The influence of maternal *Nippostrongylus brasiliensis* infection on immunity in offspring

Dunja Mrdjen

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Division of Immunology
Institute of Infectious Disease and Molecular Medicine
Department of Clinical Laboratory Sciences
Faculty of Health Sciences
University of Cape Town

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Table of Contents

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Abbreviations

AAM	Alternatively activated macrophage
ADCC	Antibody-dependent cell-mediated cytotoxicity
AP	Alkaline phosphate
APC	Antigen presenting cell; Allophycocyanin-conjugated protein
APRIL	A proliferation-inducing ligand
B cell	Bursa of fabricius Cell
BAFF	B cell activating factor
BCR	B cell receptor
CA	Central arteriole
CAM	Classically activated macrophage
CBMC	Cord blood mononuclear cell
CD	Cluster differentiation
CFU	Colony forming units
DC	Dendritic cell
dH_2O	De-ionized water
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic reticulum
FACS	Fluorescent activated cell sorting
FCS	Fetal calf serum
FDC	Follicular dendritic cell
Fig	Figure
FITC	Fluorescein isothiocyanate
FO B cell	Follicular B cell
GATA-3	GATA-binding protein 3
GC	Germinal centre
HK-STm	Heat-killed *Salmonella*
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
i.p.	Intra-peritoneally
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL-X	Interleukin X
IMDM	Iscovec's modified Eagle medium
LB	Luria broth
LPS	Lipopolysaccharide
M cell	Membranous epithelial cell
MAC	Membrane attack complex
MatAb	Maternal antibody

MDSC	Myeloid derived suppressor cell
MHC	Major Histocompatibility complex
MLN	Mesenteric lymph nodes
MMM	Marginal metallophillic macrophage
MZ B cell	Marginal zone B cell
***N.brasiliensis* or Nb**	*Nippostrongylus brasiliensis*
NES	*Nippostrongylus brasiliensis* excretory-secretory antigen
NF B cell	Newly formed B cell
NK cell	Natural killer cell
NTS	Non-typhoidal *Salmonella*
OCT	Optimal cutting temperature
OD	Optical density
Omps	Outer membrane proteins
p.i.	Post infection
PALS	Periarteriolar lymphoid sheath
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PE	Phycoerythrin
Pen/Strep	Penicillin and streptomycin
PerCP Cy5.5	Peridinin Chlorophyll Protein (Cyanin Dye)
PNP	p-Nitrophenylphosphate
PRR	Pattern recognition receptor
RAG1/2	Recombination activating gene 1/2
RES	Reticuloendothelial system
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
ST	*Salmonella enterica* serovar *Typhi*
STAT	Signal transducer and activator of transcription
STm	*Salmonella enterica* serovar *Typhimurium*
T cell	Thymus cell
T1	Transitional 1
T2	Transitional 2
T2-FP	Transitional 2 follicular precursor
T2-MZP	Transitional 2 marginal zone precursor
TAM	Tumour associated macrophage
T-bet	T-box expressed in T cells
TCR	T cell receptor
TD	Thymus-dependent
Tfh	T follicular helper cell
Th	T helper cell
TI	Thymus-independent
TI-1	Thymus-dependent type 1 antigen
TI-2	Thymus-dependent type 2 antigen
TLR	Toll-like receptor
Treg	Regulatory T cell

ABSTRACT

Helminth infections are highly prevalent in sub-Saharan Africa (SSA) where over 40% of women of child bearing age are infected with helminths. Strong associations have been made with maternal helminth infection and impaired immune response to standard childhood vaccinations. For example, generation of protective antibody to *Heamophilus influenzae* type b post vaccination is inversely proportional to the number of maternal helminth infections during pregnancy. The neonatal immune system is immature and represents a period of general increased susceptibility to infectious disease. It is known that the neonatal immune system is naturally biased towards a Th2 response and the capacity to generate a Th1 response is compromised. Mouse studies have shown that passive maternal antibodies (MatAb) influence development of the B cell repertoire of offspring. Neonates are highly dependent upon MatAb for protection against a wide range of pathogens; however MatAb can suppress an infant's primary antibody response to infection and vaccination. Areas with high helminth prevalence also have high prevalence of non-typhoidal Salmonella infections which lead to progressive bacteraemia in children aged 6-24 months. Infectious history with Nb impairs the same host's ability to control subsequent and simultaneous STm infection, and impairs efficacy of the Porins vaccine against STm. This study investigates imprinting of the murine fetal immune system by maternal infection with the helminth *Nippostrongylus brasiliensis* (Nb) prior to pregnancy and its effect on control of the *Salmonella enterica* serovar *typhimurium* (STm) in offspring. We show that maternal Nb infection in BALB/c mice results in the transfer of Nb antigen (NES)-specific IgG1 *in utero* and through breastmilk, changes in lymphocyte populations and early germinal center formation in naive offspring. Maternal Nb infection does not interfere with control of STm in offspring in BALB/c mice, but may interfere with control of STm in C57BL/6 mice. In BALB/c mice, serum opsonization experiments with serum from adult offspring born to Nb infected mothers showed impaired natural ability to opsonize STm, suggesting that maternal Nb infection has long-term functional effects in offspring. These results further our understanding of how neonatal immune systems are imprinted by maternal infectious history and how this can affect the infant responses against other pathogens in early and later life, and give reason for increased deworming, and better neonatal vaccine strategies.

1. Introduction

1.1 The Immune System

The primary function of the immune system is to protect the host from invading pathogens by distinguishing self from non-self, mounting an inflammatory response to eliminate the foreign infectious agent while maintaining tolerance to 'self'. These infectious agents range from the sub-microscopic virus and the unicellular bacteria to multicellular macroparasites. They can be intracellular: all viruses, some bacteria (e.g. *Mycobacterium leprae*) and some parasites (e.g. *Plasmodium falciparium*), or extracellular: some bacteria (e.g. *Streptococcus pneumoniae*), all fungi and some parasites (e.g. parasitic worms like *Ascaris* and *Schistosoma*).[1] While our growing understanding of the immune system has revealed its influence on a variety of different biomedical fields and shed light on many non-communicable human diseases (e.g. diabetes, multiple sclerosis), resistance to infection is still its primary role.[2]

The immune system is composed of physical and biochemical barriers such as skin, mucus, stomach acid and tears, and is maintained by various kinds of leukocytes (immune cells, also known as white blood cells) and soluble protein mediators including cytokines and antibodies.[1] Leukocytes originate from the primary lymphoid organs, the bone marrow and thymus, migrate through the blood to secondary lymphoid organs where they detect infection in the form of foreign antigen. The three major secondary lymphoid organs are the spleen which collects antigen from the blood, the lymph nodes which collect antigen from the tissues, and the mucosal-associated lymphoid tissues (MALT) which collect antigen from the epithelial surfaces of the body.[3]

1.2 Innate vs. Adaptive

The immune system protects the host by fulfilling four main tasks: immunological recognition, immune effector functions, immune regulation and immunological memory. These tasks are carried out by the co-ordinated efforts of the functionally distinct innate and adaptive responses. The innate response is the non-specific first line of defence and is responsible for detecting the pathogen, immediate and rapid control of the infection and, if unable to effectively eliminate the pathogen, induction of the adaptive response. The adaptive response is slower to provide protection to a primary infection and is characterized by its adaptive specificity to that particular pathogen, and generation of immunological memory which ensures stronger, more rapid and more specific induction upon re-encounter of that pathogen and is the fundamental principle behind vaccination.

1.3 Evolution of the Immune System

The innate immune response is much more ancient than the adaptive response. Heritable innate mechanisms of self-defence are present even in unicellular organisms, which appeared 3.5 billion years ago, and every multicellular organism, animal or plant, possesses a complex innate immune system.[4] Through the phylogenetic study of the key elements of an adaptive response, i.e. TCR, BCR, RAG1/RAG2, and MHC class I and class II genes, which make possible the recognition and specific attack of a near infinite number of different pathogens, evolutionary immunologists have shown that all jawed vertebrates possess an adaptive immune response.[5,6] The acquisition of this adaptive response is thought to have occurred between the divergence of jawed vertebrates (gnathostome) and jawless vertebrates (agnathans, of which only hagfish and lampreys still exist), over 500 million years ago.[7] Therefore, the co-evolution of the innate and adaptive immune systems over the last 500 million years has ensured their complementary and inextricably coordinated roles in mediating host protection. This interplay requires communication between the cells that make up the innate and adaptive response, by cell-to-cell contact and by the secretion and binding of cytokines to cell surface receptors.

1.4 Innate Immunity

The innate response recognizes invading pathogens, attempts to eliminate the pathogen and recruits the adaptive response if the infection becomes unmanageable. With the exception of natural killer (NK) cells,[8] innate leukocytes and soluble proteins are not pathogen specific in their response, have no memory and respond with the same in magnitude upon re-encounter with the same pathogen. Recognition occurs through the binding of Pathogen Associated Molecular Patterns (PAMPs) to germ line encoded, non-clonal Pattern Recognition Receptors (PRRs) and Toll Like Receptors (TLRs) on innate cells.[9] PAMPS include highly evolutionarily conserved molecules like LPS, bacterial CpG DNA and viral double-stranded RNA which are maintained after pathogen replication within the host.

1.4.1 Innate cells

Innate cells are hematopoietic cells originating in the bone marrow and include dendritic cells (DCs), macrophages, neutrophils, NK cells, mast cells, basophils and eosinophils.[10] From the bone marrow, immature cells move into the blood where some mature (e.g. NK cells, neutrophils) while others leak into tissues by extravasation and mature there (macrophages, DCs and mast cells). If there is an infection, cells encounter the foreign antigen and rapidly differentiate into short-lived effector cells, each with a different role in eliminating the pathogen.[1]

Professional phagocytic cells which include neutrophils, macrophages, tissue DCs and B cells bind to extracellular microbes or cell debris before engulfing and destroying them through fusion of the phagosome with acidic lysosomes into a phagolysosome.[4] Granulocytes like eosinophils and basophils release enzymes which can neutralize pathogens that are too big to be phagocytosed, including parasites like helminths.[1,11] NK cells can recognise and kill tumour cells or cells that are infected with internal pathogens by releasing cytotoxic granzymes which cause apoptosis of the target cell.[1] A set of innate serum proteins known as 'acute phase proteins', including C-reactive protein and complement proteins, are activated by antibody:antigen complexes or by pathogen surfaces and either directly kill pathogens by formation of a membrane attack complex (MAC) by or targeting them for phagocytosis.[12]

Mast cells are non-professional or occasional phagocytes and are important mediators in allergic inflammation and asthma. Here they provoke inflammation in response to innocuous antigens not associated with pathogens. Mast cells contain preformed granules of chemical mediators like histamine, which are released by mast cell degranulation upon cross linking of their high affinity receptors FcεRI with the antibody IgE.[1]

Phagocytes have multi-layered functions. Macrophages are present in virtually all tissues. They are a morphologically diverse and functionally plastic population of cells, taking on different morphologies and functions in addition to phagocytosis of pathogens and pathogen products. These functions and nomenclature depend on the tissue of residence (e.g. skin, liver, spleen, bone, brain, placenta) **(Fig 1.1)**, the type of invading pathogen (intracellular vs. extracellular) and duration of the infection (e.g. chronic vs. short-term helminth infection).[13] Their primary function is, in fact, to act as scavengers and 'janitor cells' and clear the interstitial environment of cellular debris and apoptotic cells. This occurs constantly through recognition via homeostatic receptors and without signalling from other immune cells or the production of cytokines.[14]

Macrophages also act as supervisors of the innate response and recruit other phagocytes to the site of infection through secretion of chemotactic cytokines called chemokines.[4] Importantly, macrophages function as immune surveillance cells which respond differently depending on the type of pathogen they encounter. Classically activated macrophages (CAMs) or M1 macrophages respond to intracellular pathogens like a variety of bacteria, viruses and protozoa, as well as during tumorigenesis, where they induce an inflammatory killing immune response. Alternatively activated macrophages (AAMs) or M2 macrophages respond to some extracellular pathogens and have regulatory functions. They are activated in chronic infection with helminths[15] wound healing where they have a suppressive immunomodulatory roles. They are also involved in allergic responses but their role here is controversial, some studies suggesting that they promote allergic inflammation and others suggesting a suppressive role.[16] Specialized tumour associated macrophages (TAMs) have antitumour immunity. Myeloid-derived suppressor cells (MDSCs) are a group of immature cells which include the precursors to AAMs and TAMs, exhibiting immune suppressive activity.[13]

A recently discovered population of innate cells called nuocytes has been shown to activate responses against helminths by secreting cytokines which polarize adaptive immunity towards helminth specific T cell responses.[17] DCs primarily act as highly specialized antigen-presenting cells (APCs) which internalize pathogens and foreign proteins and move to lymph nodes where they present the processed antigen and thereby signal to T cells that reinforcements are needed. DC antigen presentation to and activation of T cells, macrophage chemokine and cytokine secretion and nuocyte cytokine secretion are important links between the innate and adaptive responses.[4,18]

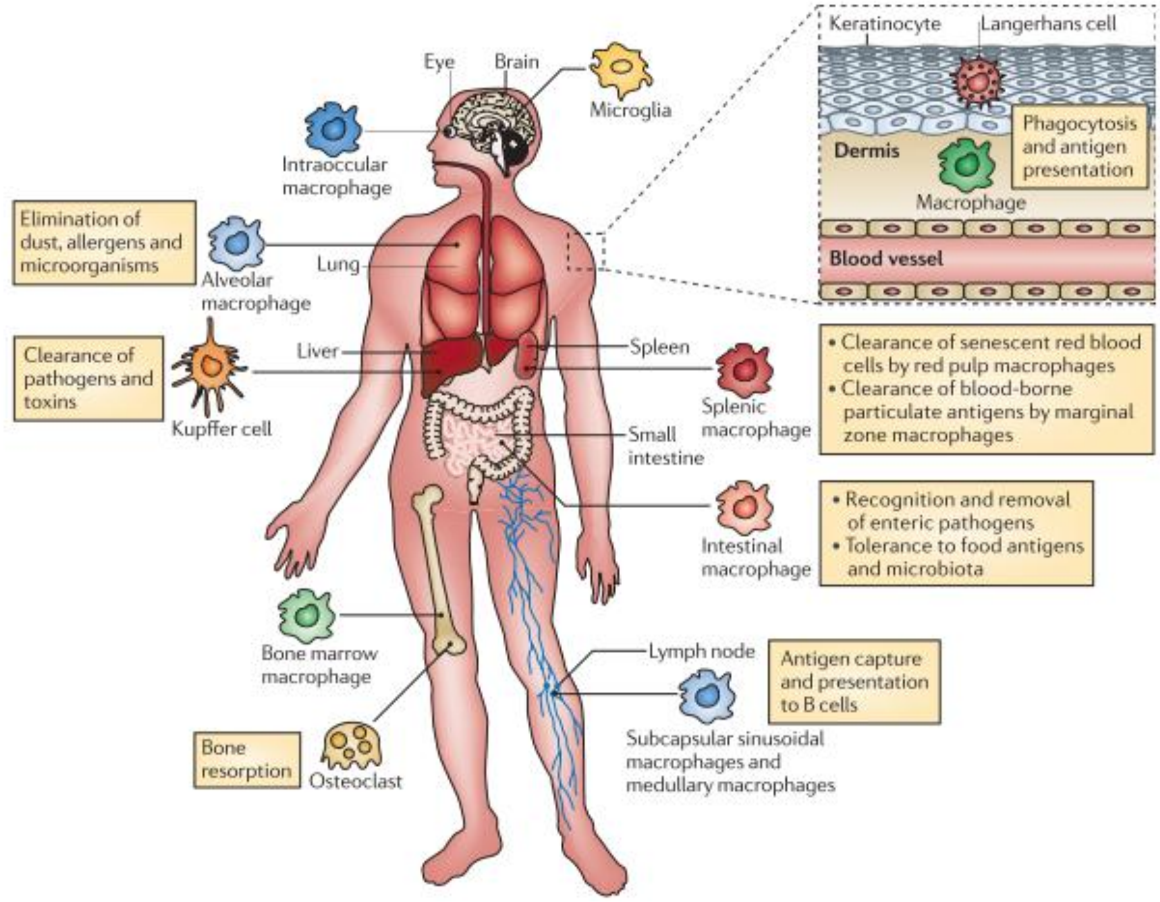


Figure 1.1 Macrophage precursors are released into the circulation from the bone marrow as monocytes. They migrate into nearly all tissues where they differentiate into specialized tissue specific mature macrophages. Various subsets are distributed at strategic locations around the body and perform immune surveillance, antigen presentation, immune regulation, clearances of cellular debris and tissue remodelling. (Image from Murray & Wynn, 2011)

1.4.2 Antigen presentation

Antigen presentation by MHCI (Major Histocompatibility Complex I, HLA molecules in humans) molecules in mice occurs on most nucleated cells. A sample of all self peptides produced in the endoplasmatic reticulum (ER) is loaded onto MHCI molecules and transported to the surface of the cell. This allows for the differentiation between self and non-self. Since viral infections are always intracellular and the virus makes use of the ER to produce its proteins, MHCI molecules also present viral peptides and therefore signal virally infected cells.[1] Antigen presentation by MHCII molecules occurs by APCs only. APCs are also phagocytes. Internalization of the pathogen or foreign complex protein is followed by its degradation into peptides in the phagolysosome and loading of the foreign peptide onto an MHCII molecule which then moves to the surface of the cell and signals to T cells of the adaptive response.[1]

1.4.3 Induction of the adaptive response

Naive T cells require two signals to be fully activated: cognate interaction with MHCI or MHCII molecules complexed to foreign peptides and the engagement of costimulatory molecules expressed on APCs, such as CD80 and CD86, with receptors on T cells, such as CD28 **(Fig 1.2)**.[18] Thus antigen presentation by innate cells functions to 1) alert the adaptive response that clonal expansion of antigen-specific cells is required to control the infection and 2) to instruct it on the nature of the pathogen and the type of adaptive response that is required.

