rα$^{-/-}$ deficiency **(Figure 3.1C).** As seen in previous results chapters, the protected group also had elevated anti-*Nb* IgG1 **(Figure 3.1D)**. PCp *Nb* infection therefore mediates increased anti-*Nb* antibody and cytokine responses via breastfeeding despite the offspring being immune-deficient and susceptible to helminth infection. Offspring breastfed by PCp *Nb* infected mothers had similar sized spleens to pups breastfed by un-infected mothers **(Figure 3.1E)**.

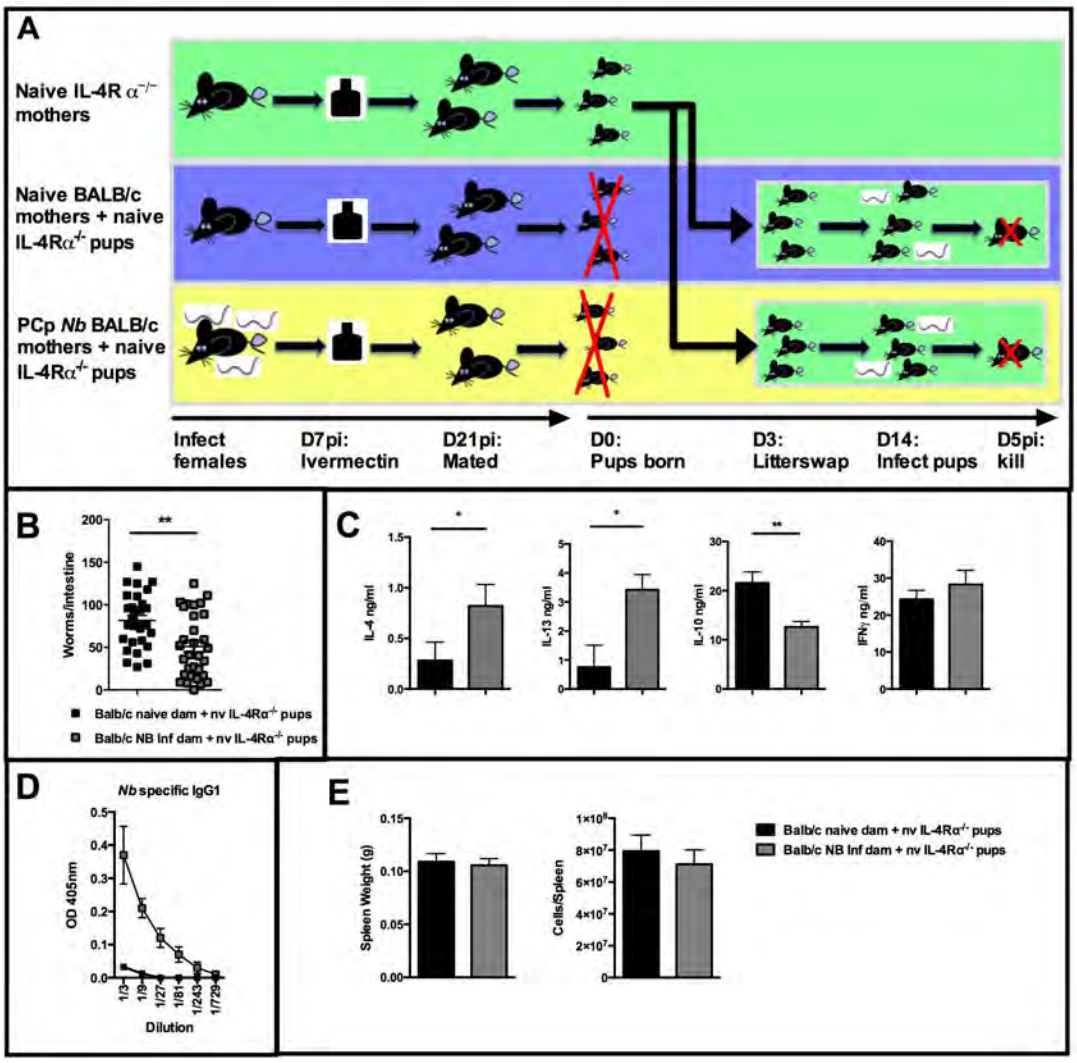



**Figure 3.1: Preconception maternal infection with *Nb* confers immune protection against *Nb* infection to IL-4Rα$^{-/-}$ offspring via breastfeeding.** Female BALB/c mice were infected with 500 L3 *Nb* and 7 days post infection (p.i.) the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later mice were mated. 3 days after the offspring were born, we transferred some pups born to naïve IL-4Rα$^{-/-}$ mothers to be breastfed by lactating PCp *Nb* BALB/c mothers. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i. **(A).** Intestinal worm burden was analysed at D5p.i. **(B).** IL-4, IL-13, IL-10 and IFNγ secretion by total splenocytes restimulated with αCD3 **(C).** Serum antigen specific IgG1 levels at D5p.i. **(D).** Spleen weight and cellularity per mouse **(E).** Data is representative (except for worm counts which are pooled)(Mean +/- SD) of 3 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

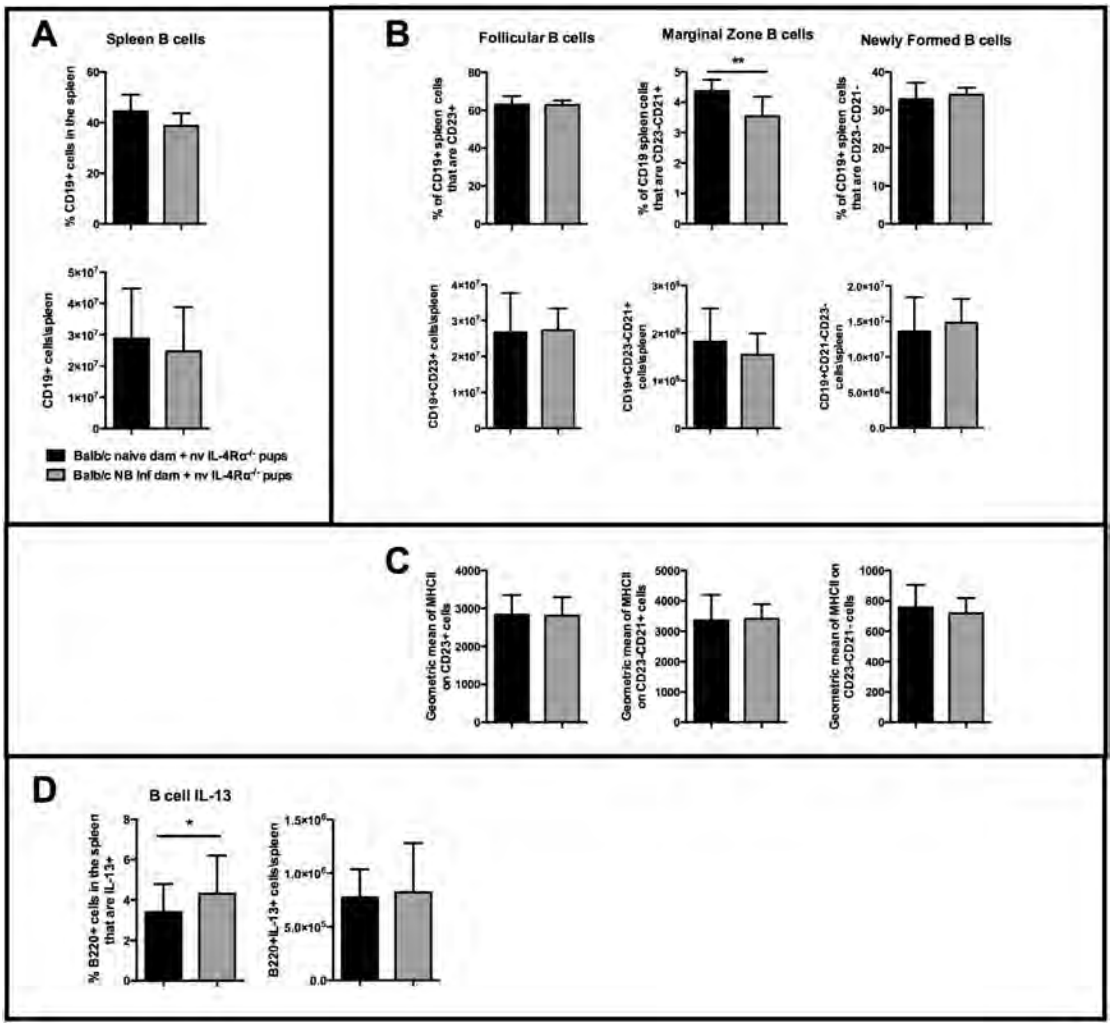



**Figure 3.2: Preconception maternal infection with *Nb* does not alter most splenic B cell populations and activation by breastfeeding.** $CD19^{+}B220^{+}$ B cell populations were analysed by FACS at D5p.i. *(Methods Figure2A)* **(A)**. B cells were stratified into FO, MZ and NF populations **(B)**. MHCII expression on FO, MZ and NF cells was analysed by FACS **(C)**. CD19+B220+ B cell populations that produce IL-13 were analysed by FACS at D5p.i. **(D)**. Data is representative (Mean +/- SD) of 3 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

As in the previous two results chapters we tested how these responses related to the immune cell populations that can confer protection against *Nb*. Splenic $CD19^{+}$ B cell populations showed similar proportions and numbers in pups breastfed by PCp *Nb* infected mice compared to those fed by naïve mice **(Figure 3.2A)**. Further analysis

showed no difference in the proportions of follicular or newly formed B cells between naïve or PCp *Nb* mice, but pups breastfed by PCp *Nb* infected mice did have reduced proportions of marginal zone B cells compared to naïve pups fed by naïve mothers in contrast to previous findings in *Figures 1.2* and *2.6* **(Figure 3.2B)**. MHCII expression was not altered **(Figure 3.2C)**. Splenic $B220^+$ B cells from PCp *Nb* infected mice had slightly elevated proportions of IL-13 producing cells in response to PMA/ionomycin stimulation compared to naïve mice but overall numbers were the same **(Figure 3.2D)**. This shows that maternal-*Nb*-linked transfer of immunity via breastfeeding or *in-utero* does not alter B cell populations considerably in the spleen when compared to pups breastfed by naïve mothers.

Furthermore splenic $CD3^+CD4^+$ T cell populations showed equivalent proportions and numbers in mice breastfed by either naïve or PCp *Nb* infected dams **(Figure 3.3A).** $CD3^+CD4^+CD44^+$ activated T cells showed equivalent numbers between groups but had increased proportions in pups born to PCp *Nb* infected mice **(Figure 3.3Bi)** and stratification of activated T cells into Teff and TCM T cells showed an increased proportion of TCMs in pups born to and breastfed by naïve mice while Teff proportions were increased in pups fed by PCp *Nb* but total numbers were not significantly altered **(Figure 3.3Bii & iii)**. Additionally expression of the IL-33 receptor (T1/ST2) was increased in pups fed by PCp *Nb* mice but numbers were similar in both naive and PCp *Nb* infected mice **(Figure 3.3C)**. Splenic $CD3^+CD4^+$ T cells from PCp *Nb* infected mice had equivalent numbers and proportions of IL-13 producing cells **(Figure 3.3D)**. This analysis shows that PCp *Nb* infection does

marginally alter CD4 T cell memory and effector phenotype activation in the spleen via breastfeeding when compared to IL-4Rα$^{-/-}$ pups breastfed by naïve mothers.