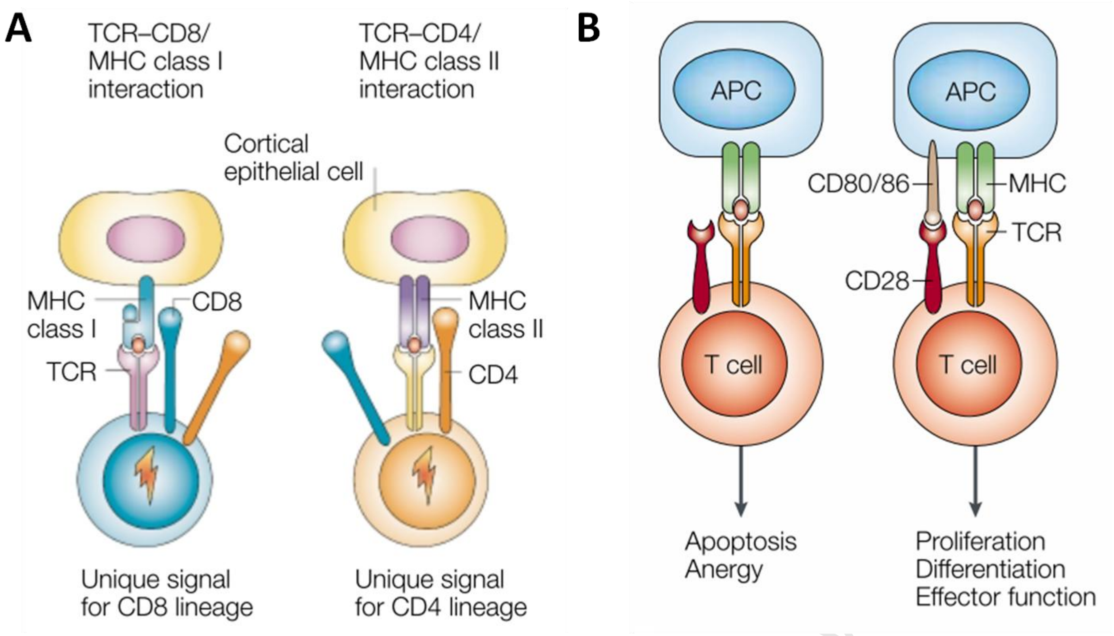


Figure 1.2 **T cell activation requires two signals. (A)** CD8+ and CD4+ T cells recognize antigen through their T cell receptors (TCR) binding to peptide antigen presented on MHCI and MHCII molecules, respectively. (Germain, 2002) **(B)** Naive T cells are activated by TCR recognition of antigen:MHC complexes on antigen presenting cells (APCs) and cognate interaction of CD28 with co-stimulatory molecules CD80 or CD86. If costimulation does not occur, the T cell undergoes apoptosis or anergy. (Alegre et al., 2001)

1.5 Adaptive Immunity

1.5.1 Lymphocytes and their antigen receptors

Lymphocytes are cells of the adaptive response and are primarily composed of T and B cells. Both originate in the bone marrow. T cells mature in the thymus whereas B cells mature in the spleen. T cells poses TCRs on their cell surfaces which recognize antigen bound to MHC molecules **(Fig 1.2 A)**, and, along with costimulatory receptor binding, activate the cell **(Fig 1.2 B)**. B cells possess BCRs (B cell receptors) which are surface immunoglobulins and bind antigen directly, internalize the antigen by phagocytosis and present it on MHC molecules. TCRs and BCRs are assembled from called the Variable, Diverse and Joining gene segments by a process called V(D)J rearrangement in the central lymphoid organs (thymus and bone marrow). The binding cleft of these receptors (encoded by the Variable gene segment, and forming the "variable region" of the receptor) is encoded by many different genes which allows the formation of a diverse set of receptors and the recognition of a large variety of antigen or antigen:MHC complexes.[1] TCRs and BCRs are essential to T and B cell survival, respectively, in their primary lymphoid organs of origin as well as in the periphery where it is thought that they both receive essential positive signals through low affinity binding of self antigen to their receptors.[19]

1.5.2 Tolerance induction

While cells of the innate system differentiate between self and foreign antigen by the germline encoded specificity of their PRRs, lymphocyte receptors undergo somatic hypermutation throughout the lifetime of the organism to adapt to the wide variety of antigen they encounter, however receptor specificity must not be directed against self antigen with high affinity. Lymphocytes undergo checkpoints during maturation to eliminate self-reactive T and B cells and prevent autoimmunity, a process called tolerance induction. T cells undergo two checkpoints of positive and negative selection during maturation in the thymus, mediated through TCR recognition of antigen:MHC complexes. A positive signal is provided by the binding of the TCR to self antigen:MHC complexes on thymus stromal cells and is required for T cell survival.[19] However, this signal must be weaker than that needed to activate a mature T cell with the same TCR, i.e. the binding must be of low affinity.[20] This is thought to be primarily required to ensure MHC restriction, that T cells only respond to antigen bound to the host's own MHC molecules.[21] TCRs that recognize self antigen:MHC complexes with high affinity are removed by clonal deletion during negative selection.[22]

Less is known about B cell tolerance induction. Two maturation checkpoints have been identified and these are both negative selection: a central checkpoint in the bone marrow and a peripheral checkpoint in the spleen.[23] B cells whose BCRs recognize self antigen with high affinity (to a multivalent antigen) either undergo receptor editing and/or clonal deletion, or if recognition is with low affinity (to a soluble, monovalent antigen), the B cell becomes anergic (discussed in more detail below).[24,25] Partly because BCRs recognize antigen directly and are not dependent upon MHC molecules on APCs, it has been difficult to determine and is yet unclear whether B cells undergo positive selection like T cells do.[26]

1.5.3 T cells

T cells have many subsets but the better characterized effector cells are CD4+ helper T cells and CD8+ cytotoxic T cells. They are MHCII and MHCI restricted, respectively **(Fig 1.2A)**.[27] Following activation, helper T cells stimulate antibody production by B cells and increased efficiency of innate responses, such as increased killing of pathogens by phagocytes.[28] CD8+ cytotoxic T cells, following activation, destroy cells infected by intracellular pathogens like viruses by inducing them to undergo apoptosis and thereby containing the infection. Importantly, cytotoxic T cells typically induce apoptosis, as opposed to necrosis, which largely contains intracellular pathogens. Helper T cells are important in *Salmonella* and helminth infections and will be discussed in greater detail.[19]

1.5.3.1 CD4+ T helper 1 vs. T helper 2

After activation or "priming", helper T cells start to proliferate and acquire effector characteristics. Helper T cells differentiate into one or more functionally distinct subsets and acquire the capacity to interact with B cells in secondary lymphoid organs and activate different immune mechanisms to eliminate the pathogen.[29] The subset that gains predominance is determined by 1) the type of APC presetting the antigen, 2) the co-stimulatory molecules, 3) the nature and dose of the pathogen and 4) the immediate cytokine environment experienced by the T cells at the time of antigen presentation.[30]

Two major subsets of the helper T cell response are the T helper cell 1 (Th1) and T helper cell 2 (Th2) response, characterized by the cytokine profile they produce and, in mice, the switched antibody isotype they induce in B cells **(Fig 1.3)**. Th1 responses induce the phagocyte-dependent host response to most intracellular pathogens. The cytokine interleukin-12 (IL-12), produced initially by APCs and particularly by macrophages, is the predominant cytokine that induces Th1 differentiation. Binding of IL-12 to its receptor on primed helper T cells results in signalling through the STAT-1 pathway and upregulation of the transcription factor T-bet.[31] Th1 cells then upregulate the IL-12 receptor (IL-12Rβ2) and release high levels of cytokines interferon gamma (IFNγ), IL-2 and tumour necrosis factor beta (TNF-β). This promotes the activation of macrophages, antibody-dependent CD8+ T cell

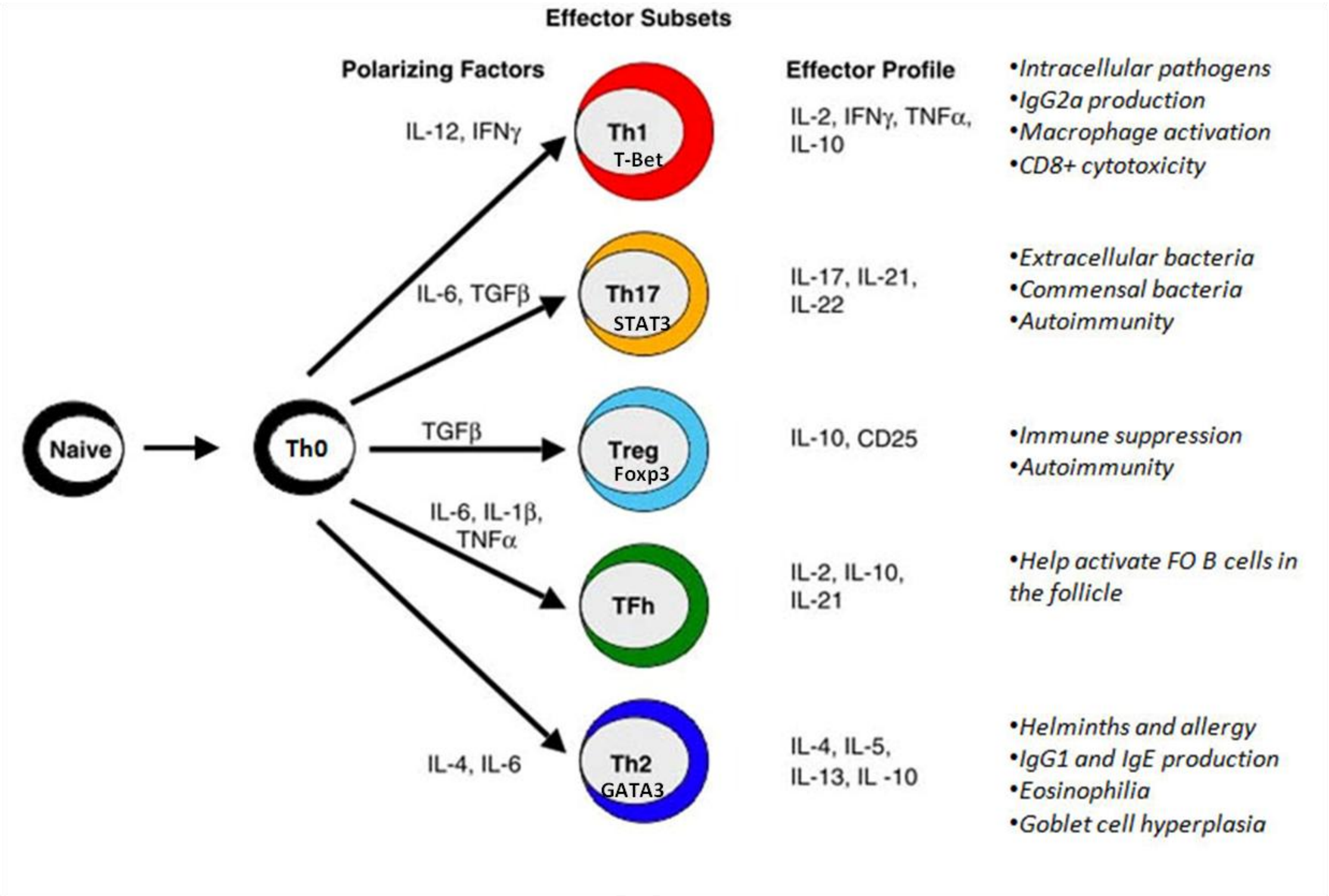


Figure 1.3 CD4+ T helper cell polarization into various effector subsets and the cytokines that induce them. Activation of naive CD4+ T helper cells in response to a particular inflammatory environment results in the polarization into a specific subset of effector T cells. They are defined by their expression of transcription factors, effector profile of cytokine secretion and activation of the adaptive response to clear the infection. Two such subsets are Th1 and Th2 cells which respond to intracellular and large extracellular pathogens, respectively, and oppose each other's functions. Other subsets include Th17, Treg and Tfh subsets. (Image adapted from Jelly-Gibbs et al., 2008)

cytotoxicity and delayed type hypersensitivity (DTH), as well as the stimulation of B cells to produce IgG2a opsonizing and complement-fixing antibodies.[32] Signalling through the STAT-4 pathway via the IL-12Rβ2 also stimulates expression of IFNγ and the IL-18 receptor, creating a positive feedback loop.

Th2 responses are humoral and phagocyte-independent, and necessary for the expulsion of extracellular pathogens such as helminths (e.g. *Nippostrongylus brasiliensis)*.[33,34] In allergic reactions, however, a harmful, exaggerated Th2 response is elicited against seemingly harmless antigens in genetically predisposed individuals which could lead to anaphylactic shock and death.[35] Th2 responses are now known to be initiated by the secretion of IL-25 and IL-33 cytokines from epithelial cells which activate innate nuocytes via their T1/ST2 receptor (a member of the TLR IL-1R). Nuocytes then produce the polarizing T cell cytokine IL-4.[36] IL-4 binding to IL-4Rα on primed helper T cells induces Th2 differentiation via signalling through the STAT-6 pathway and upregulation of the transcription factor GATA-3.[37] The T1/ST2 receptor has also been found on eosinophils and basophils,[36] which, along with the non-conventional innate-like γδ T cell subset, NK T cells and mast cells, also produce the polarizing cytokine IL-4.[38] Th2 cells then secrete predominantly IL-4 and IL-13, but also IL-5, IL-6, IL-9 and IL-10 and provide optimal help to B cells in the spleen to induce their switching to IgE and, in mice, IgG1.[39] Th2 responses also include eosinophilic inflammation, mast cell proliferation, goblet cell hyperplasia and excessive mucous production at mucosal surfaces. The details of Th2 response to helminths are discussed in Section 2.11.1 in Chapter 2.

Th1 and Th2 effector responses counter regulate each other. The IL-12 produced by a Th1 response suppresses Th2 polarisation and IL-10 blocks Th1 cells developing. This occurs at the signalling and genetic levels, where Th2 associated GATA-3 opposes Th1 associated STAT-1 signalling by blocking IL-12Rβ2 expression, whereas STAT-1 downregulates GATA-3 expression.[40]

1.5.3.2 Other CD4+ T cell subsets

The Th1/Th2 paradigm does not cover all helper T cell responses and there are other subsets which perform specific functions along with Th1 or Th2 cells **(Fig 1.3)**. Th17 cells, stimulated by IL-6 to produce IL-17, aid in immunity to extracellular bacteria, particularly on mucosal surfaces.[41] Found in the gut, Th17 cells are activated by specific commensal bacteria can promote their differentiation.[42] A major function of helper T cells is to help stimulate B cells to produce antibody to T-dependent antigens. A subset called T follicular helper (Tfh) cells reside in B cell follicles in secondary lymphoid organs and specifically enter the germinal center. Here they mediate their function by producing IL-4 and IFNγ, but no other signature cytokines, to help B cells generate antigen specific antibodies.[43] An important subset of T cells that regulates and prevents over-activity of the inflammatory response to protect host tissue from damage is the regulatory T cells (Tregs). Treg are CD4+CD25+FoxP3+, cause immuno-suppression and are induced by IL-10.[41] Deficiency in Tregs results in lethal multi-organ autoimmune syndrome and a hyper-reactivity to commensal bacteria.[44,45]

1.5.4 B cells

Mature B cells can be divided into distinct subsets which respond to T-dependent and T-independent antigens and are designated: conventional B2 B cells which comprise follicular (FO) B cells found in the follicles of secondary lymphoid organs and marginal zone (MZ) B cells found only in the spleen, as well as B1 B cells which comprise B1a and B1b B cells found in the peritoneal and pleural cavities and the gut lamina propria **(Fig 1.4)**.

1.5.4.1 Antibodies

As discussed, B cells function to present antigen and secrete cytokines, but their main function is the production and secretion of antigen specific antibodies, also called immunoglobulins (Ig), of different isotypes (IgM, IgA, IgG1, IgG2a, etc.). Antibodies neutralize the effect of a pathogen or a toxin through binding to its surface and preventing it from entering the host cell or causing damage to cells and tissues. Antibodies can also opsonize pathogens to enhance their recognition and destruction by phagocytes[46] or facilitate their destruction by a mechanism called is Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC).[47] Less prominent in mice and more relevant in cancer therapy, ADCC functions by NK T cells and eosinophils destroying antibody-coated cells without phagocytosis by the release of lysis products.[47] Another mechanism antibodies use is to activate complement proteins to attack and destroy antibody coated cells.[48] The specificity of the antibody produced by a B cell is the same as its surface Ig or BCR.

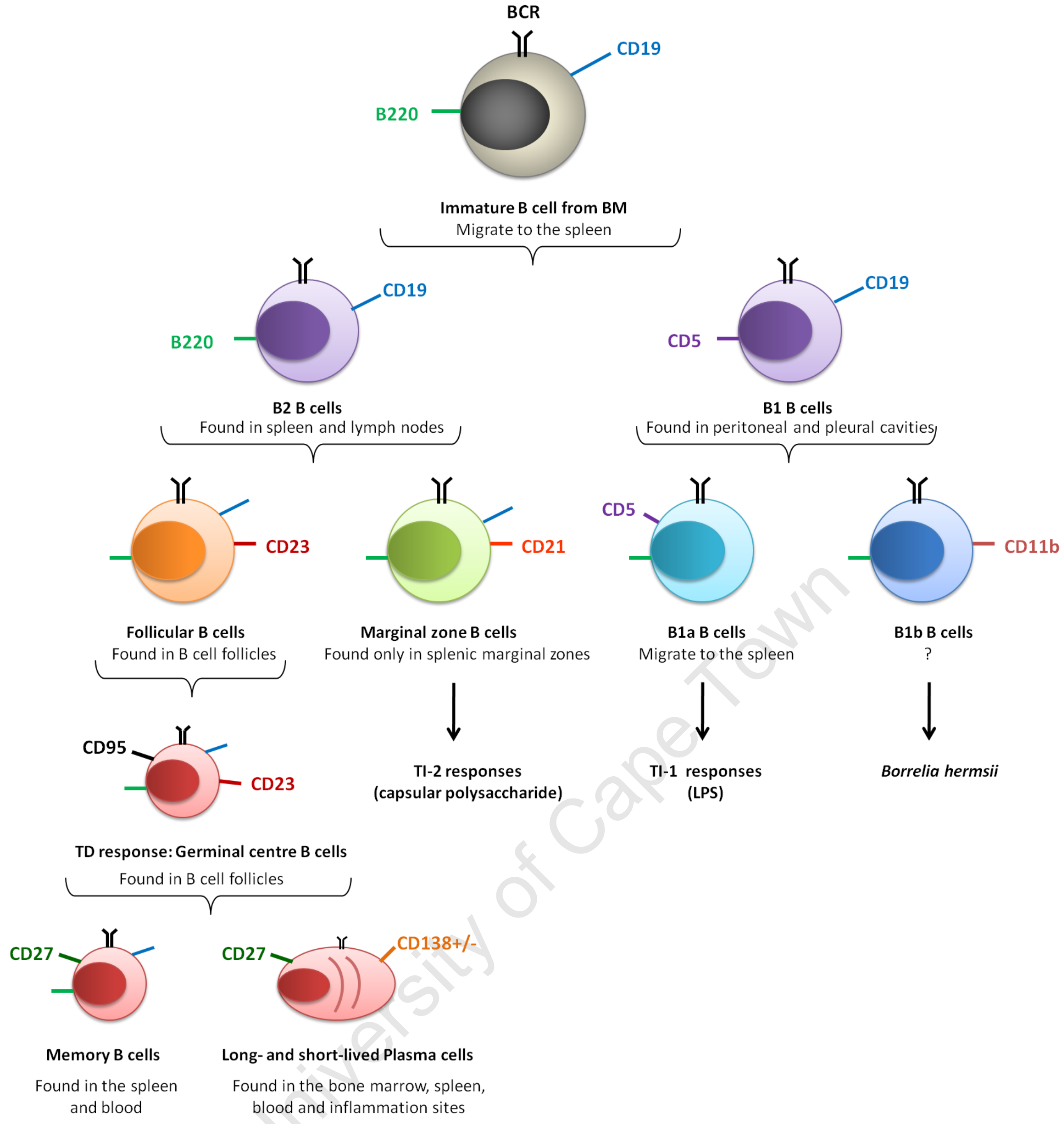


Figure 1.4 **Hierarchy of B cell populations, their surface expression of receptors and TD or TI response to antigen types.** B2 B cells represent the largest proportion of adult B cells and are subdivided into follicular and marginal zone B cells which respond to TD and TI antigens respectively. FO B cells can undergo affinity maturation in a germinal centre reaction in response to antigen which results in high affinity antibody secreting memory B cells and long- and short-lived plasma cells. Marginal zone B cells are stimulated by TI-2 antigens and form IgG3 secreting short-lived plasma cells. B1 B cells are differentiated from B2 B cells by their unique surface markers, anatomical location and their ability to self-renew. B1a B cells respond to TI-1 antigens and form IgM and IgA secreting plasma cells, as well as natural IgM antibodies. The function of B1b B cells is unknown but they are important in the TI response to relapsing fever caused by *Borrelia hermsii*. MZ and B1a 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 (APCs). BM: bone marrow, TD: T-dependent, TI: T-independent.

1.5.4.2 B cell maturation

Before birth, B cells develop in the fetal liver from pluripotent hematopoietic cells[24] and, as described in section 1.5.1, in the bone marrow after birth. B2 B cells (also called conventional B cells) then migrate to the spleen where they mature. B1 B cells, on the other hand, are innate-like B cells and complete their maturation on the bone marrow during the neonatal period. They are found in the peritoneal and pleural cavities but can migrate to the spleen, mesenteric lymph nodes and gut associated lymphoid tissues.[49]

Bone marrow development is called central B cell development and generates immature, BCR expressing B2 cells (from now on referred to as B cells) called newly-formed or transitional-1 (NF/T1) B cells. The development of B cells can be tracked based on the surface expression of various receptors **(Fig 1.4, Table 1.1).** NF/T1 B cells express high levels of early markers CD24, CD93 (also referred to as AA4.1) and surface IgM, very low levels of CD21 and have yet to acquire the B cell markers IgD and CD23 (and are therefore $CD24^{hi}CD93^{hi}IgM^{hi}CD21^{-/lo}IgD^{-}CD23^{-}$).[50] NF/T1 B cells enter the splenic follicles where they upregulate expression of IgD and mature into T2 B cells which can be divided into two separate populations, based on their subsequent lineage decision.[51] T2-FP B cells are the precursors to follicular (FO) B cells, are present in all secondary lymphoid follicles and are $CD21^{int}$. T2-MZP cells are the precursors to marginal zone (MZ) B cells, are only seen in the spleen and are $CD21^{hi}$ (complement binding receptor).[51] Both T2 populations are $CD23^{+}$. T2-FP B cells mature further into mature FO B cells, a highly differentiated population which loses expression of the early markers and is now $CD23^{hi}CD21^{int}IgM^{lo}IgD^{hi}$. T2-MZP B cells become mature MZ B cells that are $CD1d^{hi}CD23^{-}IgM^{hi}IgD^{lo}$.[26] The signals that control maturation into FO or MZ B cells include BCR signalling,[52] cognate interactions with and co-stimulation by T cells,[53] and antigen dependent selection.[50]

Few transitional B cells become mature, long-lived B cells. Out of the $2x10^{7}$ IgM^{+} B cells that develop daily in the bone marrow of the mouse, 10% reach the spleen and only 1–3% enter the mature B cell pool.[52] Mature FO and MZ B cells, now ready to become activated by stimulating antigen, respond to different types of antigen, require different activation signals and have different types of antibody responses.

<u>Table 1.1</u> B cell receptors that are used to differentiate different B cell populations. Expression of surface receptors changes throughout B cell maturation, differentiation, antigen stimulation and interaction with T cells. These receptors are used as markers to find specific B cells by flow cytometry and immunohistochemistry. CAM: cell adhesion molecule.

B cell marker	Cell type	Function	aka	Reference
B220	All B2 cells with diffuse levels of expression, excpet plasma cells	Co-stimulation	**CD45R**	*Hardy and Hayakawa (1991)*
CD19	All B cells except B1 B cells; low expression on plasmablasts	Co-receptor; part of BCR complex, regulating the BCR signal	-	*Engel et al. (1999)*
CD21	Highly expressed on MZ B cells	Complement receptor 2; binds variations of C3 protein as a co-receptor when complexed to CD19 and CD81	**CR2**	*Zandvoort and Timens (2002)*
CD23	Highly expressed on FO B cells	FcεRII: low affinity receptor for IgE; binds IgE immune complexes	**FcεRII**	*Palanichamy et al. (2009)*
CD93	Transtional B cells; re-expressed late in differentiation on PCs	Cell adhesion molecule (CAM); involved in clearance of apoptotic cells and survival of long-term PCs in the bone marrow	**AA4.1**	*Malissen et al. (2009*
CD24	Transitional B cells, GC B cells	CAM; associated with tumour cells	**HSA**	*Loder et al. (1999);* *Shinall et al. (1950)*
IgM	Transitional B cells, MZ B cells, B1 B cells	BCR	-	*Hardy et al. (2001);* *Su et al. (2004)*
IgD	Highly expressed on FO B cells	BCR	-	*Hardy et al. (2001);* *Su et al. (2004)*
CD95	GC B cells	Induces apoptosis	**Fas receptor; death receptor**	*Kondo et al. (2007)*
GL7	GC B cells	B cell activation	-	*Naito et al. (2007)*
PNA	GC B cells	Binds carbohydrate sequence Gal-β(1-3)-GalNAc	-	*Coico et al. (1983)*
CD27	Memory B cells and plasma cells	Part of the TNF receptor family; regulates B cell activation and Ig synthesis	-	*Borst et al. (2005);* *Jacobi et al. (2010);* *Qian et al. (2010)*
CD138	Plasma cells (but not all plasma cells)	Cell migration; cell adhesion; cell proliferation; internalization of HIV-1 tat protein	**Syndecan-1 (SDC1)**	*Qian et al. (2010)*
CD5	B1a B cells	Negatively regulates the BCR signal to prevent autoimmunity	-	*Berland et al. (2002)*

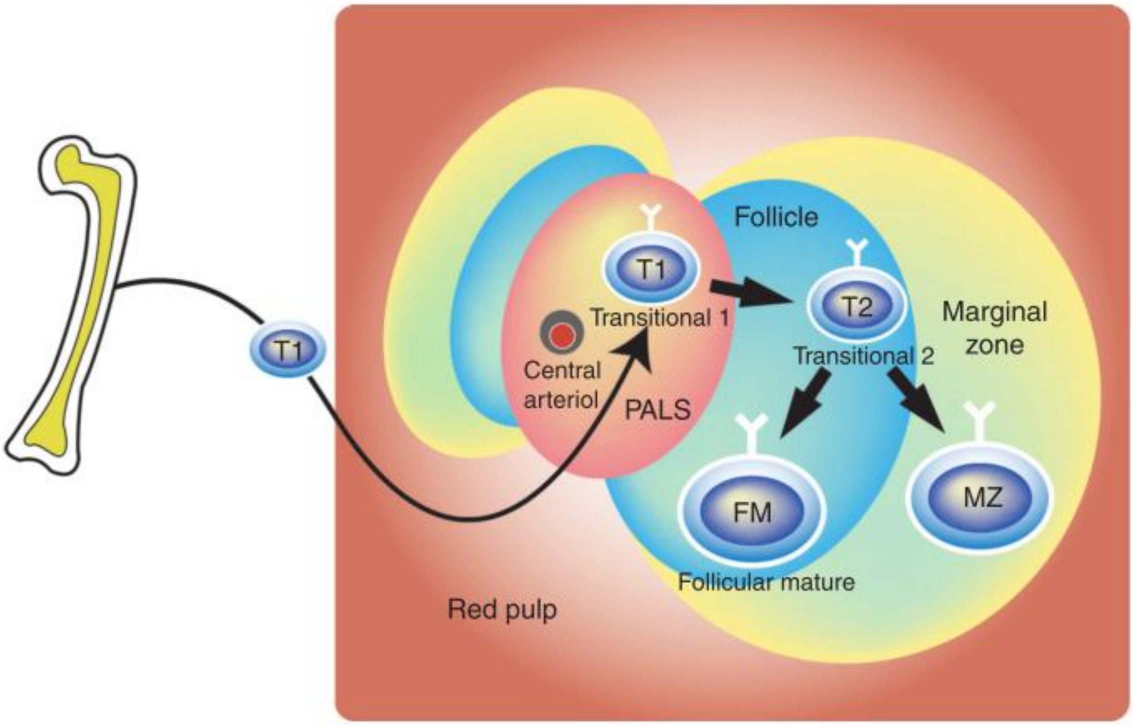
1.5.5 T-dependent vs. T-independent antibody responses

Nearly all protein antigens require cognate interaction between helper T cell and B cell along with stimulation of the BCR in order to initiate antibody responses, and are called Thymus-dependent (TD) antigens. 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 Thymus-independent (TI) antigens.[54] TI antigens have been sub-classified into: Type-I (TI-1 antigens) and Type-II (TI-2 antigens) which activate B cells by different mechanisms.[55] Naive mature B cell antigen activation occurs in secondary lymphoid organs, the spleen being the most highly organized tissue optimal for B cell stimulation with antigen, interaction with T cells and splenic APCs.

1.5.5.1 Spleen microarchitecture

The spleen is the largest secondary lymphoid organ and contains about one fourth of the body's lymphocytes, initiating immune response to blood-borne antigen. The spleen comprises mostly red pulp which contains splenic macrophages that phagocytose aged or dead red blood cells and opsonized blood-borne microbes.[56] Interspersed amidst the red pulp are discrete areas of lymphoid white pulp. The white pulp is arranged in specialized layers of cells around a central arteriole (CA) that brings lymphocytes and antigen to the spleen. These layers comprise the periarteriolar lymphoid sheath (PALS), the follicles and the marginal zone **(Fig 1.5)**.[57] The marginal sinus separates the follicle and the marginal zone and is where blood-borne antigen enters the tissue.[56]

The PALS contains mostly CD4+ T cells in the T zone, with macrophages around its edges, interdigitating DCs and migrating B cells and is the site of CD4+ T cell interaction with antigen presenting DCs. The follicle is continuous with the PALS and mainly consists of FO B cells surrounded by a network for follicular DCs (FDCs) and some macrophages.[57] The marginal zone lines the boundary between the white and red pulp and contains mainly MZ B cells and also MZ macrophages and marginal metallophillic macrophages (MMMs). Full development of the MZ B cells and the marginal zone is delayed until about 3-4 weeks after birth in rodents and 2 years in humans.[51] The organization of the white pulp of the spleen is similar to that of a lymph node, but while the spleen detects blood borne antigens, lymph nodes detect tissue localised antigens in draining lymph fluid.

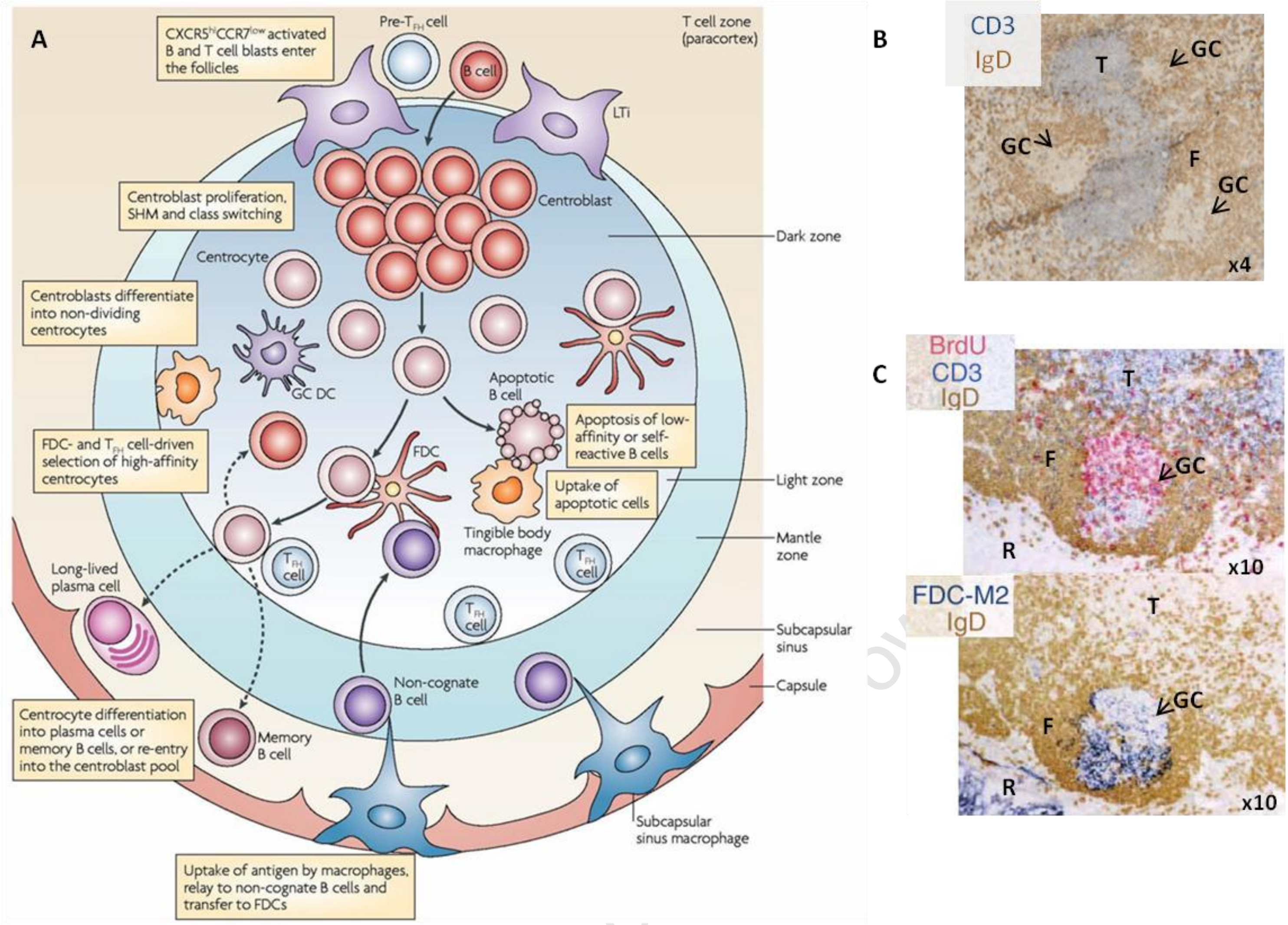


<u>Figure 1.5</u> The microarchitecture of the spleen showing B cell maturation from the bone marrow. The spleen is highly organized to allow optimal stimulation of B cells with antigen and interaction with helper T cells and APCs to promote antibody production via TD and TI responses. Amidst the red pulp are discrete areas of lymphoid tissue, called white pulp, found in layers surrounding a central arteriole (CA). The periarteriolar lymphoid sheath (PALS) comprises mostly T cells in the T zone but also some B cells and macrophages, and is an important site for lymphocyte trafficking. Immature transitional-1 (T1) B cells coming from the bone marrow enter the splenic white pulp from the CA into the PALS. Continuous with the PALS or T zone are the B cell follicles where T1 B cells mature to T2 B cells and then to follicular (FO) B cells. FO B cells are mobile and constantly migrate to and from B cell follicles in secondary lymphoid organs around the body. T2 B cells also mature into marginal zone B cells (MZ) which make up the marginal zone surrounding the follicles and the PALS and separating the white pulp from the red pulp. Between the marginal zone and the follicles is the marginal sinus (not shown here) where blood-borne antigen enters the white pulp and is captured by marginal zone macrophages and macrophages lining the outer PALS. (Su et al., 2004)

1.5.5.2 T-dependent antibody responses

Naive FO B cells represent the largest proportion of the adult B cell population. They are in a constant state of migration between the follicles of secondary lymphoid organs.[58] Migration ceases if the B cells are recruited into an antibody response. Protein antigens stimulate antibody responses from FO B cells to form highly specialized, transient germinal center (GC) reactions in follicles which are the source of high affinity antigen specific plasma cells and memory B cells. This process is initiated at the boundary between primary follicles (follicles without GCs) and the T zone where rare B and T cells specific for the same antigen are primed by antigen presented on interdigitating DCs.[59] FO B cells then migrate into the follicle where they undergo massive clonal expansion and somatic hypermutation of their Ig-variable-regions where the variable region of the BCR is further diversified by point mutations.[60] These cells form the germinal center (GC) and, as the GC matures, separate into the dark and light zones **(Fig 1.6)**.