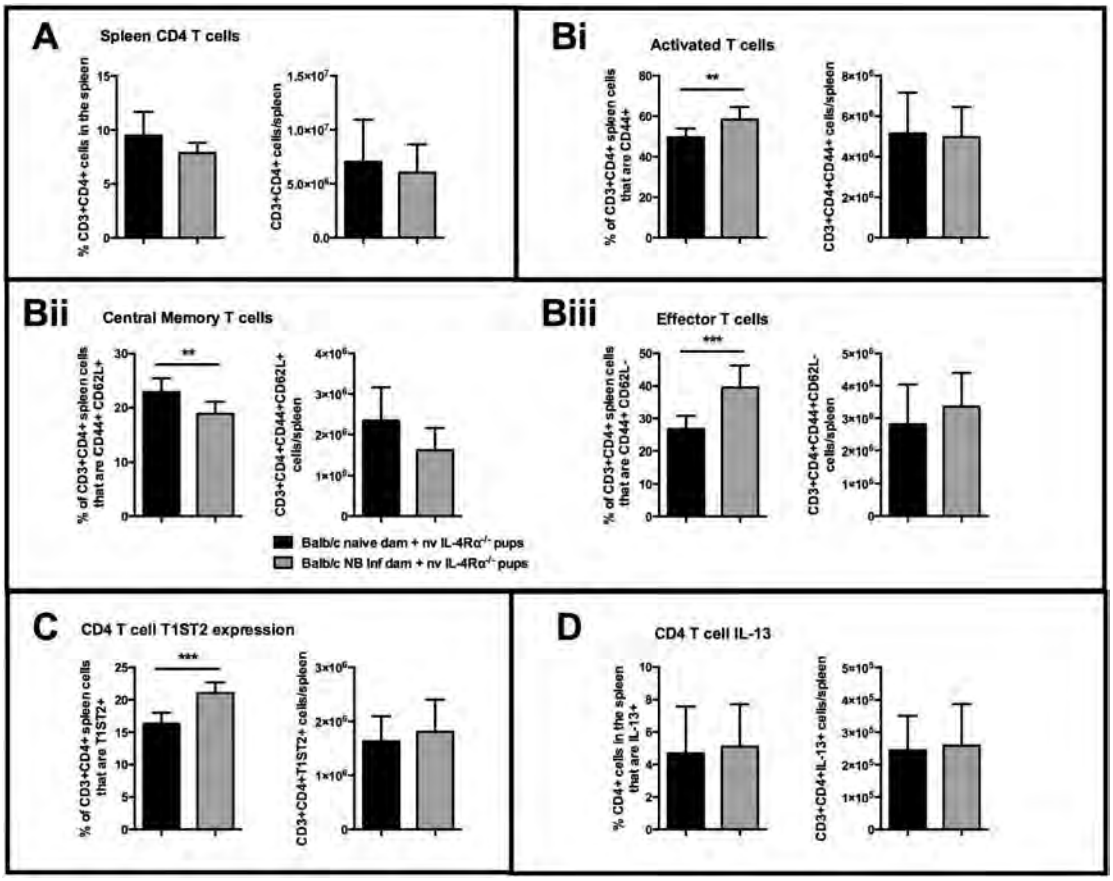



**Figure 3.3: Preconception maternal infection with *Nb* alters some splenic T cell activation through breastfeeding.** CD3$^+$CD4$^+$ T cell populations were analysed by FACS at D5p.i. *(Methods Figure2A)* **(A).** T cells were stratified into activated **(Bi)**, TCM **(Bii)** and Teff **(Biii)** populations**.** T1/ST2 expression on CD3$^+$CD4$^+$ T cells was analysed by FACS **(C).** CD3$^+$CD4$^+$ T cell populations that produce IL-13 were analysed by FACS at D5p.i. **(D).** Data is representative (Mean +/- SD) of 3 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

These data demonstrate that a PCp *Nb* infection confers a significant level of protective immunity to susceptible offspring via breastfeeding when compared to offspring breastfed by uninfected mothers. Protection was associated with higher

levels of antigen-specific antibody and protective cytokines. However most spleen B and T cell populations were not significantly altered.

**Increased lung immune responses in offspring associate with PCp-maternal-exposure-linked protective immunity to *Nb*.**

We have established that certain protection-associated markers of systemic immunity to *Nb* are elevated in IL-4Rα$^{-/-}$ pups breastfed by PCp *Nb* infected mothers when compared to those fed by naïve mothers. As in previous chapters we again looked in the lung for altered populations of localized effector cells.

Offspring breast fed by PCp *Nb* infected mothers showed significantly elevated levels of IL-13 as demonstrated by ELISA on lung tissue homogenates **(Figure 3.4A).** MST B220$^{+}$ B cells were increased in the MST of pups fed by PCp *Nb* infected mice compared to those born to naïve mice **(Figure 3.4Ci)** and have increased IL-13 producing cells **(Figure 3.4Cii)**. Total alveolar macrophages were also increased in IL-4Rα$^{-/-}$ pups breastfed by PCp *Nb* mice **(Figure 3.4D)**.

Lung CD3$^{+}$CD4$^{+}$ T cell proportions and numbers were equivalent in pups born to PCp *Nb* infected mice **(Figure 3.5A).** CD3$^{+}$CD4$^{+}$CD44$^{+}$ activated T cells showed increased proportions in pups fed by PCp *Nb* infected mice **(Figure 3.5Bi)** and stratification of activated T cells into Teff and TCM T cells showed an increased numbers of TCMs and proportions of Teff cells in pups breastfed by PCp *Nb* mice **(Figure 3.5Bii & iii)**. Expression of the IL-33 receptor (T1/ST2) on T cells was increased in pups fed by PCp *Nb* infected mice **(Figure 3.5C)**. Moreover total

$CD3^+CD4^+$ T cells in the MST from PCp *Nb* infected mice were increased **(Figure 3.5Di)** and were more likely to produce IL-13 in response to PMA/ionomycin stimulation **(Figure 3.5Dii)**. Together this analysis shows that PCp *Nb* infection increases offspring lung tissue cytokines, active lung T cells and alveolar macrophage populations, as well as IL-13 producing MST B and T cells.

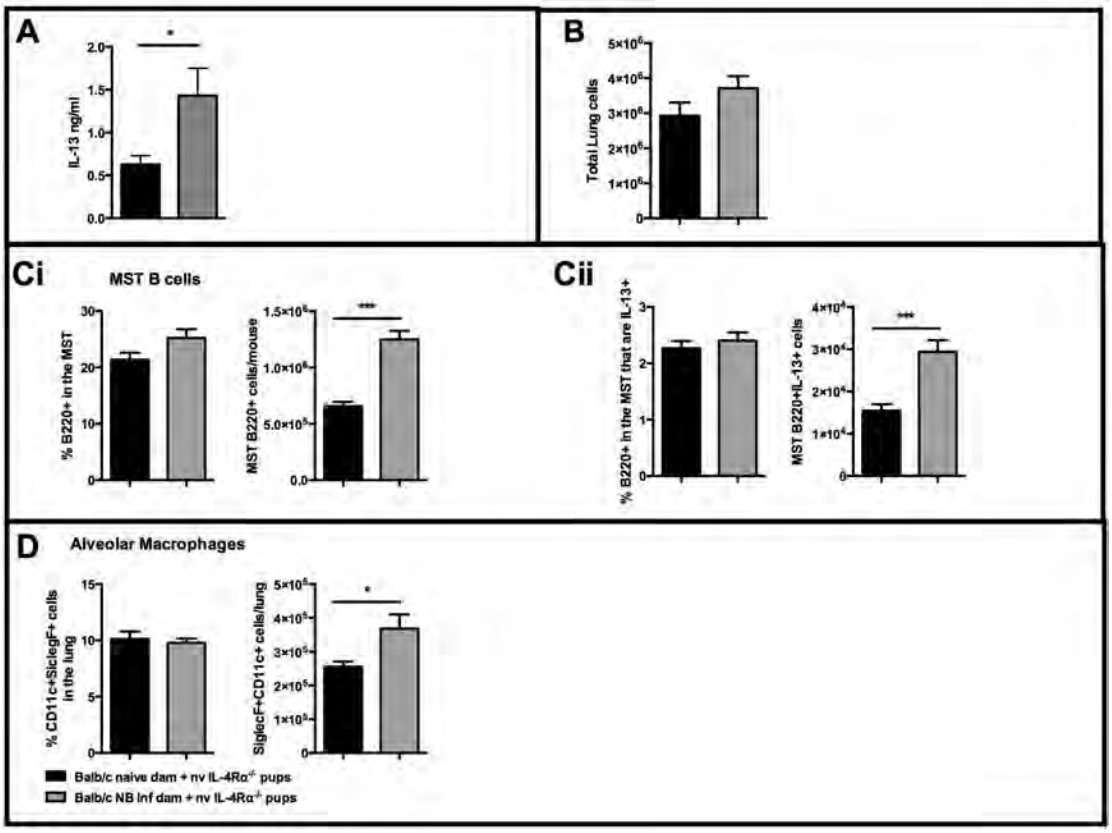



**Figure 3.4: Preconception maternal infection with *Nb* alters lung immune responses against *Nb* in IL-4Rα$^{-/-}$ offspring.** Lung IL-13 levels found in tissue homogenates at D5p.i. **(A).** Lung cellularity per mouse **(B).** MST $CD4^-B220^+$ B cell populations were analysed by FACS *(Methods Figure2A)* **(Ci).** MST $B220^+$ B cell populations that produce IL-13 were analysed by FACS **(Cii).** Alveolar macrophages ($SiglecF^+CD11c^+$) were analysed by FACS **(D).** Data is representative (Mean +/- SD) of 2 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