The dark zone is oriented towards the T zone and comprises tightly packed centroblasts undergoing rounds of replication, somatic hypermutation and antibody class switching.[56] The centroblasts then stop dividing and become small centrocytes which undergo selection in the light zone based on the affinity of their surface antibody (BCR) to the inducing antigen. Screening for self-reactive B cells also occurs in the light zone and these B cells undergo apoptosis. All apoptotic B cells in the GC are phagocytosed by tingible body macrophages.[60] The light zone, strategically oriented towards the marginal sinus and the source of foreign antigen in the spleen,[61] also contains a rich network of FDCs which are distinct from FDCs found in primary follicles.[59] These FDCs have the capacity to take up antigen and retain it for periods of over a year. This antigen can be taken up by a centrocyte through its BCR, internalized, processed and presented on its MHCII molecules to Tfh cells which recognizes it. In this process of linked recognition, the B and Tfh cells are specific for the same antigen and the B cell receives two signals which select it: antigen recognition through its BCR and co-stimulation by interaction between CD40L on the Tfh cell and CD40 on the B cell.[60]



<u>Figure 1.6</u> Germinal centre cell types and events. (A) Germinal centres (GCs) develop within B cell follicles and are seeded by a small number of activated B cells. B cells and T follicular helper (Tfh) cells specific for the same antigen establish stable interactions at the boundary between the T zone and the follicle and enter follicles, receiving survival signals. GC B cells proliferate and displace naive FO B cells, forming the two zones of the GC: the dark zone and the light zone. The dark zone contains densely packed, highly proliferative centroblasts undergoing rounds of somatic hypermutation (SHM) in their immunoglobulin variable regions. The light zone is full of follicular dendritic cells (FDCs) that bind and present antigen in the form of unprocessed immune complexes. Once they stop dividing, centroblasts move to the light zone where they are called centrocytes and are selected based on their affinity for antigen presented by FDCs and their cognate interaction with Tfh cells. Low affinity and self-reactive B cells undergo apoptosis and are phagocytosed by tingible body macrophages. Selection can lead to terminal differentiation into memory B cells or plasma cells. (Vinuesa et al. 2009) **(B)** Immunohistochemical staining of splenic white pulp: CD3 stains the T cells in the T zone (T) in blue and IgD stains the B cells in the follicles (F) in brown with unstained IgD$^-$ germinal centres (GC) within the follicles. (Bobat, 2011) **(C)** Enlarged view of GCs showing the T zone in blue, follicles in brown. Within the GC, proliferating centroblasts within the dark zone of the GC are stained in pink with BrdU and the centrocytes in the light zone are unstained. Staining of the same spleen section with FDC-M2 shows the dense FDC network within the light zone of the GC. (Allen et al., 2004)

1.5.5.3 High affinity plasma cells and memory B cells

Positively selected GC B cells are antigen specific with high affinity. A proportion of these cells moves to the red pulp and differentiates into memory B cells, plasmablasts and short-lived plasma cells. While plasmablasts and plasma cells both secrete large amounts of antibody, plasmablasts can still proliferate whereas plasma cells have stopped proliferating. Short-lived plasma cells disappear from the spleen within two weeks of infection.[62] The survival of long-lived plasma cells is ensured by the survival niche in which they reside. When activated by antigen, memory B cells can become plasmablasts which can in turn become long-lived plasma cells. Plasmablasts 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. However, once the infection and tissue inflammation has been resolved, these cells die by apoptosis.[63] Most long-lived plasma cells end up in the bone marrow where they become immobile and can survive for up to a year and account for long-term antibody production and humoral memory.[63]

In TD responses, activated FO B cells can also become plasmablasts which move to the red pulp and form plasma cells. This occurs without GC formation and is termed extra-follicular antibody response.[58]

1.5.5.4 T-independent antibody responses

Unlike TD antigens, TI antigens can directly induce B cell proliferation as the second signal can be provided by the antigen itself.[1] MZ and B1 B cells respond to TI antigens in the absence of T cell help and GC reactions and are the first to respond to foreign antigen.[58] B1a B cells constitutively produce natural IgM without exposure to any environmental antigen[58] but can respond to TI-1 antigens like LPS which trigger innate receptors on B cells.[64] These receptors are skewed towards common bacterial antigens and do not undergo somatic hypermuation.[65] They do, however, migrate to the spleen upon activation where they differentiate into plasma cells upon antigen encounter. B1a B cells also produce antigen specific IgA in the lamina propria of the gut and play an important role in host defence at mucosal surfaces.[64] B1a B cells are self-renewing, which B2 FO and MZ B cells are not. The function of B1b B cells is not clear, although they are important in the TI response to relapsing fever caused by *Borrelia hermsii.*[66]

MZ B cells are non-circulating B2 B cells found, in mice, only in the splenic marginal zone and respond to TI-2 antigens which typically include polysaccharide chains of repetitive sequences like bacterial LPS. These antigens crosslink many BCRs on the same B cell and induce a proliferative antibody response.[51] Interestingly, MZ B cells in humans recirculate freely and are identical to somatically hypermutated IgM+ memory B cells.[51] MZ B cells respond to TI antigens by differentiating into low-affinity extra-follicular plasma cells which produce germ-line encoded antibodies, the main isotype being IgG3 in mice and IgG2 in humans.[55] TI responses are essential in response to non-protein antigen and they provide immediate protection with broad specificity antibodies. Two days after immunization with TI-2 antigens, foci of plasmablasts are seen along the periphery of the PALS, expanding until 8 days post immunization after which they diminish and die by apoptosis.[62] Only 10-14 days post immunization is enough T cell help is recruited in a TD, GC response.[62] Thus the TI response is an innate-like antibody response which holds the fort until the adaptive TD response kicks in against the same infection (since microbes have many antigenic molecules). However, little is known about the mechanism by which TI responses are activated and regulated.

1.6 Neonatal Immunity

In humans the neonatal period is defined as the first 28 days after birth. The human neonatal immune system is fully developed at birth but represents a unique and highly plastic but weak period of immune defence, different to that of adults in humoral and cell-mediated immunity.[67] Due to lack of immune memory, newborns are highly sensitive to pathogens and 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. Early life infections like influenza and respiratory syncytial virus (RSV) and bacterial infections account for a significant proportion of infant hospitalizations.[68,69]

Many of these diseases should be preventable by already 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, require several booster immunizations.[68] Young infants (<3 months) are incompletely protected by vaccine schedules with pertussis vaccines which are initiated too late, no influenza vaccine is licensed for the use prior to the age of 6 months and there is no licensed RSV vaccine.[68]

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 vaccination. 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 immunizations. However, recent advances have revealed much about this unique period of immune response and show various potential strategies for the development of neonatal vaccines.

1.6.1 A Th2 biased immune system

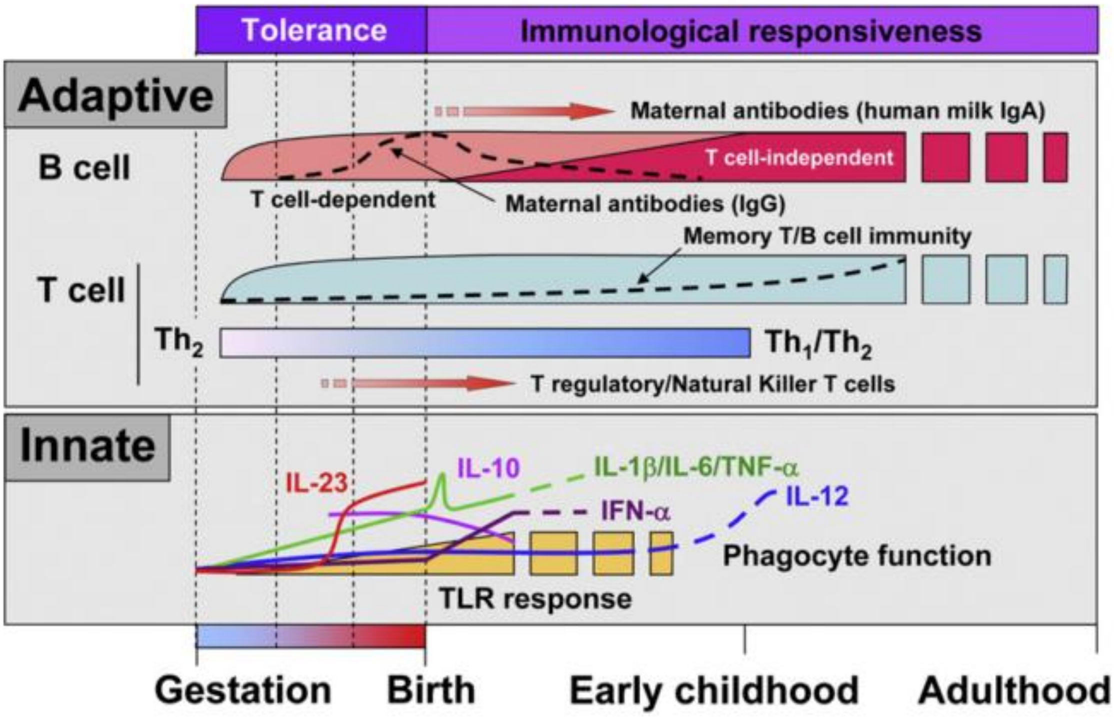
A successful pregnancy requires the maternal endometrium to accept invasion and infiltration of semiallogenic feto-paternal DNA and RNA. This type of invasion normally induces Th1 repose as in viral infections. In order for the maternal immune system to accept the fetus as an allograft and not destroy it by mounting a Th1 cytotoxic response, the maternal-fetal interface requires a suppression of the maternal inflammatory response.[70] This is brought about by the production of Th2 cytokines and repression of Th1 cytokines, largely by the placenta **(Fig 1.7)**. Proinflammatory Th1 cytokines like IFNγ and TNFα promote expression of the pro-apoptotic transmembrane protein, Fas, and thereby the sensitivity of the trophoblast cells to apoptosis. Regulatory Th2 cytokines like IL-6 and IL-10, on the other hand, repress Th1 cytokines and activation of NK cells and macrophages, promoting the implantation and maintenance of the trophopblast.[71,72] Studies in mice have shown that fetoplacental tissue spontaneously secretes Th2 cytokines, and that a Th2:Th1 ratio of cytokine production was greater than that of restimulated maternal splenic cells.[73] Thus a normal pregnancy requires a Th2 biased environment to avoid loss of the trophoblast. This regulatory immune imbalance that protects the fetus *in utero* is passed onto the fetus itself and extends into the neonatal period.[70] The neonatal immune system is therefore not under developed or immature at birth but, rather, specifically adapted for survival *in utero*. After birth the neonate encounters a new world of pathogens, many of which are viruses and bacteria. With a mostly antigen inexperienced immune system that is biased against a Th1 response, neonates are highly susceptible to viral and bacterial infections.[68]

1.6.2 Neonatal innate and adaptive immunity

Although the human neonatal immune system is fully developed at birth, it responds weakly to vaccination and infection due to an antigen inexperienced adaptive response. However, adult-like B and T cell responses have been demonstrated under specific conditions and stimulations, displaying the plastic nature of the neonatal immune system. Due to lack of antigen encounter and pre-existing immunological memory, the concentrations of soluble mediators, absolute numbers of immune cells, their ratios and activation states differ from those of adults.[74]

1.6.3 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. Once activated, T cells provide help to B cells for antibody production. Moreover, DCs produce various factors, such as IL-12, which polarize CD4+ T cells to a Th1 type response **(Fig 1.7)**.[75] Absolute numbers of neonatal mouse DCs are reduced by several logs compared to adults. The splenic T cell to DC ratio reaches adult proportions by 1 week after birth yet seems to be functionally immature in some settings.[76] This immaturity has been attributed to low expression of costimulatory molecules and IL-12. 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[77] which seems to persist for up to 1 year after birth.[78] Optimal APC function is thus a key element for overcoming the Th2 biased neonatal immune response and eliciting an adult-like Th1 response.



<u>Figure 1.7</u> The development of the neonatal innate and adaptive immune responses with time. Maternal T cell tolerance induction of the trophoblast is required for survival of the fetus. This is achieved by repression of intrauterine Th1 responses and induction of a Th2 environment and is passed onto the fetus. Humans display high proportions of Tregs and NK T cells at birth. *Adaptive immune functions* (top panel): maternal IgG is transferred *in utero* during late gestation, followed by human milk IgA (and some IgG) through breastmilk. The infant's own antibody (T-dependent, but especially T-independent) and T/B cell memory responses become fully mature later during early childhood. *Innate immune functions* (bottom panel): Pro-inflammatory Th1 and Th17 inducing cytokines (IFN-α, IL-1β, IL-6, TNF-α, IL-12 and IL-23) are attenuated in neonates, whereas anti-inflammatory IL-10 is relatively high during the late gestational phase. Phagocyte function develops fully in early childhood. (Sharma et al., 2012)

1.6.4 Neonatal complement proteins

Complement plays an important role in both innate and adaptive responses. Human[79] and mouse[80] infants have low levels of serum complement component C3, which, along with a deficiency in CD21 on B cells, impairs formation of C3d-antigen complexes and localization on MZ B cells, thereby limiting activation of the alternative pathway to polysaccharide (T-independent) antigens.

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, and pregnant women in developing countries are vaccinated in order to transfer protective IgG to their babies. With weaning and the decay of MatAb, a window period occurs where the infant is left unprotected before its adaptive response is fully matured **(Fig 1.8).**

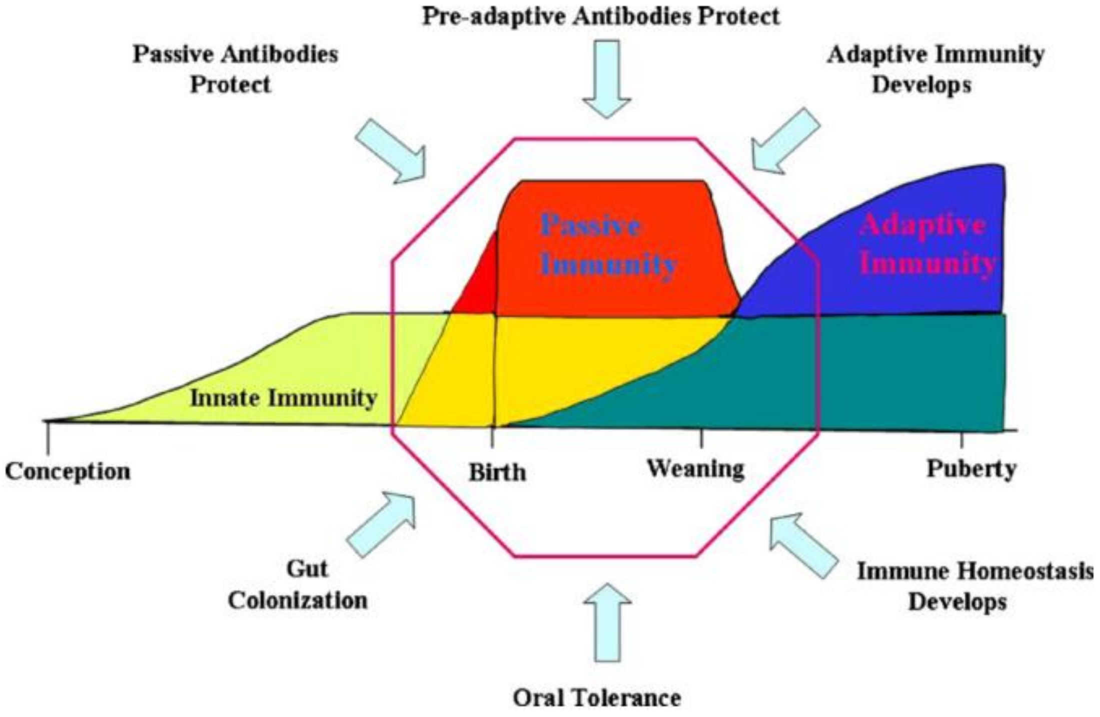


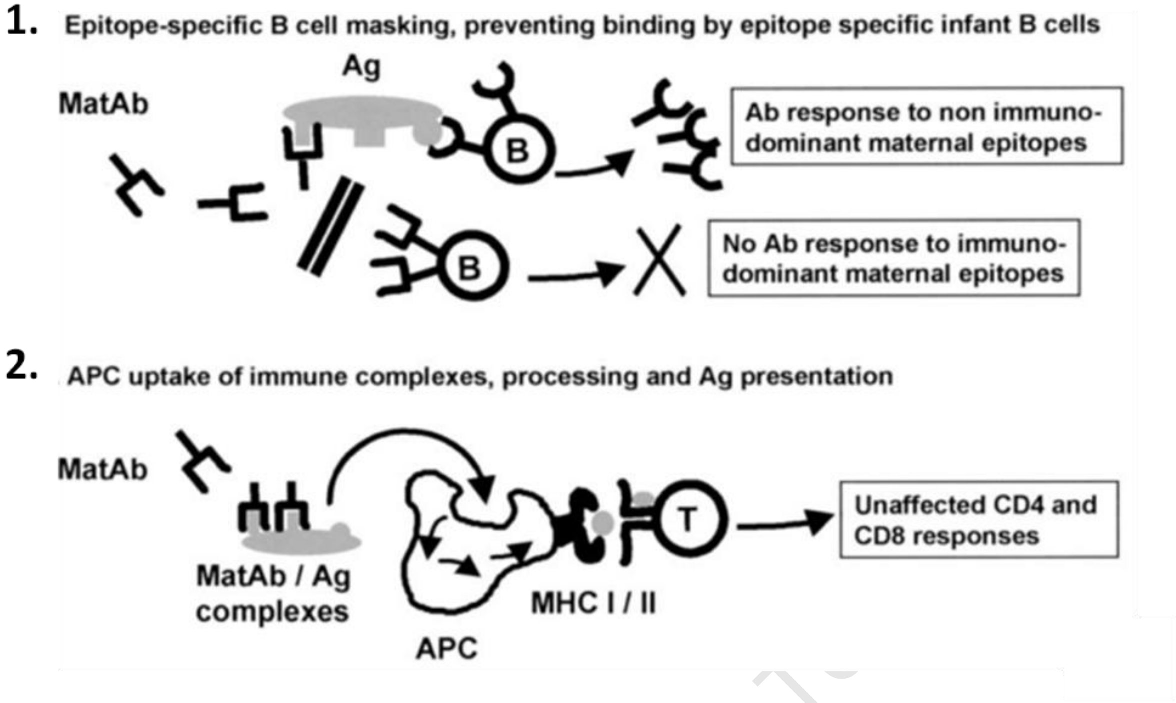
__Figure 1.8__ **Passive protection of neonates and young infants by maternal antibodies before the adaptive response develops.** Maternal antibodies are transferred *in utero* and via breastmilk and protect neonates from early infections. Breastmilk antibodies and transferred antigen in the form of 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. (Butler et al., 2009)

Paradoxically, MatAb are known to interfere with primary antibody responses to immunizations in infants.[81,82] 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 **(Fig 1.9)**.[83] 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,[84] although supporting evidence has been seen with the HIB-conjugate vaccine. MatAb specific to the protein carrier (i.e. TT) inhibit infant responses to TT, but do not inhibit infant response to the HIB polysaccharide moiety of the vaccine.[85] 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 **(Fig 1.9)**.[83]

While MatAb prevent B cell priming due to a common antigen epitope specificity, MatAb do not influence T cell priming since TCRs recognize different epitopes on the vaccine antigen.[84] This has been shown in mice and evidence from clinical studies where MatAb-inhibition of primary responses did not inhibit a priming, i.e. a T-dependent secondary response after booster immunization, suggest that this is the case in human infants.[83]

1.6.6 Neonatal B cells

Human infants have higher counts of total B cells than adults, increasing in the first weeks of life and remaining high for the first year of life.[86] There are numerous phenotypic and functional differences between neonatal and adult mouse splenic B cells, but 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 upregulate co-stimulatory molecules and MCHII upon ligation of their BCR, essential for effective interaction with T cells.[67,87] Neonatal splenic B cells have limited expression of CD21,[88] CD40, CD80, CD86 and limited interaction



<u>Figure 1.9</u> **Influence of passively transferred maternal antibodies on induction of vaccine responses in offspring.** MatAb can inhibit B cell priming of infants and thereby prevent infant production of their own humoral response to vaccinations. Two mechanisms may account for this inhibitory effect: 1. The masking by MatAb of immunodominant epitopes on the vaccine antigen and blocking access to infant B cells, and 2. The mopping up of MatAb:antigen immune complexes by infants APCs. The latter mechanism does not influence B cell antigen presentation to T cell as the APC is still able to present antigen. However, T and B cells recognize different antigen epitopes and therefore T cell responses are thought to be largely unaffected by MatAb (Image adapted from Siegrist, 2001).