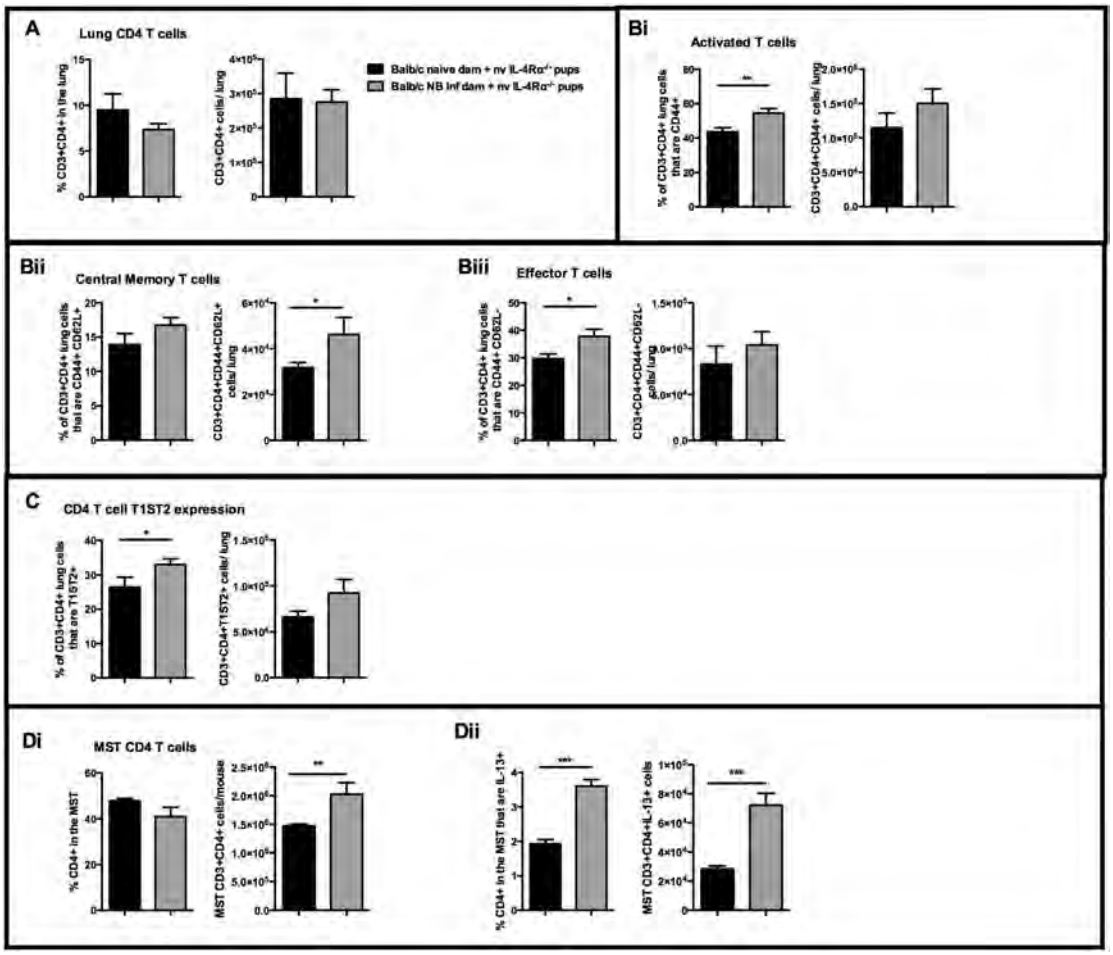



**Figure 3.5: Preconception maternal infection with *Nb* associates with increased lung and MST T cell populations and activation.** Lung $CD3^+CD4^+$ T cell populations were analysed by FACS at D5p.i. *(Methods Figure2A)* **(A).** Lung T cells were stratified into activated **(Bi)**, TCM **(Bii)** and Teff **(Bii)** populations**.** T1/ST2 expression on $CD3^+CD4^+$ T cells was analysed by FACS **(C).** MST $B220^-CD4^+$ T cell populations were analysed by FACS **(Di).** MST $CD4^+$ T cell populations that produce IL-13 were analysed by FACS **(Dii).** Data is representative (Mean +/- SD) of 2 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

## Pre-conception maternal exposure to *Nb* is sufficient to protect IL-4 and IL-13 deficient pups via breastfeeding

Having demonstrated that maternal PCp *Nb* can override susceptibility in IL-4Rα$^{-/-}$ pups we also tested if this was also the case for pups deficient for the cytokines IL-4 and IL-13 which signal through receptors containing IL-4Rα. Female BALB/c mice

were infected with 500 L3 *Nb* and 7 days p.i. the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later the BALB/c and naive IL-4$^{-/-}$ or IL-13$^{-/-}$ mice were mated. 3 days after the offspring were born, pups born to naïve IL-4$^{-/-}$ or IL-13$^{-/-}$ mothers were transferred to naïve or PCp *Nb* BALB/c lactating mothers. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i.**.** Pups breastfed by mothers who had a preconception (PCp) *Nb* infection had reduced intestinal numbers of adult *Nb* when compared to pups breastfed by un-infected mothers for both the IL-4$^{-/-}$ and IL-13$^{-/-}$ pups **(Figure 3.6).**

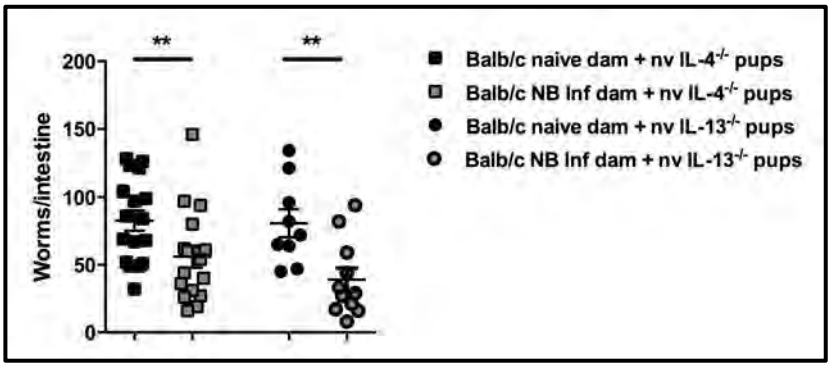



**Figure 3.6: Preconception maternal infection with *Nb* confers immune protection against *Nb* infection to IL-4$^{-/-}$ or IL-13$^{-/-}$ offspring via breastfeeding.** Female BALB/c mice were infected with 500 L3 *Nb* and 7 days post infection (p.i.) the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later mice were mated. 3 days after the offspring were born, we transferred some pups born to naïve IL-4$^{-/-}$ or IL-13$^{-/-}$ mothers to be breastfed by lactating PCp *Nb* BALB/c mothers. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i.**.** Intestinal worm burden was analysed at D5p.i.**.** Data is pooled (Mean +/- SD) from 2 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

*Discussion*

Our analysis shows that maternally derived protection against *Nb* is able to override offspring susceptibility; acquired immunity was protective in IL-4Rα deficient pups as well as pups unable to produce endogenous IL-4 or IL-13. This agrees with Letterio et al.'s finding using TGF-β 1 deficient mice [383].

Protection in IL-4Rα$^{-/-}$ pups is associated with an increase in lung T lymphocyte and innate alveolar macrophage populations and activation, as well as systemic type 2 cytokines and antibodies. The production of type 2 cytokines by re-stimulation of endogenous offspring T and B cells in the spleen and MST suggests that competent cells may be transferred by breastfeeding from wild type mothers exposed to PCp *Nb* infection. The potential for mother to child cell transfer will be examined in the next chapter.

But there is another potential mechanism for the protection we have demonstrated. Esser von Bieren et al. have found that antigen-specific-antibodies are able to trap tissue migrating helminth larvae by driving IL-4Rα-independent alternative differentiation of macrophages using a *H. polygyrus* model [384]. So far, protection has been associated with both higher levels of anti-*Nb* IgG1 and increased alveolar macrophage populations; this suggests that we may be observing a similar mechanism to that seen by Esser von Bieren et al. This needs to be confirmed in future work.

# Chapter 6: Results 4

## What maternal immune components are transferred to protect offspring from an *Nb* infection?

*Introduction:*

IL-4Rα is an essential component of the IL-13 signalling pathway that drives host resolution of nematode infections such as *N. brasiliensis*. In results chapter 3 we demonstrated that the enhanced immunity derived by offspring from PCp infected mothers could overcome susceptibility found in IL-4Rα$^{-/-}$ offspring. Others have shown that prior maternal helminth exposure can influence offspring immunity in a number of other ways depending on the immune components transferred [248, 289, 306, 369, 373]. Here we explore the immune components required by the mother to transfer protection seen previously to offspring, including the importance of maternal IL-4R responsiveness.

In the following chapter we address the following:

- Is maternal IL-4Rα required for transfer of protection to offspring?
- The importance of maternal cytokine production
- Are maternal B and T cell responses required for this protection?
- Are requirements for antibody mediated maternal protection as significant for maternal protection against *Nb* as for control of *H. polygyrus* infections [320]?
- Can the transfer of cells to offspring contribute to maternally derived protection against *Nb*?

*Results:*

**Maternal PCp *Nb* protection of offspring is dependent on maternal IL-4Rα responsiveness and IL-13 production.**

Female BALB/c or IL-4Rα$^{-/-}$ mice were infected with 500 L3 *Nb* and 7 days p.i. the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later BALB/c and IL-4Rα$^{-/-}$ mice were mated. 3 days after offspring were born, the pups born to naïve BALB/c mothers were transferred to naïve or PCp *Nb* BALB/c or PCp *Nb* IL-4Rα$^{-/-}$ lactating mothers. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i. **(Figure 4.1A).**

Pups breastfed by PCp *Nb* BALB/c mothers showed significantly reduced intestinal numbers of adult *Nb* when compared to pups breastfed by un-infected BALB/c mothers or PCp *Nb* IL-4Rα$^{-/-}$ mothers **(Figure 4.1B).** As seen in results chapter 2 the protected group also had more anti-*Nb* IgG1 than both other groups **(Figure 3.1C)**. Interestingly the unprotected BALB/c pups breastfed by PCp *Nb* IL-4Rα$^{-/-}$ mothers also had elevated anti-*Nb* IgG1 compared to pups breastfed by un-infected BALB/c mothers. Moreover protected pups breastfed by PCp *Nb* BALB/c mothers had elevated levels of IL-13 in response to CD3 stimulation of total splenocytes **(Figure 4.1D).** Offspring breastfed by BALB/c mothers had similar sized spleens to pups breastfed by IL-4Rα$^{-/-}$ mothers **(Figure 4.1E)**. Furthermore splenic CD3$^{+}$CD4$^{+}$ T cell populations showed equivalent proportions and numbers in mice breastfed by either BALB/c or IL-4Rα$^{-/-}$ dams **(Figure 4.1E).** And CD3$^{+}$CD4$^{+}$CD44$^{+}$ activated, Teff and TCM T cells showed equivalent proportions and numbers between groups **(Figure 4.1F)**.