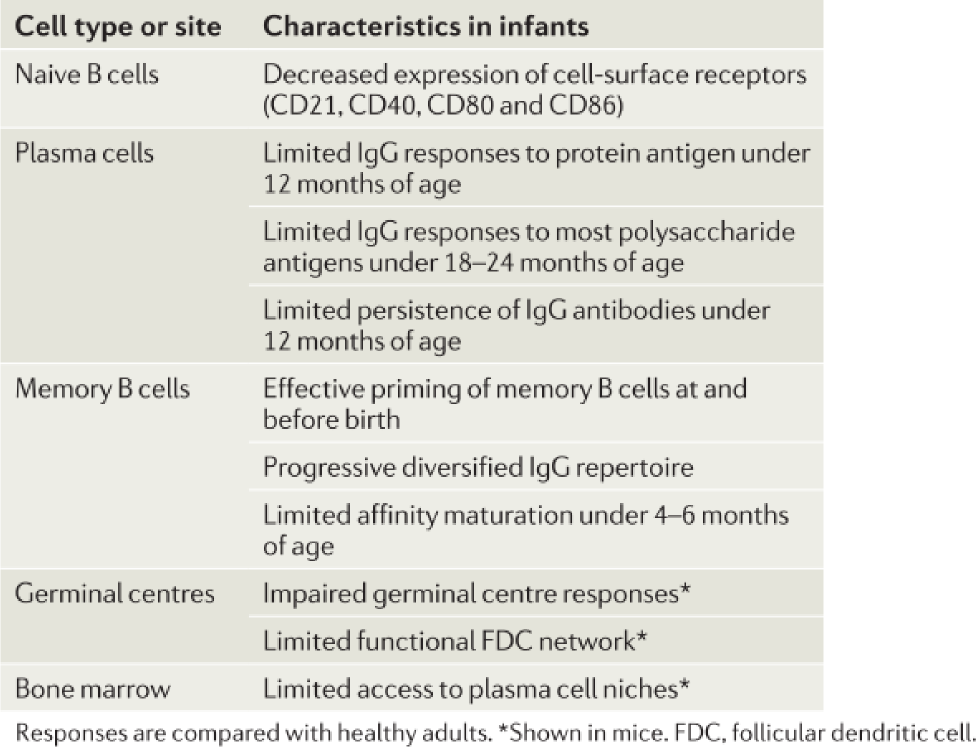
with ligands such as B cell activating factor (BAFF) and/or proliferation-inducing ligand (APRIL), all of which are plasma cell supporting factors.[89]

1.6.6.1 Neonatal germinal centres

Neonates have impaired GC formation. In humans, GCs only appear at about 4 months after birth.[90] With the appearance of GCs in mice at 3 weeks of age, they also acquire the ability to induce adult-like antibody responses.[91] This delayed GC reactivity is due to delayed maturation of the essential FDC network, resulting from FDC precursors failing to respond to

B cell lymphotoxin-α signalling. This has been shown in mice[91], even with potent adjuvants that induce adult-like B and T cell interaction.[89,91]

<u>Table 1.2</u> Human infant B cell responses. Neonatal and infant B cells respond poorly to antigen stimulation and interaction with T cells, resulting in weak primary and secondary antibody responses. (Table adapted from Siegrist and Aspinall, 2009)



Cell type or site	Characteristics in infants
Naive B cells	Decreased expression of cell-surface receptors (CD21, CD40, CD80 and CD86)
Plasma cells	Limited IgG responses to protein antigen under 12 months of age
	Limited IgG responses to most polysaccharide antigens under 18–24 months of age
	Limited persistence of IgG antibodies under 12 months of age
Memory B cells	Effective priming of memory B cells at and before birth
	Progressive diversified IgG repertoire
	Limited affinity maturation under 4–6 months of age
Germinal centres	Impaired germinal centre responses*
	Limited functional FDC network*
Bone marrow	Limited access to plasma cell niches*

Responses are compared with healthy adults. *Shown in mice. FDC, follicular dendritic cell.

1.6.6.2 Neonatal marginal zone B cells

Neonatal splenic marginal zone B cells are deficient in mice and humans.[92] In mice they 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 TI antigens.[93] Human neonatal spleens contain fewer marginal zone macrophages and these cells differ in their capacity to produce cytokines required for B cell activation.[94]

1.6.6.3 Bias towards a memory B cell response

The most important difference is that the neonatal response favours B cell memory induction rather than high affinity antibody production through plasma cell development. This preference has been attributed to the combination of many factors **(Table 1.2)** that are highly conserved (at least between mice and humans), and thought to be a protective adaptation of the naive neonatal immune system from a prolonged state of heightened inflammation in response to overwhelming exposure to antigen which may prove toxic to normal development and even survival.[67] This has been demonstrated in mice where IL-12 treatment was used to subvert the Th2 bias and enhance a Th1 response but caused weight loss and even death at higher doses.[95–98]

The preferential differentiation into memory B cells is indicated by the inability to elicit a specific primary antibody response with vaccination at birth, while priming a secondary response to a boost vaccination. It has been shown that neonatal post-GC plasma cells home readily to the bone marrow but fail to establish themselves as long-lived plasmablasts due to insufficient differentiation and survival signals from bone marrow stromal cells.[99,100] The impaired ability to generate GC reactions and long lived plasmablasts has implications for vaccination with TD antigens and is important in vaccination schemes.

1.6.7 The use of rodent and human cord blood models

The neonatal immune system has been studied using human umbilical cord blood mononuclear cells (CBMCs) as stand-ins for those of the newborn, murine models and other mammalian species. The degree of development of soluble and cellular components of neonatal immune systems varies widely between mammalian species.[74] 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 partuition.[74]

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 experiments used varying immunisation time points (≤24 h, ≤48 h and ≤7 days), yielding confusing results.[78] Highly variable results were obtained from immunisations within the first 3-4 days of life, suggestive of the individual variation in appearance of DCs and T cells in peripheral lymphoid organs during the first week of life.[101,102] Even after neonatal priming at ≤48 h after birth, secondary antibody responses were weak. Together these initial experiments suggested that murine neonatal immune maturation was delayed compared to humans.[78] 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.[78,103,104]

1.7 Experimental Infection Models

1.7.1 Helminths

More than 2 billion people worldwide are infected with parasitic helminths and infections are particularly prevalent in developing countries of sub-Saharan Africa, parts of Asia and South America.[105] Infection usually occurs directly through the skin by walking barefoot through contaminated faecal matter.[106] Infection is chronic and may last 1-5 years for the hookworms *Nector americanus* and about 6 months for *Ancylostoma duodenale*.[107] High rates of morbidity rather than mortality are the major health concerns associated with helminth infections. Chronic infections often lead to anaemia and protein malnutrition and this has been related to poor cognitive development in children[108] and can affect maternal *in utero* fetal imprinting.[109,110] Strong associations have been made with maternal helminth infection and reduced childhood vaccine efficacy in offspring.[111] The antihelminthic drug albendazole is administered through global strategies to control these infections. However, human host inability to develop effective immune memory and the development of drug resistance results in re-infections and confounds these efforts.[112] To date no anti-helminth vaccine exists but is being actively investigated.[113,114]

1.7.1.1 *Nippostrongylus brasiliensis*

N. brasiliensis is a gastro-intestinal (GI) rodent parasite, analogous to the human infectious hookworms *Ancylostoma duodenale* and *Necator americanus*. *N.brasiliensis* serves as an excellent model to study human hookworms and a highly polarized Th2 immune response in mice. It is easy to handle in the laboratory as it does not require an intermediate host for the maintenance of its life cycle, is not infectious to humans and not fatal in rodents.[115]

1.7.1.2 The life cycle of *N.brasiliensis*

The life cycle of *N.brasiliensis* consists of a free-living and a parasitic stage **(Fig 2.3)**. Infection occurs by the free-living infectious L3 larvae which penetrate the skin of the rodent. In experimental models, the L3 larvae are injected subcutaneously. Once under the skin, L3 larvae enter the circulation and migrate to the lung over a period of 24-48 hours.[116] Here they get trapped in capillaries, undergo maturation and moulting to become L4 larvae which breach the capillaries and enter the pulmonary alveoli.[117] L4 larvae are then coughed up and enter the oesophagus through swallowing.[118] L4 larvae then migrate through the oesophagus and stomach to the small intestine where the final moult to the L5 stage takes place and larvae mature into adult worms at day 4-5 post infection. Adult worms establish themselves here, lodging in the intestinal epithelium. By day 6 post infection, adult worms are now able to mate and produce eggs which leave the host through faeces.[117] In BALB/c and C57BL/6 mice egg production stops at about day 9 post infection when the worms are expelled due to the robust immune response of the host.[116,119]

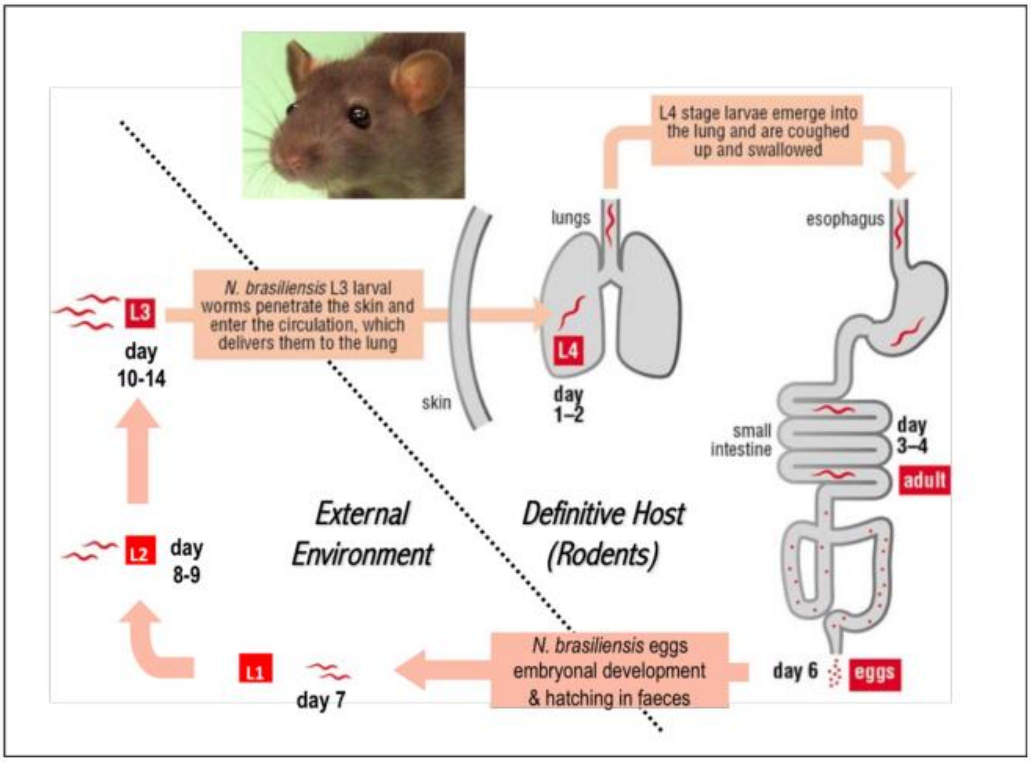


Figure 1.10 The life cycle of *N.brasiliensis*. There are six developmental stages in the cycle, four larval stages L1-L4 of which L1-L3 are free-living, L4 and the adult L5 are parasitic and require a rodent host, and the egg which is produced in the small intestine and leaves the host through the faeces. Two main areas in the host undergo pathological changes due to infection: the lung and the small intestine. The L3 larva is the infectious stage. (Image adapted from Murray Selkirk, Imperial College London)

1.7.1.3 Primary immune response to *N.brasiliensis*

The immune response to helminths is a highly polarized Th2 response which includes the recruitment of basophils, eosinophils and mast cells, goblet cell hyperplasia (differentiation and proliferation) and mucous secretion, CD4+ T cell dependant generation of IgE and isotype switching of B cells to produce IgG1.[107,115,117,118] Entry of L3 larvae into the lung upon primary infection with *N.brasiliensis* leads to pulmonary inflammation and induction of this Th2 response and causes airway hyper responsiveness.[120] This is further characterized by the secretion of cytokines IL-4, IL-5, IL-9 and IL-13.[15]

It is primarily IL-13 that drives the Th2 response to *N.brasiliensis* and has a wide range of Th2 related functions on a variety of cell types. IL-13 is produced by T and B cells, mast cells, basophils, DCs and NK cells.[121,122] Expulsion of *N.brasiliensis* from the small intestine in a primary infection is dependent on the expression of IL-4Rα, part of the heterodimeric receptors for IL-4 and IL-13 on the cell surface of hematopoietic, muscle, epithelial, endothelial cells, fibroblasts and neurons.[123] Surprisingly, this expulsion is not due to effects of IL-13 signalling on immune cells but, rather, on non-haematopoietic cells such as epithelial, goblet and intestinal smooth muscle cells.[124–128] It is likely that a combination of increased smooth muscle contractility and mucous production creates a hostile environment to the adult worms in the small intestine and interferes with their ability to feed properly and bind securely to the intestinal wall.[118,125,128]

1.7.1.4 Secondary immune response to *N.brasiliensis*

Humans are unable to develop an effective protective Th2 memory response to helminth infections while rodents are. Re-infection studies with nematodes *Trichuris muris*[129], *Heligmosomoides polygyrus*[130] and *N.brasiliensis*[131] have demonstrated an enhanced memory response that rapidly clears the secondary infection.

Studies with *N.brasiliensis* show that the lung is the key site of host secondary responses.[132,133] Although IL-4Rα expression on immune cells is not essential to expulsion of the worms in a primary infection, it has been shown to increase the magnitude of the host Th2 response through signalling on CD4+ T cells during a primary infection.[127] 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 (manuscript in preparation). These data imply an important role for adaptive immunity in the memory response to *N.brasiliensis*.

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 altered immune components in offspring are acquired from the mother *in utero* and via breastmilk.

1.7.2 *Salmonella*

1.7.2.1 The species *Salmonella enterica*

Salmonella is a facultative intracellular, non-spore forming Gram-negative bacillus. The species of *Salmonella* are divided based on DNA sequences and include *S.enterica* and *S.bongori*. These are further divided into subspecies called serovars and defined by their surface antigen structure and shared genotype in accordance with the Kauffman-White classification scheme.[134] *S.bongori* primarily infects cold blooded animals while *S.enterica* is clinically relevant and causes disease in humans.[135] *Salmonella enterica* serovar *Typhus* (ST) and *Salmonella enterica* serovar *Typhimurium* (STm) are almost 98% identical in DNA sequence, however 20% of their genes are different and unique to the species.[136,137] Where ST has an immunodominant antigenic capsular polysaccharide called Vi, used in the generation of the Vi vaccine, STm does not. Currently, there is no vaccine against STm. ST causes typhoid fever, a serious systemic infection which can be fatal if untreated. STm is a non-typhoidal *Salmonella* (NTS) and causes self-limiting gastroenteritis in developed countries.[138] However, in sub-Saharan Africa there is a high prevalence of invasive NTS disease caused by STm isolates, resulting in severe symptoms in the absence of gastroenteritis.[139–141] STm disease is discussed in further detail in Chapter 4.

1.7.2.2 Entry and colonisation of the host

Salmonella disease progression has been characterized based on ST infection. Naturally, bacteria are ingested through contaminated food or water and enter the GI tract. The severity of disease depends on the ability of the bacteria to disseminate and colonize sites remote from where the infection occurred. Bacteria that survive the acidic stomach spread rapidly along the GI tract, cross the intestinal epithelium, primarily through membranous epithelial cells (M cells) **(Fig 2.4)**.[142,143] *Salmonella* can also enter the penetrate the GI lining inside phagocytes and through epithelial cells.[144] After penetration, bacteria colonize the lamina propria and Payer’s patches where they invade host cells like macrophages and DCs and, if host defence responses fail to control the infection, initiate systematic dissemination through the lymphatics into deeper tissues. Bacteria translocate to the mesenteric lymph nodes (MLN) where they multiply and enter the blood through the thoracic duct. This leads

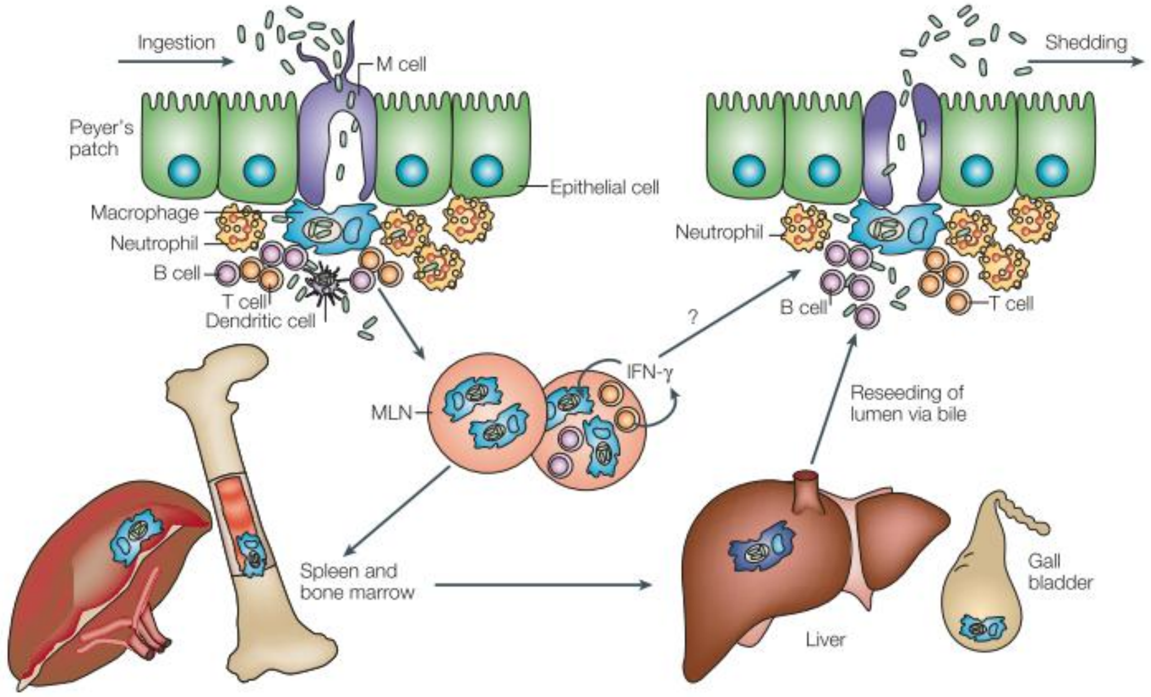


Figure 1.11 Colonisation of the host by *Salmonella enterica* serovar *Typhi* resulting in persistent infection. Ingested bacteria translocate to the GI tract and cross the mucosal barrier of the intestine through M cells and enter the Payer's patches. *Salmonella* may then target DCs and macrophages which transport them to the MLNs and deeper tissues. This then leads to dissemination to the spleen, bone marrow, liver and gall bladder. Bacteria can persist in the MLNs, bone marrow and gall bladder for life with periodic reseeding of the intestinal lumen.[145,147] (Image taken from Monack et al., 2004)

to transient bacteraemia which is cleared by the reticuloendothelial system (RES).[145] As bacteria continue to replicate within the cells of the RES, secondary bacteraemia results and spread to the spleen, bone marrow and non-lymphoid organs such as the liver occurs. Here bacteria invade and replicate within macrophages.[146] From the liver, bacteria spread to the gall bladder which reseeds the lumen of the intestine through bile. Bacteria can persist in the MLNs, bone marrow and gall bladder for life with periodic reseeding of the mucosal surface.[147]

1.7.2.3 Immune response to *Salmonella enterica* serovar *Typhimurium*

STm infection in mice can resemble typhoid fever in many respects and used as an experimental model for studying human typhoid and NTS.[148] The effective immune response to STm in the mouse model is a highly polarized Th1 CD4+ T cell response which effectively clears the infection after 35 days post infection.[149–151] The infection kinetic begins with the early phase of infection (the first week) where innate cells attempt to kill the colonizing bacteria. These cells include neutrophils, macrophages and NK cells **(Fig 2.5)** which act to restrict bacterial multiplication and dissemination before the adaptive response is established.[142,145,152] STm gastroenteritis infections in humans are completely cleared by innate cells. However, in the mouse model of NTS caused by STm, the innate response to infection only restricts initial growth but is not able to clear the infection and therefore dissemination and bacteraemia occurs.

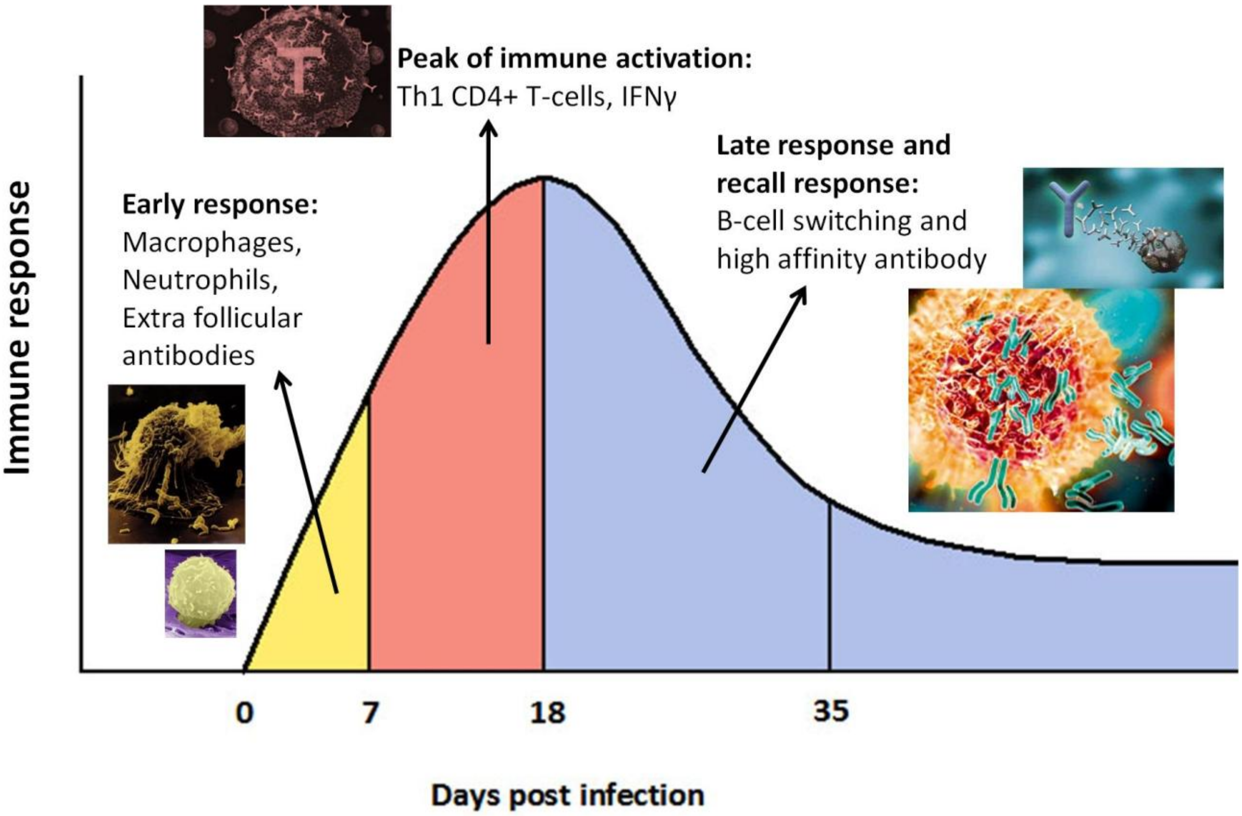


Figure 1.12 The immune response to *Salmonella enterica* serovar *Typhimurium* in mice. The early response to infection includes phagocytosis and killing of bacteria by innate cells and an extrafollicular low-affinity IgM response, lasting 7 days post infection.[145,150] Once the adaptive response is established, Th1 CD4+ T cells are recruited and a peak of immune activation is reached at 18 days post infection.[151] Late in the response to STm, germinal centre formation produces high affinity OMPS-specific IgG2a.[150] Optimal immunity and recall response to vaccination against STm requires both Th1 CD4+ T cells and high affinity IgG2a production.[153]

Clearance of infection requires the recruitment of the adaptive response which is discussed in more detail in Chapter 4. STm transitions from an extracellular to and intracellular phase where the bacteria reside in specialised Salmonella-containing vacuoles (SCVs).[144] This enables evasion of host antimicrobial defences by preventing phagolysosomal fusion,[144] and delaying vacuole acidification.[154] This new intracellular location of STm requires Th1 cell mediated control while, with invasive strains of STm, the extracellular dissemination and bacteraemia require humoral control. However, optimal immunity including a memory response and vaccine efficacy require both Th1 T cell and antibody mediated immunity.[153]

1.8 Objectives

In this study we aim to elucidate the effects of maternal infection with the helminth Nippostrongylus brasiliensis on adaptive immunity in offspring. 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. Helminth co-infection studies with malaria105 or bacterial infections like Salmonella106 have suggested an impaired protective response to both pathogens. We further investigated the effects of maternal N.brasiliensis infection on offspring control of a highly polarized Th1 inducing pathogen, namely Salmonella enterica serovar Typhimurium aroA-/- strain SL3261.

2. Materials and Methods

2.1 Animal Work

2.1.1 Mice used, animal unit and ethics

This study made use of BALB/c and C57BL/6 mice. Adult females and males used for matings were bred by the Animal Unit of the University of Cape Town, and all mice were housed in specific pathogen-free conditions in the animal unit facilities. All experimental procedures performed on mice were in accordance with protocols 011/008 and 008/009 approved by the Faculty of Health Sciences Animal Ethics Committee from the University of Cape Town. Mice

2.1.2 Matings and litters

Female mice aged between 7 and 8 weeks were mated with male mice at one male per cage of four 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 until pups were weaned at 3 weeks of age and separated from their mothers according to sex.

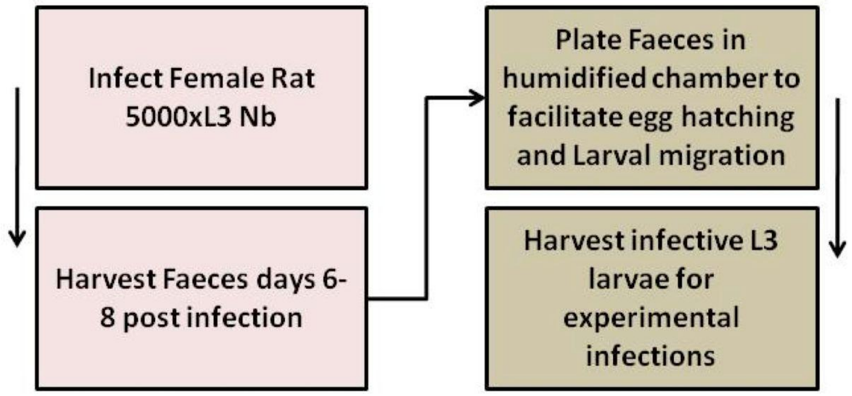
2.1.3 Experiment end point

At specific time points mice were killed by inhalation of halothane. Peripheral blood was taken by cardiac puncture and spleens removed by incision under sterile conditions. Blood was collected in plasma separation tubes and spun at 8,000rpm for 20min to separate plasma from red blood cells. Samples were stored at -20°C. Spleens were weighed whole, cut into segments which were weighed separately and used for either flow cytometry, *in vitro* splenocyte restimulation for cytokine assessment by ELISA, immunohistochemistry and bacterial culture to determine bacterial burden.

2.2 Working with *Nippostrongylus brasiliensis*

2.2.1 Cycling of *N.brasiliensis*

The life cycle of *N.brasiliensis* (Nb) was maintained by passage through Wistar rats and collection of worm eggs in their faecal pellets **(Fig 2.1)**. Naive 6 week old female rats were infected subcutaneously with 5,000 L3 larvae in 1ml of 0.9% NaCl. Rat faecal pellets were collected on days 6, 7 and 8 post infection (p.i.) and emulsified in autoclaved water with 5µg/ml fungizone (Gibco® Life Technologies). Emulsified faeces was spread on moist filter paper over gauze (moistened with fungizone water) in petri dishes which were kept in humidified boxes at room temperature. After 7 days, the larvae hatched and migrated to the edge of the filter paper, where they were harvested at the infective L3 stage for future infections of rats (for stock maintenance) and mice (for experimental infection studies).



<u>Figure 2.1</u> Maintenance of the N.brasiliensis life cycle in Wistar rats.
See Section 2.2.1 for details. (Diagram from W.G. Horsnell)

2.2.2 Primary infection with Nb

Nb L3 larvae were washed off filter paper in 0.9% NaCl solution from pieces of filter paper. The larvae were counted under a dissecting microscope and then resuspended at a concentration of 2500 L3 larvae/ml in 0.9% NaCl. The infections were carried out in the Animal Biosafty Level 2 procedure room in the animal unit (Faculty of Health Sciences, University of Cape Town) where mice were injected s.c. with 500 x Nb L3 larvae in 200µl of

0.9% NaCl using a 21G needle (Braun, Melsungen, Germany), making sure that the larvae were in suspension at the time the syringe was filled.

2.2.3 Clearance of Nb

While Nb worms are naturally cleared from BALB/c and C57BL/6 mice at approximately 9 days p.i., to ensure that all worms were expelled mice were treated with 10 mg/ml Ivermectin in their drinking water from day 7 to 14 p.i. to clear the pathogen.

2.3 Working with *Salmonella enterica* serovar *Typhimurium*

2.3.1 Growth of *S.typhimurium* (STm) bacteria

The attenuated *Salmonella enterica* serovar *Typhimurium AroA*$^{-/-}$ SL3261 (STm) strain was used throughout this study. STm was stored at -80°C and inoculated into 10ml autoclaved 3% w/v Bacto^{TM} Tryptic Soy Broth (BD Bacto^{TM}) supplemented with 0.5% w/v yeast extract in dH_2O (LB media) the day before infection of mice. Bacteria were grown overnight in LB media at 37°C with agitation at 180rpm.

2.3.2 Infection with STm

The infectious dosage of STm bacteria was harvested by the Miles and Misra method,[155] where, providing that bacteria are harvested at a similar OD reading and diluted accordingly, only modest differences in bacterial numbers are found. Bacteria were harvested at late log phase with an OD_{600nm} of 1.0. A volume of 1ml of bacterial broth was centrifuged at 10,000rpm for 5min at 4°C prior to resuspension in 1ml sterile PBS and washing. A volume of 1µl of this initial bacterial PBS suspension was taken to equal $5x10^5$ CFU. Bacteria were diluted to give the appropriate infectious dose in sterile 1 x PBS. This was designated as neat concentration. Pups were infected intra-peritoneally (i.p.) with a dose of $1x10^5$ CFU in 200µl sterile 1 x PBS ($5x10^5$ CFU/ml) and adult mice were infected i.p. with a dose of $5x10^5$ CFU in 200µl sterile 1 x PBS ($2.5x10^6$ CFU/ml). To check bacterial numbers of the does given to the mice, a volume of 100µl diluted bacteria was plated after infection onto LB agar plates

(Bacto^{TM} Tryptic Soy Broth enriched with yeast extract as described (Section 2.3.1), with added 1.2% w/v Bacto^{TM} Agar) at 1/10 dilutions from neat down to 1/10,000. Plates were incubated overnight at 37°C and CFU were counted the following day to ensure correct dosage.

2.3.3 Infection with serum-opsonized STm

For opsonisation studies the serum to be tested was heated at 56°C for 30min to inactivate complement prior to use. In each experiment at least 6 samples of sera per group were pooled before coating bacteria. After heat-inactivation, complement-inactivated sera were mixed with bacteria which had previously been harvested, washed and the bacterial concentration adjusted to 2.5x10^{6} CFU/ml with sterile 1 x PBS. Serum was added at 10% v/v of total PBS-bacteria-serum mix and mixed by gentle agitation for 30min at room temperature. Adult mice were infected with 5x10^{5} CFU in 200µl sterile 1 x PBS i.p. Bacteria were then plated onto LB agar plates and grown overnight at 37°C to check CFU counts of the infection dose.

2.4 Vaccination

2.4.1 Vaccination with heat-killed STm

Pups were vaccinated with heat-killed STm bacteria which were harvested and washed as described (Section 2.3.2). After washing, the 1ml suspension of STm was heat killed by incubation at 72˚C for 1 hour after which the bacterial concentration was adjusted to 2.5x10^{6} CFU/ml in sterile 1 x PBS. Pups were vaccinated with 1x10^{5} heat-killed CFU in 200µl sterile PBS. Bacteria were plated and grown overnight at 37°C to confirm that all bacteria were non-viable.

2.4.2 Vaccination with porins

Purified porins (OmpC, OmpD and OmpF) from STm (strain ATCC 14028) were generously donated by our collaborator Dr. Adam Cunningham at the MRC Centre for Immune Regulation, University of Birmingham, UK. Porins were generated by Dr. Cristina Gil-Cruz through repeated extraction with sodium dodecyl sulphate (SDS). The detailed protocol is described in Appendix B. Porins were diluted to 100µg/ml in sterile 1 x PBS and adult mice were immunised i.p. with 20 µg in 200µl.