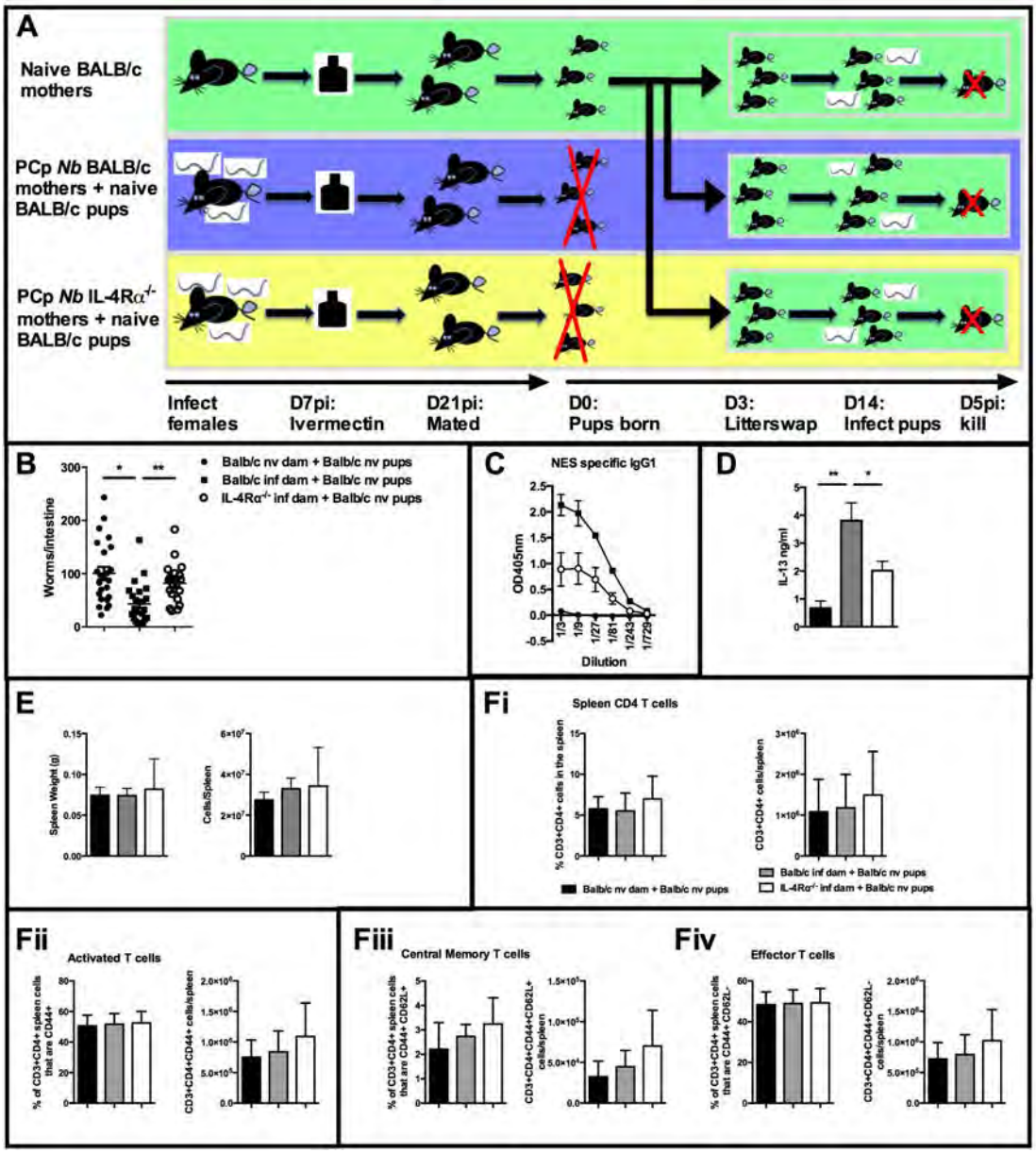



**Figure 4.1: Maternal PCp *Nb* linked protection of the offspring is dependent on maternal IL-4Rα responsiveness.** Female BALB/c or IL-4Rα$^{-/-}$ mice were infected with 500 L3 *Nb* and 7 days post infection (p.i.) the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later BALB/c and IL-4Rα$^{-/-}$ mice were mated. 3 days after the offspring were born, the pups born to naïve BALB/c mothers were transferred to naïve or PCp *Nb* BALB/c or PCp *Nb* IL-4Rα$^{-/-}$ lactating mothers. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i. **(A).** Intestinal worm burden was analysed at D5p.i. **(B).** Serum antigen specific IgG1 levels at D5p.i. **(C).** IL-13 secretion by total splenocytes restimulated with αCD3 **(D).** Spleen weight and cellularity per mouse **(E).** Spleen CD3$^{+}$CD4$^{+}$

T cell populations were analysed by FACS at D5p.i. **(Fi).** T cells were stratified into activated **(Fii)**, TCM **(Fiii)** and Teff **(Fiv)** populations**.** Data is representative (except for worm counts which are pooled)(Mean +/- SD) of 3 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

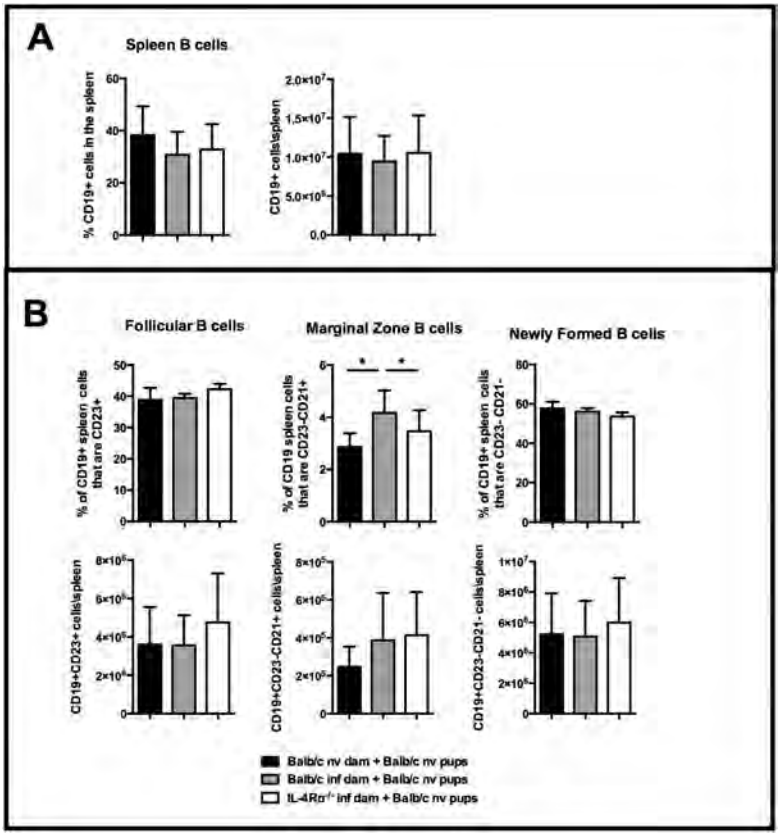



**Figure 4.2: Preconception maternal infection with *Nb* does not alter most splenic B cell or innate cell populations.** $CD19^{+}B220^{+}$ B cell populations were analysed by FACS at D5p.i. **(A)**. B cells were stratified into FO, MZ and NF populations **(B)**. Data is representative (Mean +/- SD) of 2 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

Splenic $CD19^{+}$ B cell populations showed similar proportions and numbers in pups breastfed by BALB/c mice compared to those fed by IL-4R$\alpha^{-/-}$ dams **(Figure 4.2A)**. Further analysis showed no difference in the proportions of follicular or newly formed B cells between groups, but pups breastfed by PCp *Nb* infected BALB/c mice did

have increased proportions of marginal zone B cells compared to naïve pups fed by naïve BALB/c or IL-4Rα$^{-/-}$ mothers **(Figure 4.2B)**.

This analysis shows that transfer of PCp *Nb* infection linked immunity to offspring is dependent on maternal IL-4Rα responsiveness. However maternal IL-4Rα responsiveness does not affect offspring activation of CD4 T cell or B cell populations in the spleen except for MZ cell populations. Protection was associated with higher levels of antigen-specific antibody, protective cytokines and increased proportions of marginal zone B cells.

Maternal cytokines have also been shown to affect offspring immunity [383]. We investigated the role of production of the two key TH2 cytokines, namely IL-4 and IL-13, produced during *Nb* infection on offspring protection. Female BALB/c, IL-4$^{-/-}$ or IL-13$^{-/-}$ mice were infected with 500 L3 *Nb* and 7 days p.i. the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later BALB/c, IL-13$^{-/-}$ and IL-4$^{-/-}$ mice were mated. 3 days after offspring were born, pups born to naïve BALB/c mothers were transferred to naïve or PCp *Nb* BALB/c, IL-4$^{-/-}$ or IL-13$^{-/-}$ lactating mothers. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i. **(Similar to Figure 4.1A).**

Pups breastfed by PCp *Nb* BALB/c mothers showed significantly reduced numbers of intestinal adult *Nb* when compared to BALB/c pups breastfed by un-infected BALB/c, PCp *Nb* IL-4$^{-/-}$ **(Figure 4.3A)** or PCp *Nb* IL-13$^{-/-}$ mothers **(Figure 4.3B).** This showed that maternal IL-4 and IL-13 production is important to generate the immune

components required for offspring immunity to *Nb*. However IL-13 was not found at detectable levels in breastmilk (taken from the stomach of the offspring) **(Figure4.3C)** so whether these cytokines are directly transferred to offspring is unclear.

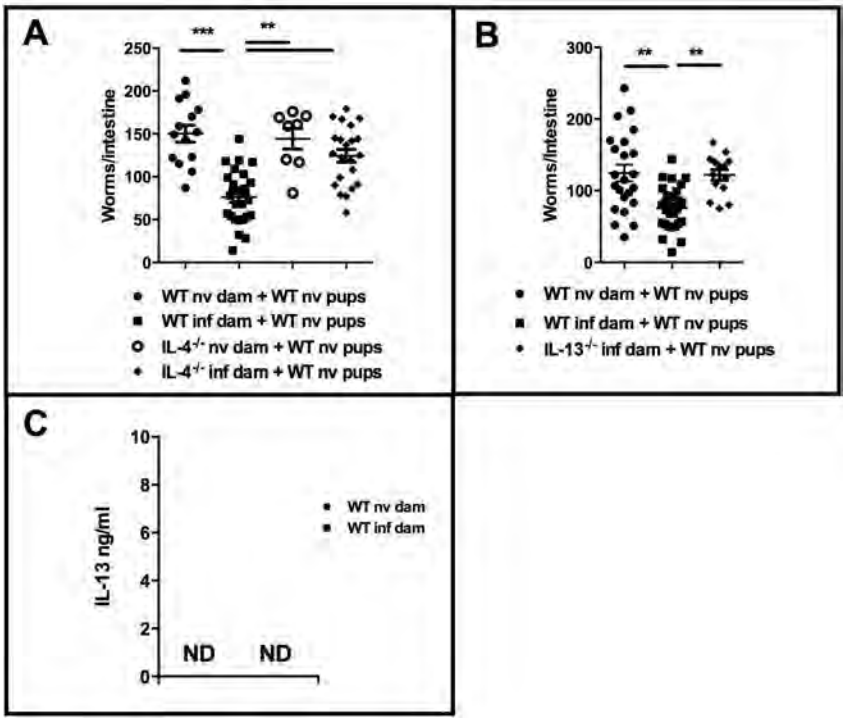



**Figure 4.3: Maternal PCp *Nb* linked protection of the offspring is dependent on maternal IL-4 and IL-13 production.** Female BALB/c, IL-13$^{-/-}$ or IL-4$^{-/-}$ mice were infected with 500 L3 *Nb* and 7 days post infection (p.i.) the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later BALB/c, IL-13$^{-/-}$ and IL-4$^{-/-}$ mice were mated. 3 days after the offspring were born, the pups born to naïve BALB/c mothers were transferred to naïve or PCp *Nb* BALB/c, IL-4$^{-/-}$ or IL-13$^{-/-}$ lactating mothers. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i.**.** Intestinal worm burden was analysed at D5p.i. **(A & B).** IL-13 was not detected in breastmilk (taken from the pups' stomach) by ELISA **(C).** Data is representative (except for worm counts which are pooled)(Mean +/- SD) of 2 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

**Protection in offspring associates with a competent maternal B cell response.**

So far we have established that IL-4Rα and certain maternal protection-associated cytokines are related to immunity against *Nb* in pups. Transfer of antibody in breast milk is widely accepted as an important source of offspring immunity and has previously been shown to be important for maternally derived protection against *H. polygyrus* [320]. We next addressed how maternal B cell responses, including antibody production may contribute to offspring protection against *Nb.*

First we characterized maternal splenic B cell populations 19 days after giving birth (approximately 9 weeks post infection). $CD19^+$ B cell proportions and numbers were equivalent between PCp *Nb* infected and naïve mothers **(Figure 4.4A)** but B cells from PCp *Nb* infected mothers showed increased capacity for IL-13 production following PMA/ionomycin stimulation **(Figure 4.4B)**. To demonstrate if maternal B cells were essential for offspring protection, female BALB/c, B cell-deficient (μMT) and B cell-specific IL-4Rα knock out $MB1^{cre}$IL-$4R\alpha^{-/lox}$ mice were infected with 500 L3 *Nb* and 7 days p.i. the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later mice were mated. 3 days after the offspring were born, pups born to naïve BALB/c mothers were transferred to naïve or PCp *Nb* BALB/c, μMT or $MB1^{cre}$IL-$4R\alpha^{-/lox}$ lactating mothers. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i. **(Similar to Figure 4.1A).** Pups breastfed by PCp *Nb* BALB/c mothers showed significantly reduced numbers of intestinal adult *Nb* when compared to BALB/c pups breastfed by un-infected BALB/c, PCp *Nb* μMT **(Figure 4.4C)** or PCp *Nb* $MB1^{cre}$IL-$4R\alpha^{-/lox}$ mothers **(Figure 4.4D).** This

shows that maternal B cells are an important immune component contributing to offspring immunity to *Nb*.

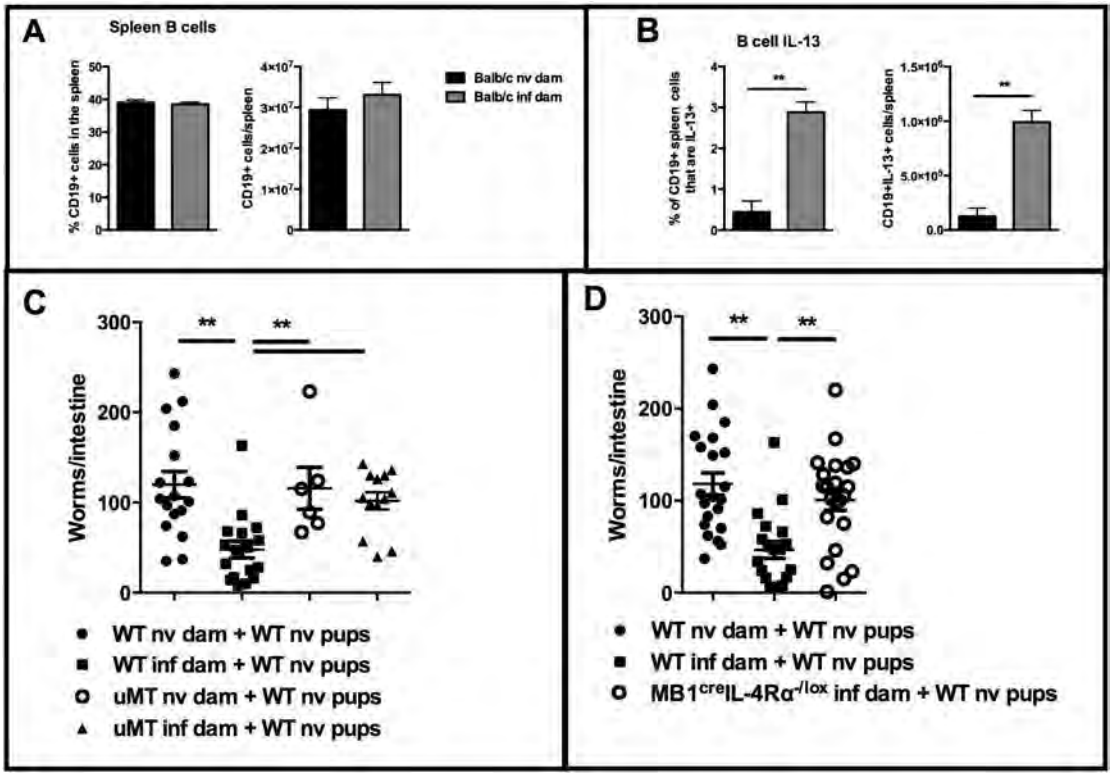



**Figure 4.4: Maternal B cell function is important for offspring immunity.** Maternal spleen CD19+ B cell populations were analysed by FACS at 19 days after birth. **(A)**. Maternal CD19+ B cell populations that produce IL-13 were analysed by FACS **(B)**. Female BALB/c, µMT or MB1$^{cre}$IL-4Rα$^{-/lox}$ mice were infected with 500 L3 *Nb* and 7 days post infection (p.i.) the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later the mice were mated. 3 days after the offspring were born, the pups born to naïve BALB/c dams were transferred to naïve or PCp *Nb* BALB/c, µMT or MB1$^{cre}$IL-4Rα$^{-/lox}$ lactating dams. Offspring were infected when 14 days old with 250xL3 *Nb* and killed D5p.i.**.** Intestinal worm burden was analysed at D5p.i. **(C & D).** Data is representative (except for worm counts which are pooled)(Mean +/- SD) of 2 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

B cell produced antibodies are a major effector component of the immune system and especially of maternally derived immunity in the offspring; high levels of antigen

specific antibodies have been associated with protection throughout this study. But as pointed out in the literature review, B cells can play a multi-functional role as they can also be a source of cytokines that induce *Nb* protective T cell activation [109]. To establish the specific role that the maternal B cells are playing we investigated if maternal antibody contributed to offspring immunity to *Nb*.

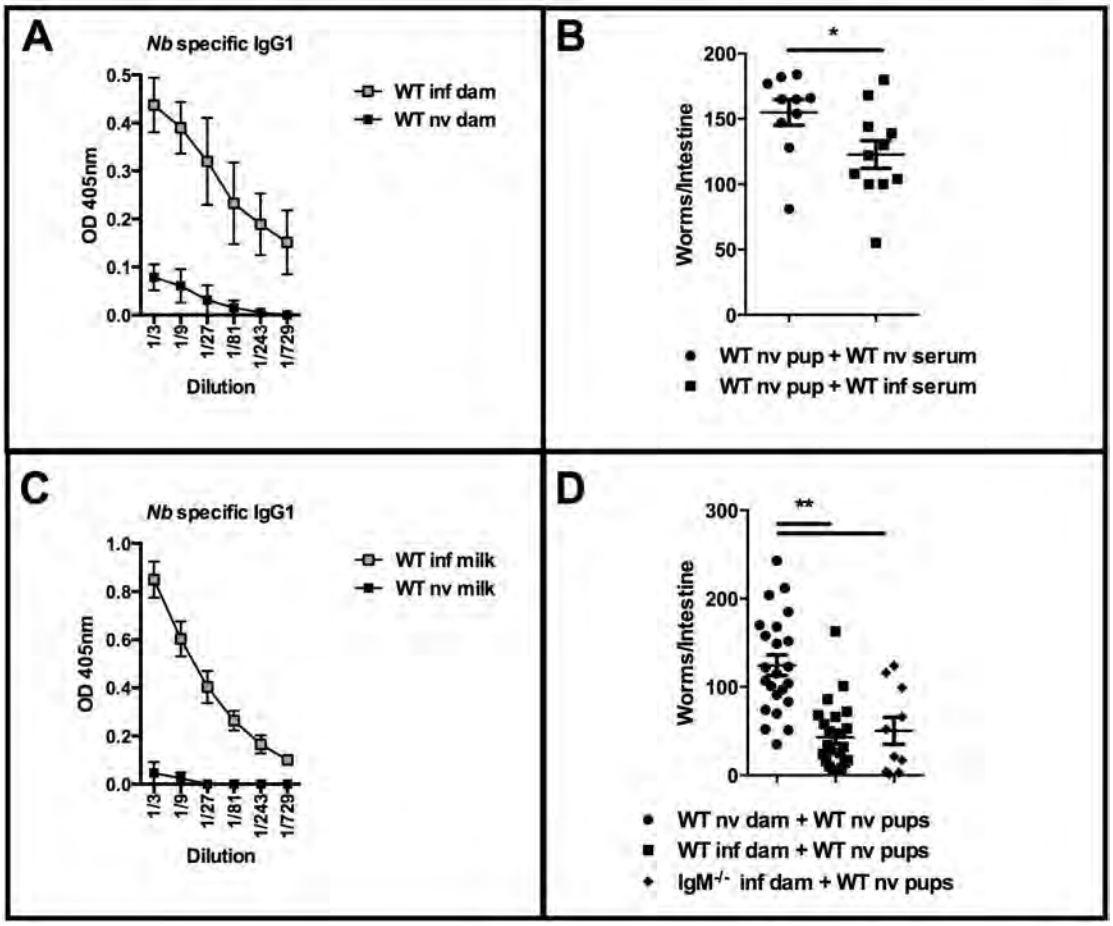



**Figure 4.5: Maternal PCp *Nb* linked protection of the offspring is associated with but not dependent on maternal antibody production.** Maternal serum antigen specific IgG1 levels at 19 days after birth **(A).** 150µl of heat-inactivated serum from either naïve or *Nb* mothers was given to 14 day old naïve BALB/c pups intra-venously. The pups were infected the following day with 250xL3 *Nb* and killed 5 days p.i.. Intestinal worm burden was analysed at D5p.i. **(B)**. Antigen specific IgG1 levels in breastmilk taken from the stomach of the pups **(C).** Female BALB/c or $IgM^{-/-}$ mice were infected with 500 L3 *Nb* and 7 days p.i. the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later the mice were mated. 3

days after the offspring were born, the pups born to naïve BALB/c dams were transferred to naïve or PCp *Nb* BALB/c or $IgM^{-/-}$ lactating dams. Offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i.**.** Intestinal worm burden was analysed at D5p.i. **(D).** Data is representative (except for worm counts which are pooled)(Mean +/- SD) of 2 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

First we characterized the levels of maternal serum IgG1 present 19 days after birth. PCp *Nb* dams still had elevated anti-*Nb* IgG1 **(Figure 4.5A)**, even though it was approximately 9 weeks post infection. Then we took some of this serum and heat inactivated it to destroy the complement proteins and cytokines, but leaving antibodies intact. We transferred 150µl of heat-inactivated serum from either naïve or *Nb* mothers to 14 day old naïve BALB/c pups intra-venously (i/v). The pups were infected the following day with 250xL3 *Nb* and killed 5 days p.i.. Pups receiving *Nb* maternal serum showed a small but significant reduction in numbers of intestinal adult *Nb* when compared to BALB/c pups that received serum from un-infected mothers **(Figure 4.5B).** We also found elevated anti-*Nb* IgG1 in the milk (taken from the stomach of the offspring) from *Nb* mothers compared to the milk from naïve mothers showing antibody is definitely transferred via breastfeeding **(Figure 4.5C)**. Together this data shows that maternal antigen specific antibody is transferred to the offspring and that it can provide a small but significant protection against infection. This may be a similar passive immunizing effect to that reported by Nieuwenhuizen et al. [216].