2.5 Antigen Generation

2.5.1 *Nippostrongylus brasiliensis* excretory-secretory antigen (NES)

NES is *N. brasiliensis* excretory-secretory antigen and comprises the soluble fraction of cellular proteins from Nb L3 larval tissue. L3 larvae were washed from the edge of the filter paper, as described (Section 2.2.2), into autoclaved water to which antibiotics Penicillin & Streptomycin (Pen/Strep) were added at 50U/ml and 50µg/ml respectively to kill most contaminating bacteria. This mixture was left for 1 hour at room temperature to allow the larvae to settle at the bottom of the tube. Once settled, the supernatant was aspirated off and the larvae were washed twice in Pen/Strep water. The larvae, which are at an unknown concentration, were allowed to settle once more. Once settled, the pellet of larvae was concentrated into 2 ml of distilled water, transferred to an eppendorf and dipped into liquid nitrogen to snap freeze the larvae, thereby disrupting cell walls and membranes and releasing the cell contents which include intracellular proteins. Subsequently, disrupted larvae were homogenized, centrifuged to pellet out the large insoluble cellular and tissue debris and the supernatant, containing the soluble fraction of the larval proteins (including NES), was taken for protein quantification. Protein concentration was determined by Nanodrop and adjusted to a working concentration of 500µg/ml in volumes of 200µl per eppendorf and stored at -80°C for future use. NES was used as coating antigen for antigen specific antibody ELISAs at 10µg/ml in 50µl per well or splenocyte restimulating antigen for cytokine analysis at 10µg/ml in 200µl per well.

2.5.2 STm outer membrane proteins (OMPS)

OMPS comprise the entire outer membrane of STm, largely consisting of beta-barrel proteins (including porins) but likely also containing LPS and lipoprotein. OMPS were prepared by our collaborating lab (Dr. Adam Cunningham, University of Birmingham) and generously donated for this study. The detailed protocol for their preparation is described in Appendix B. OMPS were used as coating antigen for antigen specific antibody ELISAs at a concentration of 10µg/ml in 50µl per well.

2.5.3 Heat-killed STm

HK-STm was prepared as described (Section 2.4.1) for vaccination purposes. The protein concentration was determined by Nanodrop, adjusted to a working concentration of 500µg/ml and stored at -80°C. HK-STm was used for *in vitro* splenocyte restimulations for cytokine analysis at 10µg/ml.

2.5.4 Porins

Porins were obtained from our collaborators (see Section 2.4.2) and used as coating antigen for antigen specific antibody ELISAs at 5µg/ml.

2.6 Cell and Tissue Processing

2.6.1 Splenocyte resuspension

Spleen segments to be used for flow cytometry and *in vitro* splenocyte restimulations were kept in complete media comprising Iscovec's modified Eagle medium (IMDM) (Invitrogen) supplemented with 10% heat-inactivated filter-sterilized foetal calf serum (FCS) and 100U/ml penicillin G and 100µg/ml streptomycin. Single cell resuspension of splenocytes was achieved by passing the spleen segment through a 40µm nylon cell strainer (Becton-Dickson, NJ) using a 2ml syringe plunger. Cells were pelleted (1,200rpm for 5min at 4°C), the media discarded and the contaminating red blood cells removed by resuspending the cells in 1ml red cell lysis buffer for 1-2min after which the lysis buffer was neutralized with 2ml complete media. Cells were pelleted again and resuspended in complete media, the cells now comprising mostly splenic lymphocytes and macrophages. A volume of 10µl was diluted and stained in Trypan blue to exclude dead cells and viable cells were counted under a light microscope using a haemocytometer slide. Cells were then resuspended at a working concentration of $1x10^7$ cells/ml in complete media, now ready for plating for *in vitro* splenocyte restimulation and flow cytometry.

2.6.2 Bacterial culture to determine splenic bacterial burden

For the assessment of splenic bacterial burden, spleen segments for this purpose were kept in IMDM media (Invitrogen) with 10% FCS but without penicillin or streptomycin to ensure that STm bacteria were not killed by the antibiotic. Single cell suspensions of splenocytes were obtained as described (Section 2.6.1), without resuspension in red cell lysis buffer (the presence of red blood cells and cell counting was not important here). The cells were resuspended in 1ml antibiotic free media and serially diluted at 1/10, 1/100 and 1/1000. The diluted suspensions were plated onto LB agar plates at 100µl per plate and incubated overnight at 37°C. Bacterial CFUs were counted and the total CFU per spleen and per gram of spleen was determined based on whole spleen mass and dilution of splenocytes.

2.6.3 *In vitro* restimulation of cells

Single cell suspensions of splenocytes in complete media (see Section 2.6.1) were plated at $1x10^6$ cells per well in round-bottomed 96 well plates (Nunc, Maxisorp) and restimulated with optimal anti-CD3 (prepared by our lab), NES and/or HK-Tm at 10µg/ml, and/or sub-optimal anti-CD3 at 1µg/ml mixed with NES at and 10µg/ml in a total volume of 200µl complete media. Cells restimulated with optimal anti-CD3 (10µg/ml) were incubated for 36 hours. Cells restimulated with other antigens 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.

2.7 Flow Cytometry

2.7.1 The basic method

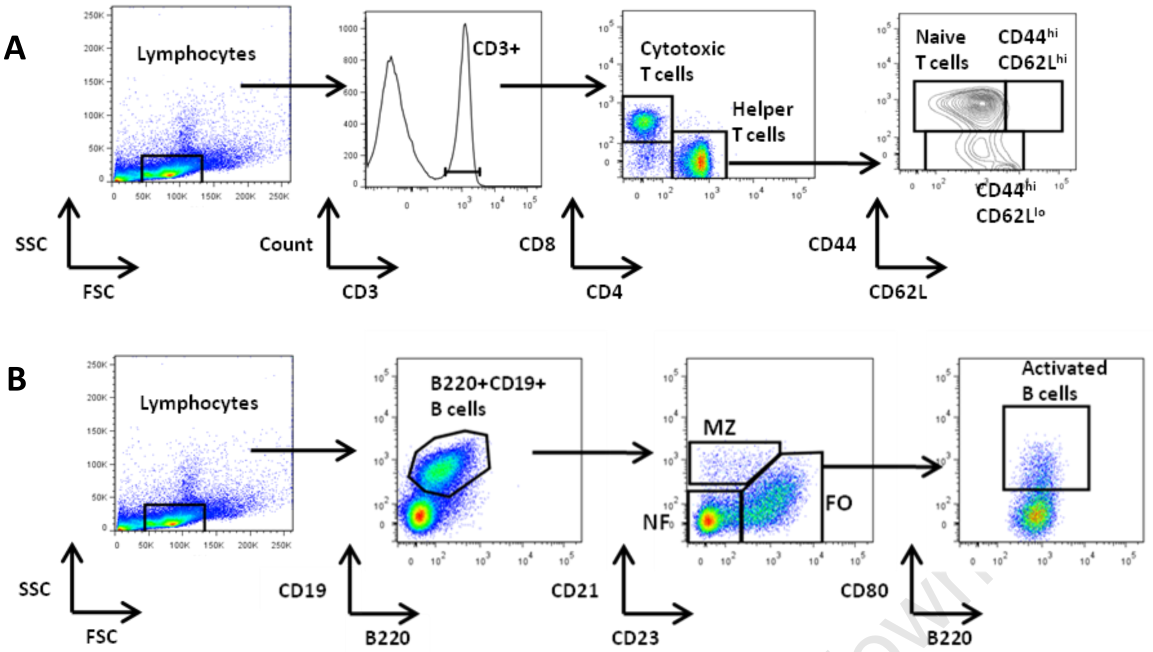
Flow cytometry (or fluorescent activated cell sorting; FACS) was used to analyse splenic lymphocytes for cognate cell surface receptor expression in order to quantify the different populations of T and B cells and their maturation and activation statuses. Single cells were stained at 1x10^6 cells/well/sample, in 96-well round-bottom plates (Nunc, Maxisorp). Cells were stained with 25µl antibody master mix in MACS buffer (Appendix A) containing 2% rat serum and 2% anti-FcγII/III to block non-specific binding, and the required mono-clonal antibodies diluted to the correct concentration (Table 2.1) for 30min on ice. Single stain controls were prepared with FACS CompBeads (BD, Biosciences) instead of cells. Cells were pelleted (1,200rpm for 5min at 4°C) and the antibody mix was washed off with MACS buffer. If a secondary antibody stain was required, cells were stained again for 30min at 4°C, washed, streptavidin-linked Qdot605 diluted at 1/640 in MACS buffer was added and cells were incubated for 20-30min at 4°C. Cells were then washed again and resuspended in MACS buffer and read by a 4 laser BECTON DICKINSON FACS FORTESSA machine and the data was collected by DIVA© BD software (San Diego, CA). Flow cytometry data was analysed by FlowJo© Treestar (Ashland, OR) and graphed with GraphPad Prism software (La Jolla, CA).

2.7.2 Antibodies and gating strategies

All flow cytometry antibodies (from BD Pharmingen) used in this study and their working concentration are listed in **Table 2.1**. T and B cell populations and sub-populations were determined using two main gating strategies **(Fig 2.2)**, with some variations for different experiments (outlined in the specific results chapters). Unstained cells and CompBeads were used to set parameters on the Facs Fortessa machine.

Table 2.1 The monoclonal antibodies used for analysis of T and B cell populations by flow cytometry, their fluorophore, clone, isotype control and working concentrations.

Antibody	Fluorophore	Clone	Isotype Control	Working concentration
CD3	PE	500A2	IgG2a	1/320
CD4	PerCP	RM4-5	IgG2a	1/1280
CD8a	V500	53-6.7	IgG2a	1/160
CD44	FITC	IM7	IgG2b	1/320
CD62L	APC	MEL-14	IgG2a	1/1280
B220	FITC	RA3-6B2	IgG2a	1/370
CD19	PerCP-Cy 5.5	ID3	IgG2a	1/640
CD21	APC	7G6	IgG2b	1/640
CD23	PE	B3B4	IgG2a	1/1280
CD80	V450	16-10A1	IgG2	1/320
IgG	Biotin	Polyclonal	Ig	1/80
IgDa	FITC	11-26c.2a	IgG2a	1/320
IgMa	PE	RMM-1	IgG2a	1/20



<u>Figure 2.2</u> Flow cytometry gating strategies for splenic T cells and B cells. Raw data is gated for lymphocytes based on size (FSC) and granularity (SSC), then CD3+ cells are separated into cytotoxic (CD3+CD8+) and 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 **(A)**. In this case, naive mice were used so central memory T cells are a very small proportion of total helper T cells. Lymphocytes are also separates into B220+CD19+ B cells, whose maturation status is assessed in terms of newly-formed (NF) (CD21-CD23-), follicular (FO) ($CD23^{hi}CD21^{int-lo}$) and marginal zone (MZ) ($CD21^{hi}CD23_{lo}$) B cells, and activated B cells as CD80+ FO B cells **(B).**

2.8 Enzyme-linked Immunosorbent assay (ELISA)

2.8.1 Antibody ELISA

Relative antigen specific serum antibody levels were determined by ELISA. Briefly, flat-bottomed 96 well plates (Nunc, Maxisorp) were coated with the antigen of interest at the required concentration (see Antigen Generation in Section 2.5) diluted in 1 x PBS in a total volume of 50µl per well overnight at 4°C. After coating, plates were blocked for 1 hour at 37°C with 200µl blocking buffer (Appendix A) and then washed 3 times in wash buffer (Appendix A). Serum was diluted to a starting concentration of 1/30 or 1/50 in 150µl dilution buffer (Appendix A), and serially diluted at 1/3 across six wells. Plates were then incubated for 1 hour at 37°C. After incubation, plates were washed and probed with AP-conjugated goat anti-mouse IgM, IgG, IgG1 and IgG2a secondary antibodies at a dilution of 1/1000 (Southern Biotech). Plates were then washed and the signal detected using the substrate p-Nitrophenylphosphate (PNP) (Sigma-Aldrich) powder at 1mg/ml in substrate buffer (Appendix A). Plates were incubated with the substrate at 37°C until the desired colour intensity was observed, after which the absorbance was read at λ405 nm using the Softmax Pro programme on a VersaMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA). Relative antigen specific antibody levels were plotted as dilution graphs or as titres in dot plots. All antibody ELISA sandwich antibodies were from BD Pharmingen.

2.8.2 Cytokine ELISA

Cytokine levels were quantified from supernatants taken from antigen-restimulated splenocytes (Section2.6.1). For all solution and antibody details for cytokine ELISAs, refer to Appendix A. Briefly, IFNγ and IL-13 levels were quantified by coating flat-bottomed 96 well plates (Nunc, Maxisorp) with IFNγ or IL-13 coating antibody, respectively, 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 1/3 from 100ng/ml across 11 wells. Plates were then incubated overnight at 4°C. 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.

2.9 Immunohistochemistry

2.9.1 Spleen sectioning

Spleen segments that were to be used for immunohistochemistry (IHC) were frozen in liquid nitrogen and stored at -80°C until needed. Sectioning was done in the cryostat. Spleen segments were mounted onto a chuck on top of a bed of OCT TissueTek compound (Dako) which froze the tissue onto the chuck. Sections 7µm in thickness were cut on the cryostat and mounted onto four-spot slides (CA Hendley Essex, Ltd). Slides with four sections each were left to dry for one hour before fixation in acetone for 20min at 4°C and storage in tin foil at -20°C.

2.9.2 Staining of spleen sections

IHC was carried out as described (Toellner et al., 1996). In brief, slides were labelled with the stains to be used, brought up to room temperature and hydrated in Tris buffer (pH 7.6) (Appendix A). Sections were subsequently kept in this buffer throughout the staining procedure, ensuring that they never dry (or the tissue would crack). Primary antibodies were diluted in Tris buffer pH7.6 at the optimum dilution and added to sections which were incubated for 1 hour at room temperature and then washed twice for 5 minutes in fresh buffer. Secondary antibodies were first adsorbed with 10% normal mouse serum to block non-specific binding to mouse sections, and then added to the slides and incubated for 45min at room temperature. All antibodies used are listed in Table 2. Slides were then washed as before and streptavidin-complex with alkaline phosphatase (AP) (Dako) was added for 30 minutes. Horse-Radish Peroxidase (HRP) and AP reagents were developed sequentially using DAB and napthol AS-MX phosphate with Fast Blue salt (all from Sigma-Aldrich), respectively (Appendix A). After development slides were washed in Tris buffer pH 7.6 and then dH_2O to remove buffer salts which may cause tissue cracking upon drying. Slides were dried at room temperature prior to mounting with coverslips using Immu-mount (Thermo Electron Corporation). Photographs were taken using a Leica CTR6000 microscope (Leica, Milton Keynes, UK) and QCapture software. Images were processed using Adobe Photoshop.

Table 2.2 The antibodies used for dual IHC stains, their chromospheres, isotypes and working concentrations.

Antibody (anti-mouse)	**Chromophore used**	**Raised in**	**Working concentration**	**Company**	**Clone**
Primary antibodies					
IgD	HRP	Sheep	1/1000	Abcam	Polyclonal
CD3	AP	Rat	1/1000	Abcam	KT3
PNA	AP	n/a	1/5000	Vector Labs	n/a
IgM	AP	Rat	1/500	Serotec	LO-MM-9
IgG1	AP	Rat	1/500	Abcam	LO-MG1-13
IgG2a	AP	Rat	1/150	Serotec	LOMG2a7
Antibody	**Linked to**	**Raised in**	**Working concentration**	**Company**	**Clone**
Secondary antibodies					
Anti-rat	Biotin	Rabbit	1/500	Abcam	Polyclonal
Anti-Sheep	HRP	Donkey	1/100	Abcam	Polyclonal

2.10 Statistics

The results shown in this study are either from individual mice or as group means ± standard error of the mean (SEM). P values and significances were determined using the Mann-Whitney T-test, non- parametric one-way ANOVA or standard two-way ANOVA with Bonferroni post-tests (GraphPad Prism software; La Jolla, CA). Groups were judged to be statistically significant when $p<0.05$ and were indicated with by an asterisk.

3. The effect of maternal *N.brasiliensis* infection on immune development in offspring

3.1 Introduction

Neonates and infants can be highly susceptible to infection due to their poorly developed immune memory and an immature B cell repertoire, which can result in relatively weak primary antibody responses to infection and vaccination.[67] However, the neonatal immune system does have a full complement of immune cells at birth. Moreover, the neonatal immune system can also mount T and B cell responses comparable to those of an adult (for example, with the use of specific adjuvant formulations, supplementation with adult DCs or the use of DNA vaccine delivery systems).[67,78] Irrespective, neonatal cell populations are immature with an impaired ability to launch maximal immune responses. To a large extent this immaturity is overcome through antigenic exposure, beginning with exposure to maternal immune components *in utero* and transferred antigen and immune components through breastmilk,[156] colonization of the gut with commensal microbes,[157,158] and exposure environmental antigen.[159] This experience gradually leads to the development of a pool of experienced memory B and T cells and expansion of the infant's antibody repertoire. [86,160] Until the infant's B cell repertoire has developed sufficiently, neonates and infants rely on passively acquired maternal antibodies (MatAb) for protection for the first 3-24 months of age after which MatAb decline below protective thresholds.[161] While MatAb are largely protective, they can also inhibit generation of some protective neonatal and infant antibody responses (as shown 60 years ago[162]), preventing the use of several vaccines during infancy, such as measles vaccine, oral polio, varicella-zoster, influenza and human rotavirus vaccines (reviewed here[83]).

Since neonatal T and B cells can induce adult-like responses to specific *in vitro* stimulations,[67,78] it may be that the fetus can generate its own antibody response following in utero antigen stimulation. Evidence that maternal infection or *in utero* exposure to soluble antigen can result in fetal immunologic sensitization has been reported in offspring from mothers infected during pregnancy with mumps[163] or toxoplasmosis.[164] Moreover, antigen specific recall responses to childhood vaccines have been reported in babies whose mothers were vaccinated during pregnancy with tetanus toxoid[165], streptococcal[166] or meningococcal vaccines.[167] The presence of parasite-specific IgE and IgM in cord blood, isotypes that do not normally cross the placenta, suggests that prenatal sensitization to chronic parasite infections such as schistosomiasis[168] and filiriasis,[169,170] can occur. Stimulation of antigen specific B cell immunity and helminth antigen specific IgG has also been shown in children whose mothers had chronic helminth infections during pregnancy.[171] Maternal infection with *Ascaris lumbricoides* has also been associated with newborn T cell sensitization to *A.lumbricoides.* Here, antigen stimulated cord blood from newborns born to infected mothers had increased frequencies of CD4+ T cells expressing antigen specific IFNγ and IL-4.[172]

Helminth-specific immunity acquired *in utero* by infants born to infected mothers has been shown to persist into childhood and bias the immune response to BCG vaccination against the protection associated IFNγ response.[173] In developing countries, women of child-bearing age can frequently be infected with one or more helminth parasite.[111] Chronic helminthiasis can affect immune responses to unrelated antigens within the same host, promoting Th2 cytokine production and down regulating effector responses by inducing regulatory T cells and anti-inflammatory cytokines.[174–176] These changes have been associated with diminished childhood vaccine efficacy, more rapid progression of and increased susceptibility to unrelated infections (reviewed here[177]).