Then we looked at whether $IgM^{-/-}$ mothers, which have a normal IgD response but produce significantly less antigen-specific class-switched antibodies [112] could

provide protection to pups. Female BALB/c or IgM$^{-/-}$ mice were infected with 500 L3 *Nb* and 7 days post infection (p.i.) the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later mice were mated. 3 days after the offspring were born, pups born to naïve BALB/c mothers were transferred to naïve or PCp *Nb* BALB/c or IgM$^{-/-}$ lactating mothers. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i. **(Similar to Figure 4.1A).** Pups breastfed by both PCp *Nb* BALB/c mothers and PCp *Nb* IgM$^{-/-}$ mothers showed significantly reduced numbers of intestinal adult *Nb* when compared to BALB/c pups breastfed by un-infected BALB/c, **(Figure 4.5D).** Thus although *Figure4.5A-C* shows that transferred antibody can be protective it appears that it is not essential to this protection, confirming the findings of Horsnell et al. [109] that antigen specific antibody is not necessary to resolve a *Nb* infection. This suggests another protective B cell driven effect may be taking place that is not dependent on antibody affinity maturation and is able to respond to the offspring infection independently.

**Protection in offspring associates with maternal T cell responses and transfer.**

We have established that maternal B cell function, IL-4Rα-responsiveness and certain maternal protection-associated cytokines are related to immunity against *Nb* in pups. But data suggesting the possibility of an antibody-independent protective mechanism warranted further investigation. In order to examine this we analysed the other maternal immune cell population that could form protective memory cells, namely T cells.

Again we characterized the population of maternal $CD3^+CD4^+$ T cells present in the spleen 19 days after they had given birth (approximately 9 weeks post infection). $CD4^+$ T cell proportions and numbers were equivalent between PCp *Nb* infected and naïve mothers **(Figure 4.6A)** as were $CD44^+$ activated T cells and TCMs **(Figure 4.6Bi & iii).** But effector T cells were increased in PCp *Nb* infected mothers **(Figure 4.6Bii)** and T cells from PCp *Nb* infected mothers have the capacity for increased IL-13 production after PMA/ionomycin stimulation **(Figure 4.6C)**.

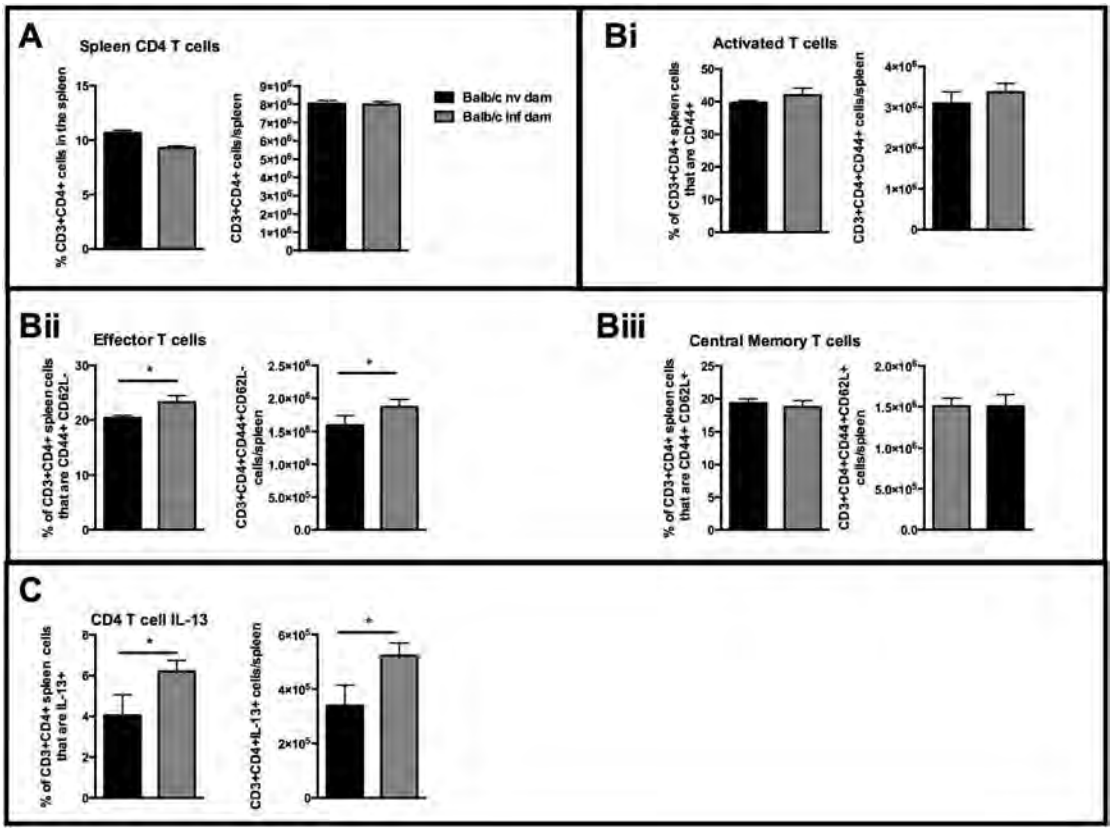



**Figure 4.6: Maternal T cell function is important for offspring immunity.** Maternal spleen $CD3^+CD4^+$ T cell populations were analysed by FACS at 19 days after birth. **(A)**. Spleen T cells were stratified into activated **(Bi)**, TCM **(Biii)** and Teff **(Bii)** populations**.** Spleen $CD3^+CD4^+$ T cell populations that produce IL-13 were analysed by FACS at day 19 post birth **(C).** Data is representative (Mean +/- SD) of 2 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

Next in order to show whether maternal T cells were essential to offspring protection, female BALB/c or T cell specific IL-4Rα knock out ilck$^{cre}$IL-4Rα$^{-/lox}$ mice were infected with 500 L3 *Nb* and 7 days p.i. the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later mice were mated. 3 days after the offspring were born, pups born to naïve BALB/c mothers were transferred to naïve or PCp *Nb* BALB/c or ilck$^{cre}$IL-4Rα$^{-/lox}$ lactating mothers. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i. **(Similar to Figure 4.1A).** Pups breastfed by PCp *Nb* BALB/c mothers showed significantly reduced numbers of intestinal adult *Nb* when compared to BALB/c pups breastfed by un-infected BALB/c or PCp *Nb* ilck$^{cre}$IL-4Rα$^{-/lox}$ mothers **(Figure 4.7A).** Moreover protected pups breastfed by PCp *Nb* BALB/c mothers had elevated levels of IL-13 in response to CD3 stimulation of total splenocytes **(Figure 4.7B).** This shows that maternal T cells are an important part of the immune components required for offspring immunity.

But how were the maternal T cells affecting the offspring? Previously we found anti-*Nb* IgG1 but not IL-13 in the breastmilk we were able to isolate from the stomachs of nursing pups *(figures 4.3 and 4.5)*. We were not able to detect cells in the milk taken from the offspring stomachs, probably due to the effects of the stomach acid and enzymes. However, direct isolation of milk from the mother revealed a population of CD3$^{+}$CD4$^{+}$ T cells **(Figure 4.7C).** There is a precedent for this finding with T cells having been detected in breastmilk before [261, 262, 280, 282-284, 307].

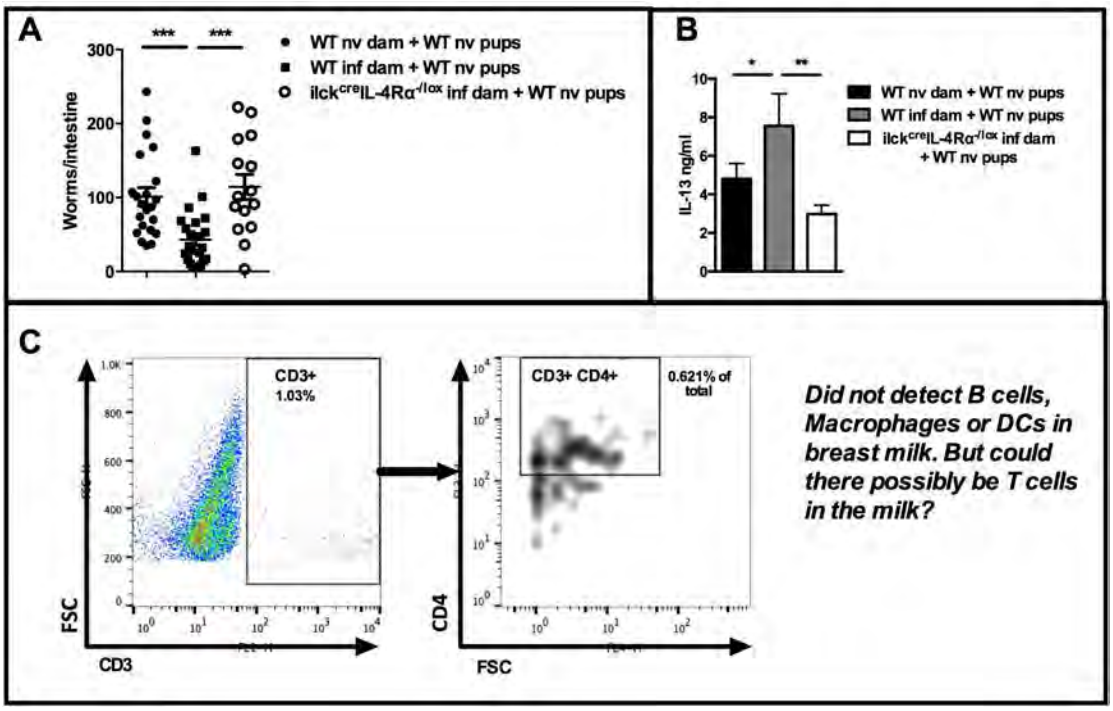



**Figure 4.7: Maternal T cells play a role in offspring immunity.** Female BALB/c or ilck$^{cre}$IL-4Rα$^{-/lox}$ mice were infected with 500 L3 *Nb* and 7 days post infection (p.i.) the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later the mice were mated. 3 days after the offspring were born, the pups born to naïve BALB/c dams were transferred to naïve or PCp *Nb* BALB/c ilck$^{cre}$IL-4Rα$^{-/lox}$ lactating dams. Offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i.**.** Intestinal worm burden was analysed at D5p.i. **(A).** IL-13 secretion by total splenocytes re-stimulated with αCD3 **(B).** T cells detected in BALB/c breastmilk isolated by breast-pump **(C).** Data is representative (except for worm counts which are pooled)(Mean +/- SD) of 1 or 2 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

In order to confirm whether maternal T cells could be transferred to offspring to mediate protection, female Thy1.1 mice were infected with 500 L3 *Nb* and 7 days p.i. the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later mice were mated. 3 days after the offspring were born, pups born to naïve Thy1.2 BALB/c mothers were transferred to naïve BALB/c or naïve or PCp *Nb* Thy1.1 lactating mothers. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i. **(Figure 4.8A).**

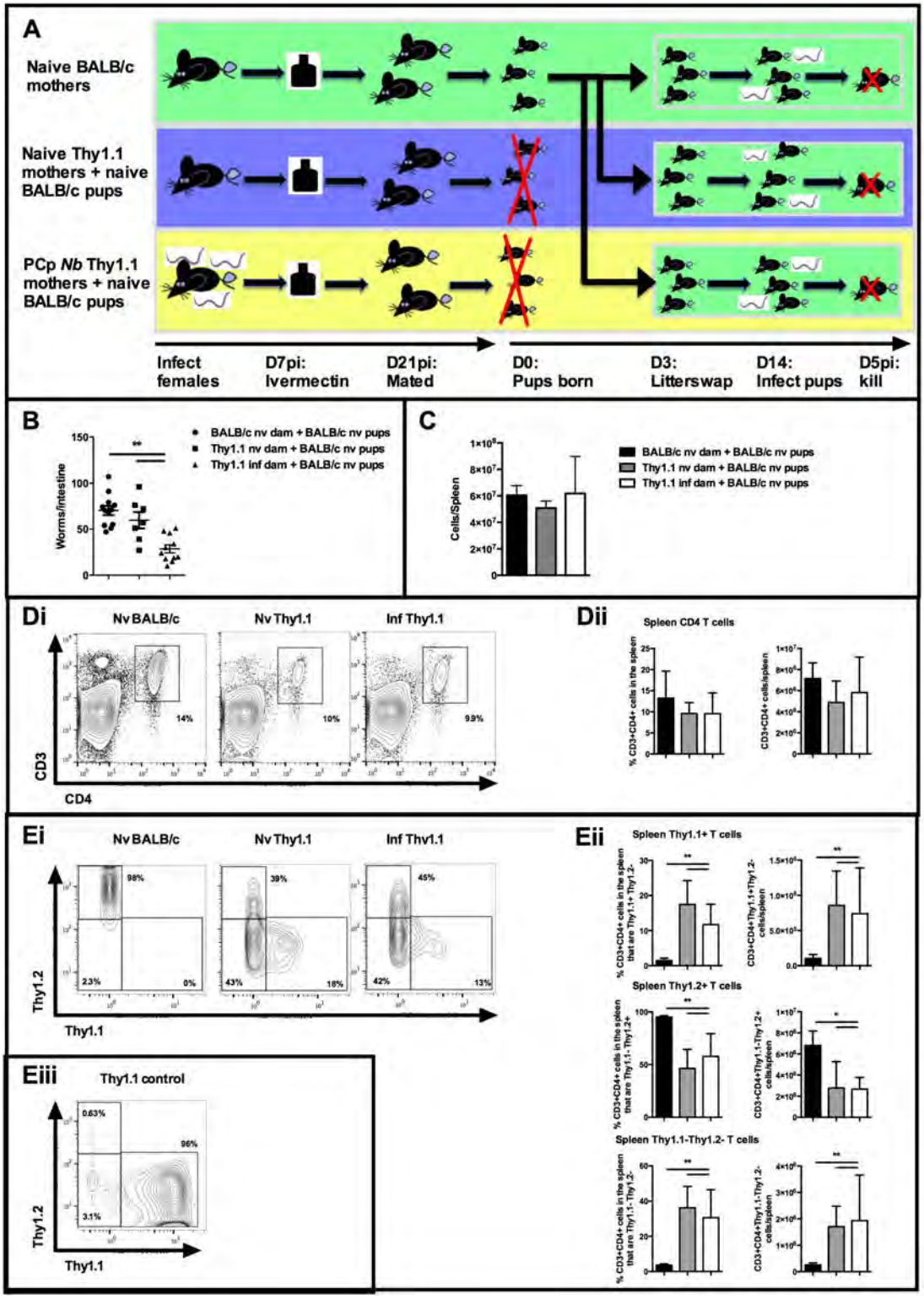



**Figure 4.8: Maternal T cells are transferred to offspring in the breastmilk.** Female Thy1.1 mice were infected with 500 L3 *Nb* and 7 days post infection (p.i.) the infection was cleared by a 5 day oral treatment with ivermectin; 14 days later mice were mated. 3 days

after the offspring were born, pups born to naïve Thy1.2 BALB/c mothers were transferred to naïve BALB/c or naïve or PCp *Nb* Thy1.1 lactating mothers. The offspring were infected when 14 days old with 250xL3 *Nb* and killed 5 days p.i. **(A).** Intestinal worm burden was analysed at D5p.i. **(B).** Spleen weight and cellularity per mouse **(C).** Spleen $CD3^{+}CD4^{+}$ T cell populations were analysed by FACS at D5p.i. **(D).** T cells were stratified into $Thy1.2^{+}Thy1.1^{-}$, $Thy1.2^{-}Thy1.1^{+}$ and Thy1.2-Thy1.1- populations **(E).** Data is representative (Mean +/- SD) of 2 independent experiments. n=6-8 offspring per group which stem from between 2 and 4 mothers.

BALB/c pups breastfed by PCp *Nb* Thy1.1 mothers showed significantly reduced numbers of intestinal adult *Nb* when compared to BALB/c pups breastfed by un-infected BALB/c or Thy1.1 mothers **(Figure 4.8B).** Offspring breastfed by BALB/c mothers had similar sized spleens **(Figure 4.8C)** and total $CD3^{+}CD4^{+}$ T cell populations **(Figure 4.8D)** to pups breastfed by Thy1.1 mothers. Significantly, while total T cells in Thy1.2 BALB/c pups breastfed by Thy1.2 BALB/c mothers were $Thy1.2^{+}Thy1.1^{-}$, T cells in Thy1.2 BALB/c pups breastfed by Thy1.1 mothers were split into three groups, namely $Thy1.2^{+}Thy1.1^{-}$, $Thy1.2^{-}Thy1.1^{+}$ and $Thy1.2^{-}Thy1.1^{-}$ **(Figure 4.8E).** Since $Thy1.1^{+}$ CD4 T cells were detected in Thy1.2 BALB/c pups, T cells must indeed be transferred from mother to offspring. Approximately 15% of splenic T cells in $Thy1.2^{+}$ offspring were $Thy1.1^{+}$.

*Discussion*

Our analysis shows that the maternally derived protection against *Nb* transferred to offspring by breastfeeding from mothers exposed to PCp *Nb* infection is IL-4Rα dependent, which is in agreement with current literature [109]. Protection is also associated with IL-4 and IL-13 production by the mothers, but it is unclear if these cytokines are directly passed to the offspring since we were not able to detect them in breastmilk. This is perhaps not very surprising considering the time elapsed between maternal infection and birth. Our data does show that mothers maintain an increased potential for launching a type 2 immune response following lymphocyte stimulation and this may be important for transfer of immunity to offspring.

We also show that offspring protection is associated with maternal B cell function; the absence of a competent B cell response precludes the transfer of this protection. We also show that direct transfer of maternal antigen specific antibody to offspring may contribute partially to this effect. But B cells play a multi-functional role in conferring immunity to *Nb*; for example we have shown that they are important in driving a protection via antigen presentation and cytokine production [109]. Our work and others [119] have shown that such protection can be significantly mediated by B cell priming of CD4 T cells. The findings presented by us here demonstrate a new paradigm about the importance of B cell activation of T cells, and how this regulates protection. We show that maternal B cells are required for transfer of protection but this is not critically dependent on antibody. Instead we suggest that the overarching transfer of lymphocyte-mediated protection is via transfer of B cell educated CD4 T cells to the offspring.

Previous studies have found the presence of maternal stem cells and T cells in the breastmilk [261, 280, 282-284]. T cell memory is essential for protection against *Nb* reinfection [35, 209] and the transfer of functional memory T cells to the offspring could compensate for offspring protection even in the absence of antigen specific antibodies or in genetically vulnerable organisms. This could therefore be a mechanism by which BALB/c mothers could provide protection to IL-4Rα$^{-/-}$ offspring that would override susceptibility. This could also be a method that would provide longterm protection depending on whether the transferred T cells were fully assimilated into the offspring's own immune structure. The findings presented here support the hypothesis of maternal T cell transfer. The use of Thy1.1 syngeneic mothers demonstrates that maternal T cells are transferred from mother to offspring via breastfeeding. Thus we show that maternal T cell expression of IL-4Rα is required for offspring protection, we show the presence of CD4 T cells in breastmilk and finally we show that syngeneic maternally derived CD4 T cells are significantly incorporated into offspring lymphocyte compartments, at least in the short term.

# Chapter 7: Concluding Remarks

## 7.1 Summary of results

In sub-Saharan Africa pregnant women are often infected with one or more helminth during pregnancy. Maternal infection is strongly correlated with impaired vaccine efficacy of standard vaccinations like *HiB* in their children [365]. This has many important implications on both maternal and neonatal/infant health in general, and specifically on child immune development and responses to homologous and heterologous infections and co-infections, especially since areas endemic for helminths usually overlap with areas with a high prevalence of serious bacterial and viral infections. Maternal helminth infection may further exacerbate the high susceptibility of young children to these infections resulting in respiratory disease, progressive bacteremia and even death.

Our study has showed that, in mice, preconception maternal infection with the helminth *N. brasiliensis* impacts on immune development of offspring by increasing innate lung cell, T cell and B cell population development and proliferation via breastfeeding, resulting in greater anti-*Nb* offspring immunity. This is achieved by a wide range of mechanisms including antibody transfer of certain isotypes and immune cells via breastfeeding; however this does not preclude the potential for the transfer of cytokines, immune complexes or other soluble molecules, or additional trans-placental effects not yet investigated which may also affect offspring immunity.

Pups born to *N. brasiliensis* exposed mothers had increased populations of total lung and splenic CD4 helper cells, as well as higher sub-populations of central memory

and effector CD4 cells compared to pups born to naive mothers **(Table 7A)**. Many developing and mature B cell populations were also increased in pups born to previously infected mothers and analysis of the spleen showed an increase in a number of B cell populations, most notably MZ cells **(Table 7A)**. Furthermore alveolar macrophage and neutrophil populations were increased by PCp *Nb* **(Table 7A)**. Our data suggests that these innate, B and T cells are more sensitive to stimulation and are better able to respond to *N. brasiliensis* infection, by increased activation and IL-13 production. Transfer of PCp *Nb* maternally mediated protection was able to override IL-4Rα-deficient susceptibility in offspring.

**Table 7A:** Summary of findings from results chapters 1-3; the immunological factors associated with offspring protection against *N. brasiliensis.*

| | | | Analysed at D5 post infection (19 days old) | | | |
|---|---|---|---|---|---|---|
| | | | ***In-utero* and breastfeeding** | ***In-utero*** | **Breastfeeding** | **IL-4Rα$^{-/-}$ pups** |
| **Protected** | | | *Yes* | *No* | *Yes* | *Yes* |
| ***Nb* specific IgG1** | | | ++ | 0 | ++ | ++ |
| Spleen | IL-4, IL-13 | | ++ | | ++ | + |
| | IL-10 | | + | | | -- |
| | IFNg | | + | | | 0 |
| | B cells | Total | + | 0 | 0 | 0 |
| | | FO | 0 | 0 | 0 | 0 |
| | | MZ | ++ | 0 | + | - |
| | | NF | 0 | 0 | 0 | 0 |
| | | MHCII | ++ | 0 | 0 | 0 |
| | T cells | Total | + | 0 | 0 | 0 |
| | | Eff | + | 0 | 0 | + |
| | | TCM | + | 0 | 0 | - |
| | | T1/ST2 | + | 0 | 0 | 0 |
| Lung | IL-4, IL-13 | | + | | + | + |
| | B cells | | 0 | | + | 0 |
| | T cells | Total | + | ++ | ++ | 0 |
| | | Eff | ++ | ++ | ++ | + |
| | | TCM | + | 0 | + | + |
| | | T1/ST2 | + | + | + | + |
| | ILC | Total | + | | + | |
| | | IL-7R | 0 | | + | |
| | | T1/ST2 | 0 | | + | |
| | Alv Macs | Total | ++ | ++ | ++ | + |
| | | Relmα | ++ | | + | |
| | | YM-1 | ++ | | + | |
| | Neutrophils | Total | ++ | | ++ | |
| | | Relmα | 0 | | 0 | |
| | | YM-2 | + | | - | |
| MST | B cells | | + | | - | + |
| | T cells | | ++ | | + | + |
| | IL-4, IL-13 | | ++ | | ++ | + |
| | IL-10 | | ++ | | | |
| | IFNg | | ++ | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ++ / + | Increased in PCp pups | 0 | No Difference | - / -- | Decreased in PCp pups | | Data not yet available |

Protection was dependent on IL-4Rα maternal-responsiveness, associated with increased offspring antigen-specific serum antibody levels and IL-13 production. Moreover we found that maternal IL-4Rα-competent B and T cells contribute to *N. brasiliensis* priming by nursing. When we compared pups nursed by B or T cell specific IL-4Rα knockout mothers to pups nursed by BALB/c mothers we found that that only pups nursed by PCp *Nb* BALB/c mothers showed protection against *N. brasiliensis* infection.

Protection was also associated with IL-4 and IL-13 production by the mothers. Helminth-associated type 2 cytokine responses, particularly IL-4 activation of transcription factor STAT6, are classically associated with antibody class switching in B cells to produce IgG and IgE. Indeed, IL-4 was originally termed B cell-stimulatory factor-1 (BSF-1) based on its ability to enhance IgE and IgG1 production in LPS (lipopolysaccharide)-stimulated B-cells [385]. Throughout most of the study, protection was associated with elevated levels of anti-*Nb* IgG1, and anti-*Nb* IgG1 was found in breastmilk of PCp *Nb* BALB/c mothers. Since antibody has previously been shown to passively protect against *H. polygyrus* in a maternal model [320] and against *Nb* in adult mice [216] this provides a potential mechanism for maternally-derived protection against *Nb* in pups. The study by Esser von Bieren et al. showing that antigen-specific-antibodies drive anti-helminth IL-4Rα-independent alternative macrophage differentiation in a *H. polygyrus* model [384] further suggests anti-*Nb* antibody as the mechanism of protection. This needs to be confirmed in future work.

However we found that the absence of a competent antibody response in $IgM^{-/-}$ mothers does not hinder transfer of protection to her offspring. This indicates that there may be another protective mechanism independent of antibody. Besides production of antibody, B cells can be an important source of cytokines and induce T cell activation during *Nb* infection [109]. Previous studies show T cell transfer in the breastmilk [261, 280, 282-284], a finding we confirmed. T cell memory is essential for protection against *Nb* reinfection [35, 209] and the transfer of functional memory T cells to the offspring could compensate offspring for genetic susceptibility or the absence of antigen specific antibody, and could have long-term effects. This is therefore a second mechanism by which BALB/c mothers may provide protection to IL-$4R\alpha^{-/-}$ offspring. Our findings support this hypothesis; using Thy1.1 syngeneic mothers we demonstrated that maternal T cells are transferred from mother to offspring via breastfeeding. This is an avenue of work that needs to be explored further in the future.

In summary this study demonstrates the principle role of IL-4Rα-responsive maternal immunity in transferring preconception *N. brasiliensis*-mediated protection to *Nb* via breastfeeding to her offspring. Protection is associated with offspring antigen specific antibody levels, type 2 cytokine production and cellular responses. Although the presence of maternal antibody was not essential to protection, we have shown antibody transfer may play a role in offspring disease resolution. On the other hand we have established the potential of transfer of functional T cells in the breastmilk as an antibody independent mechanism of protection, which could have widespread and long-term effects. These are significant observations that are important in

understanding immunity to human nematodes with analogous lifecycles, and their clinical implications in pregnancy especially in regions where helminth infections are common.

## 7.2 Future work

While this body of work is interesting and shows a significant amount of evidence there are still a few questions that remain unanswered or have not been fully explored. We justify our conclusion that antibody is not playing a role in the maternally derived protection transferred from $IgM^{-/-}$ mothers on the basis of previous research showing they cannot generate a competent antibody switched response [112]. We need to confirm this by testing anti-*Nb* IgG1 antibody levels in both $IgM^{-/-}$ maternal and offspring serum as well as in maternal $IgM^{-/-}$ breastmilk.

Furthermore in order to fully explore the role of transferred antibody mediated protection versus transferred T cell mediated protection we need to carry out several experiments. The differentiation of macrophages that have been cultured *in vitro* with serum high in anti-*Nb* antibody would indicate the importance and function of antibody to the protection described here. If there is a significant amount of alternative activation then that would suggest that the mechanism of protection we are investigating is similar to that of Esser von Bieren et al. [384]. This could also be established in an *in vivo* model by repeating our serum transfer experiment from *Figure4.5* and examining the immune responses of mice that received serum high in anti-*Nb* antibody. Again high alternative activation of macrophages would further suggest an antibody-mediated mechanism of protection.

Our finding showing the presence of T cells in breastmilk is very interesting despite the fact that this has been shown before because it provides us with a potential mediator for longterm effects, something we show in *Figure1.7.* If we could show increased activation of the transferred syngeneic cells from *Figure4.8* found in protected pups in response to *Nb* stimulation, then that would demonstrate the importance of transferred maternal T cells in offspring immunity. We need to examine the transferred syngeneic cells from *Figure4.8* for their CD44/CD62L memory profile, IL-13 production and their T1/ST2 expression.

An interesting point to note is that *N. brasiliensis* antigens and excretory/secretory products are not fully characterised, and neither are their properties and interactions with immune cells which are likely to be complex and multi-factorial. Also the L3 stage of the larvae sheds its chitin sheath in the host lung in order to moult into L4 as part of its natural life cycle. As a tough, nitrogenous polysaccharide it would break down slowly in the mother and may provide persistent circulating antigen for a long period after active infection has ceased. It is possible that the protective effect we see in the offspring is due to direct antigen transfer from mother to offspring that results in offspring immune priming in an effect similar to that seen with OVA by Verhasselt et al. [301]. In order to test this we would need to test the breastmilk for antigen. It would also be interesting to see whether the mother can still provide protection in the interval after the 21 day timeframe we allowed between infection and mating, maybe several weeks or months post infection.

Finally it would be beneficial to explore the breastmilk in depth, for antibodies, cytokines, cells, antigen and even potentially DNA/RNA and commensal bacteria. We managed to get a limited amount of breastmilk directly from the mothers using a makeshift breast-pump but this proved a very difficult undertaking. From the small quantity we did get directly from the mothers, FACS revealed a population of T cells, but unfortunately the quantity of milk acquired was not enough sample to test by ELISA. Previously we found anti-*Nb* IgG1 but not IL-13 or immune cells in the breastmilk we were able to isolate from the stomachs of nursing pups *(figures 4.3C and 4.5C)* using a method established by Verhasselt et al. [301]. In the future we may again attempt to study murine breastmilk taken directly from the mother using techniques, such as microarray, which do not need large quantities of sample.

While some of the conclusions we draw on the data presented in this study do need some confirmation, the results are reproducible and we hope to corroborate and substantiate them further in future using the techniques discussed, and eventually fully establish and explain the role that preconception maternal *Nippostrongylus brasiliensis* infection plays in altering offspring immune development and protection against disease.

# Chapter 8: Appendices

## Appendix A - Antibodies used

**TableA:** The monoclonal antibodies used for analysis of T, B cell and innate populations by flow cytometry.

| Antibody | Flurofore | Clone | Isotype control |
|---|---|---|---|
| CD4 | PerCP | RM4-5 | IgG2a |
| CD44 | FITC | IM7 | IgG2b |
| CD62L | APC | MEL-14 | IgG2a |
| CD3 | PE | 500A2 | IgG2 |
| T1/ST2 | FITC | DJ8 | IgG2a |
| B220 | V500 | RA3-6B2 | IgG2a |
| CD19 | PerCP | ID3 | IgG2a |
| CD21 | APC | 7G6 | IgG2b |
| CD23 | PE | B3B4 | IgG2a |
| MHCII | FITC | 2G9 | IgG2a |
| SiglegF | PE | E50/2440 | IgG1 |
| CD11c | APC | HL3 | IgG2a |
| GR-1 (Ly6G) | APC-Cy7 | RB6-835 | IgG2b |
| ICOS | APC | 7E.17G9 | IgG2a |
| CD127 | PE-Cy7 | SB/199 | IgG2b |
| Relmα | FITC | E19 | IgG2a |
| YM-1 | PerCP | ECF-L | IgG2b |

| | | | |
|---|---|---|---|
| IL-13 | PE-Cy7 | ebio13a | IgG1 |
| IL-4 | FITC | 11B11 | IgG1 |
| IL-10 | A700 | JES5-2A5 | IgG2b |
| IFNγ | APC | R4-6A2 | IgG1 |
| **Lineage mix:** CD3, CD19, CD11b, FceR1, Ter119, CD4, CD8, B220, Ly6G/6C | PE | Biolegend | |

**TableB:** The monoclonal antibodies used for analysis of cytokines and antibodies by ELISA.

| Cytokine | Coating/capture antibody | Detection antibody | Protein standard | Sensitivity |
|---|---|---|---|---|
| **IFNγ** | **Rat anti-mouse IFNγ** | **Biotinylated rat anti-mouse IFNγ** | **Recombinant IFNγ** | 46pg/ml |
| Concentration | 1/500 | 1/1000 | 100ng/ml | |
| Company | BD-Pharmingen | BD Biosciences | BD Biosciences | |
| Clone | R4-6A2 | XMG1.2 | n/a | |
| **IL-13** | **Rat anti-mouse IL-13** | **Biotinylated rat anti-mouse IL-13** | **Recombinant IL-13** | 46pg/ml |
| Concentration | 0.5mg/ml (1/250) | 0.1mg/ml (1/500) | 100ng/ml | |
| Company | R&D Systems, Germany | R&D Systems | BD Biosciences | |
| Clone | 38213.11 | Polyclonal | n/a | |
| **IL-4** | **Rat anti-mouse IL-4** | **Biotinylated rat anti-mouse IL-4** | **Recombinant IL- 4** | 46pg/ml |
| Concentration | 2.2mg/ml (1/500) | 0.5mg/ml (1/1000) | 250ng/ml | |
| Company | BD-Pharmingen | BD-Pharmingen | Pepro Tech EC Ltd. London | |
| Clone | BVD4-1D11 | BVD6-24G2 | n/a | |

## Appendix B - General Buffer recipes

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

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

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

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

*Complete media (Iscove's Modified Dulbeco's Medium (IMDM)):*
1 tube IMDM (Gibco)
750 ml ddH2O
81.7 ml $NaHCO_3$ (37g/L)
2 ml Penicillin/streptomycin (500X)
Adjust the pH to 7.2 – 7.4
Make up to 1L with ddH2O and filter sterilize

*FACS Buffer:*
0.1% BSA (Roche)
0.05% NaN3 (Merck)
Made up in 1X PBS

*MACS Buffer:*
2mM EDTA
0.5% BSA
Dissolve the above in a final volume of 1000ml of 1X PBS and store at 4$^{o}$C.

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

*Permeabilisation Buffer:*
0.5g Saponin
0.055g $CaCl_2$
0.0625g $MgSO_4$
0.25g $NaN_3$
0.5g BSA
10mM Hepes
Dissolve in a final volume of 500ml of 1 X PBS and store at 4$^oC$.

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

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