In the following chapter we present data demonstrating how maternal priming by infection with the rodent specific helminth, *Nippostrongylus brasiliensis*, prior to pregnancy impacts on the offspring’s immune system. In particular we examined how this infectious influence altered the T and B cell responses of the offspring.

3.2 Results

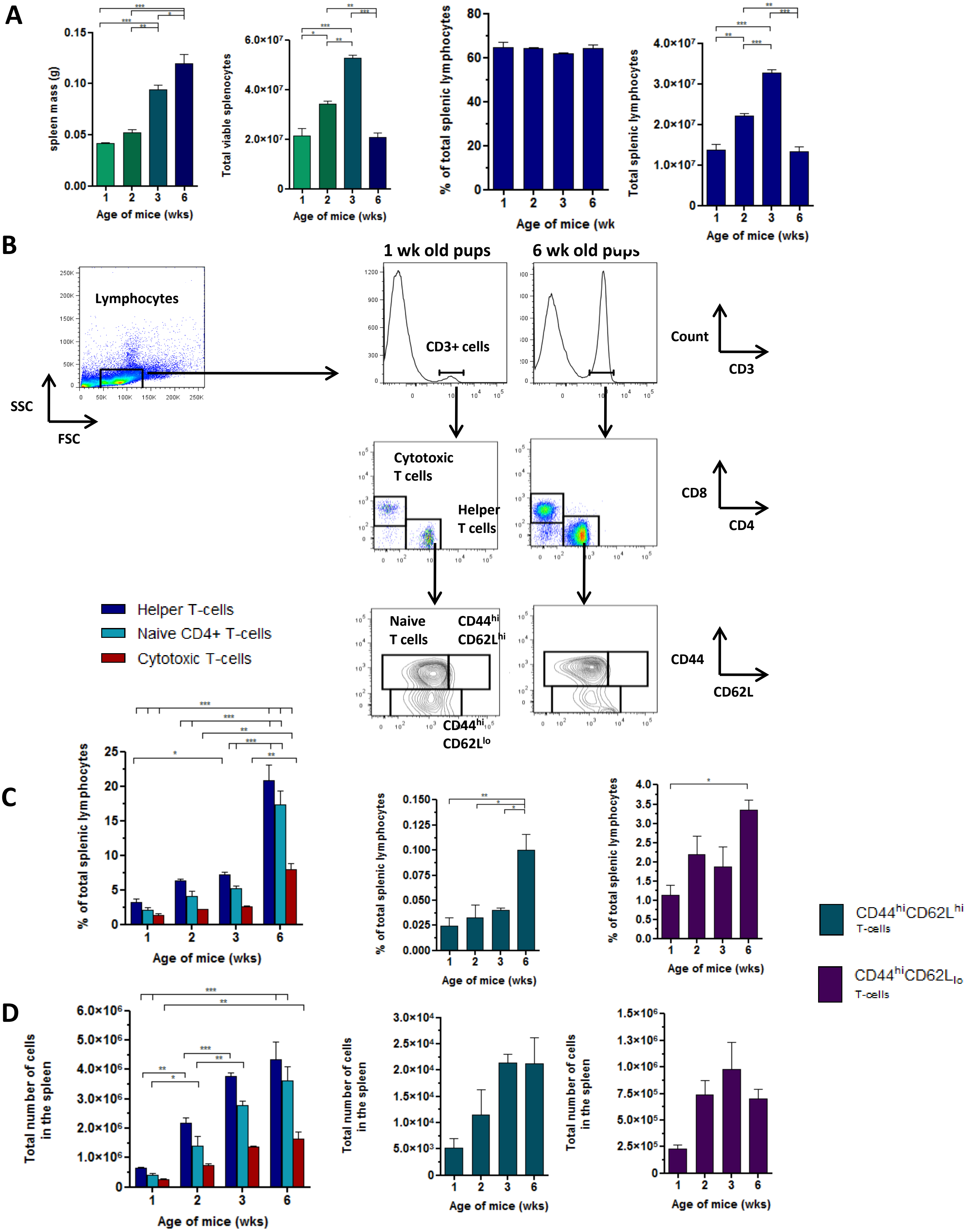
3.2.1 Maturation of splenic lymphocyte populations with age in naive BALB/c pups

To demonstrate if T and B cell populations in the spleen change with age in neonatal and infant BALB/c mice, we analysed the proportions and numbers of these cells in 1, 2, 3 and 6 week old BALB/c mice born to naive mothers. This data provides an outline as to how these cell populations change from the neonatal stage (1 week old) to the young adult (6 week old).

As expected both spleen mass and cellularity increased with age. The average spleen mass of a 1 week old neonate was 0.042g (±0.0008) increasing to an average of 0.12g (±0.0085) in adult 6 week old mice. Splenocyte numbers increased from an average of $2.13\text{x}10^7$ ($\pm3.087\text{x}10^6$) splenocytes in 1 week old neonates, peaked at $5.28\text{x}10^7$ (±1.36x106) at 3 weeks, after which they declined to $2.08\text{x}10^7$ ($\pm1.92\text{x}10^6$) splenocytes in adult 6 week old mice. Total lymphocyte proportions remained constant from the neonate to the adult. Total lymphocyte numbers changed in accordance with total splenocyte numbers (which contain lymphocytes, macrophages and DCs), from an average of $1.36\text{x}10^7$ ($\pm1.49\text{x}10^6$) in the neonate, doubled and peaked at $3.26\text{x}10^7$ ($\pm7.81\text{x}10^5$) at 3 weeks and dropped to $1.34\text{x}10^7$ ($\pm1.17\text{x}10^6$) in the adult.

This pattern conforms to changes in splenic lymphocyte numbers in the peripheral blood of other species, namely rabbits, calves, piglets, kittens, and puppies, all of which have low numbers at birth which increase rapidly in the first weeks of life and decline in adulthood.[74] However, Spear *et al.* showed an increase in the splenic nucleated cells of Swiss-L mice from approximately $2.5\text{x}10^7$ cells per spleen at 1 week to approximately $1\text{x}10^8$ cells per spleen at 5 weeks which was sustained in 10 week old naive mice.[160] Swiss-L mice are an outbred strain whereas BALB/c mice are inbred, which may account for the differences we see.

Humans have high total lymphocyte numbers at birth, unusual compared to other species, but numbers also decrease in adulthood.[86] Most studies in the literature assessed circulating



<u>Figure 3.1</u> **Maturation of spleen mass, splenocyte and lymphocyte number and splenic T cell populations with age in BALB/c pups. (A)** Spleen mass, total splenocytes, proportions and total numbers of splenic lymphocytes in 1, 2, 3 and 6 week old naive BALB/c pups born to naive mothers. **(B)** T cell populations were assessed. **(C)** Percentages of CD4+ helper T cells, naive CD4+ T cells and CD8+ cytotoxic T cells, CD44^{hi}CD62L^{hi} central memory T cells and CD44^{hi}CD62L$_{lo}$ effector helper T cells, calculated from total lymphocytes. **(D)** Total numbers of T cell populations and sub-populations. *$p<0.05$. **$p<0.01$. **$p<0.001$. N=3. Experiment was carried out once.

lymphocytes in domestic animals and humans. To the best of our knowledge this data represents the first study to examine splenic lymphocyte development with age in BALB/c mice. Spleen size and cellularity are one of the indicators of the infection or immunization status of mice and humans.

Examination of T and B cell populations revealed age related increases in the proportions of these cell populations; however, total numbers of B cells were significantly reduced in the adult spleen when compared to the 3 week old spleen.

3.2.1.1 T cell development

T cell populations were analyzed as previously described **(Fig. 3.1B)**.The percentage of splenic helper T cells (CD3+CD4+) increased with age from 3% in neonatal mice to 20% in adult 6 week old mice **(Fig.3.1C)**. Cytotoxic T cells (CD3+CD8+) increased from 1.3% in neonatal mice to 7.9% in 6 week old mice. Spear *et al.* defined a splenic Thy-1^+ (θ^+) population in spleens of Swiss-L mice which was already at 20%±3.6% at birth and increased slightly to 27.3% ± 3.9% at 10 weeks, conflicting with our results and evidence in other species where peripheral blood lymphocyte numbers are low at birth and then increase.[160] Stobo and Paul showed that murine splenic T cell numbers increase with age.[178]

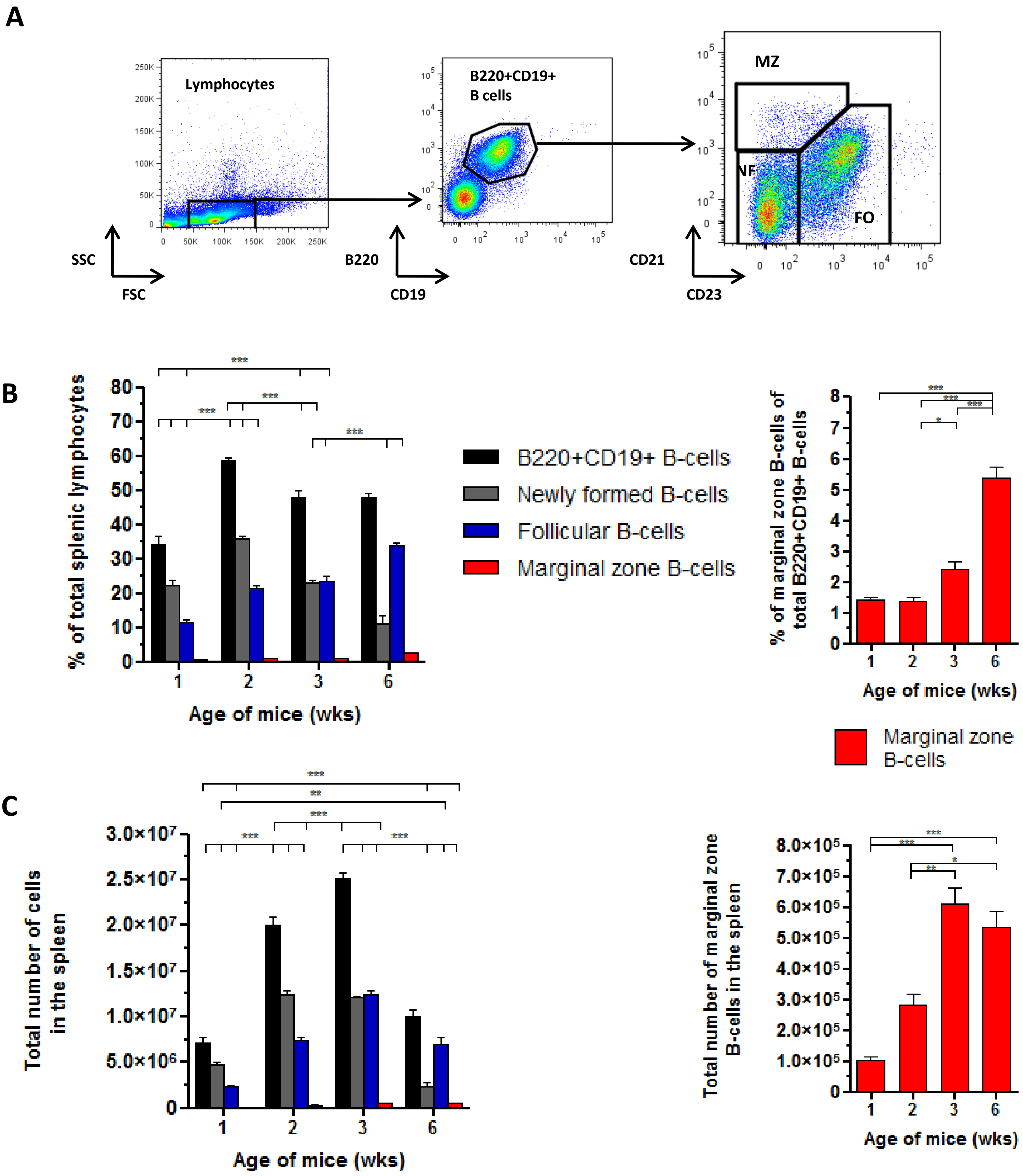
Dissection of the activation status of CD3+ CD4+ T cells showed the percentage of naive CD4+ T cells increased with age from 2% in the neonate to 17% in the adult. Effector T helper cells (CD3+CD4+CD44^{hi}CD62L$_{lo}$) increased from 1.1% at 1 wk to 3.4% at 6 weeks of age, and central memory T cells (CD3+CD4+CD44^{hi}CD62L^{hi}) also increased from 0.024% at 1 wk to 0.100% at 6 wks. However, it is interesting to note that activated T cell populations comprised a small percentage of the T helper cell repertoire, the majority of cells being classed as naïve. This could be expected since these mice are likely to be largely antigen-inexperienced.

Total T cell numbers of all populations increased with age although the dramatic increase in T cell proportions seen between 3 week and 6 week old mice was not apparent in the changes in total numbers, suggesting that T cell numbers stabilized soon after 3 weeks while other lymphocyte populations decrease in numbers.

3.2.1.2 B cell development

The maturation of splenic B cell populations was assessed in the same 1, 2, 3, and 6 week old naive BALB/c mice as previously described: B220+CD19+ B-cells and sub-populations of newly formed B cells (NF) (B220+CD19+ CD21-CD23-), marginal zone B cells (MZ) (B220+CD19+ $CD21^{hi}CD23_{lo}$) and follicular B cells (FO) (B220+CD19+ $CD23^{hi}CD21^{int-lo}$) **(Fig. 3.2A)**. Populations were quantified as percentages of total splenic lymphocytes **(Fig. 3.2B left)** and MZ B cells were also quantified as a percentage of B220+CD19+ B cells **(Fig. 3.2B right)**. Proportions of total B220+CD19+ B cells increased from 34% of total splenic lymphocytes at 1 wk to 58% at 2 wks and then stabilized at 47% at 3 and 6 wks. Gelfand *et al.* also showed that Ig^+ splenic lymphocytes reached adult proportions at between 2 and 4 weeks.[179] Nossal and Pike showed that splenic and peripheral B cells increase rapidly after birth, more slowly thereafter and plateau at 5 weeks of age.[180]

In our study, NF B cells (22%, as a percentage of total splenic lymphocytes) were initially greater in proportions than FO B cells (11%) and peaked at 2 wks (36%) with an increase in FO B cells (22%), but then decreased at 3wks, along with total B220+CD19+ B cell proportions, where NF and FO were equal. NF further decreased in adulthood at 6 wks to 11% and FO increased to 34% of total splenic lmphocytes, representing the maturation of already present NF B cells in the spleen and a decline in their migration from the bone marrow to the spleen.[181] MZ B cells comprised a small overall proportions at 1 wk and increased steadily from 1.4% of total splenic lymphocytes to 5.4% into adulthood, corresponding to other studies and coinciding with the ability to mount a response to T-independent antigens.[93] Total splenic B cell numbers of all populations were quantified **(Fig. 3.2C)** and MZ B cells were plotted together **(left)** and apart **(right)** from other populations due to their low numbers. Total splenic B cell numbers followed a similar pattern of development; however the peak of total B220+CD19+ B cells, NF and FO B cells was reached at 3 wks whereas the peak in proportions of total splenic lymphocytes was reached earlier at 2 wks. MZ B cell total cell numbers plateaued at 3 wks.



<u>Figure 3.2</u> Maturation of splenic B cell populations with age in BALB/c pups. (A) Developmental B cell populations were assessed in 1, 2, 3 and 6 week old BALB/c pups by flow cytometry. Percentages of B cell populations were quantified **(B)** as a percentage of total lymphocytes (left). Marginal zone B cells were quantified as a percentage of total lymphocytes (left) and B220+CD19+ B cells (right). **(C)** Total numbers of B cell populations. Total numbers of marginal zone B cells as compared to total B cells , newly formed B cells and follicular B cells (left), and alone (right). *$p<0.05$. **$p<0.01$. **$p<0.001$. N=3. Experiment was carried out once.

Overall, in naive mice splenic T cell populations increase in proportion to total splenic lymphocytes until adulthood but their absolute numbers plateau at or soon after 3wks, with the exception of central memory CD4+ T cells which decreased again to numbers seen at 2 wks.

Splenic T cell sub-populations maintain their relative proportions throughout. Splenic B cell populations, on the other hand, stabilize in proportion to total lymphocytes at 3 wks, but FO B cells become dominant in adulthood where NF B cells were dominant at 1 wk. Absolute numbers reach 2.5 times that of adult numbers at 3 wks but then decrease at 6 wks, a consistent pattern reported in domestic animals but not in humans, which have 2.2-2.6 times the count of adult lymphocytes at birth.[74,86]

In contrast to splenic T cells, non MZ B cells decrease in number in adulthood but remain constant in proportion to total splenic lymphocytes. NK cells have also been shown to decrease steadily from high numbers at birth in human cord blood to 1/5 of this number in adulthood.[86] The decline in B cell numbers, and possibly NK cell numbers, within the decline in total splenic lymphocytes in adulthood, accounts for the increase in T cell proportions without a corresponding increase in T cell numbers and therefore the increase in the T:B cell ratio with age in naive BALB/c splenic lymphocytes. This increase in T:B cell ratio into adulthood has been reported in Swiss-L mice although neonatal and infant Thy-1$^+$ cell proportions were much higher than reported here.[160]

3.2.2 Maternal Nb infection results in an increase in lymphocyte populations in offspring

After defining normal spleen mass and splenic lymphocyte development of neonatal, infant and adult naive mice, we assessed how a maternal history of infection with the helminth *N.brasiliensis* (Nb) influenced offspring. Unlike other studies in the literature (Reviewed here[111]), the maternal infection was cleared *before* mating and pregnancy and there was thus no parasite present in the mother during fetal development. However, the presence of parasite antigen cannot be excluded.

Mothers were infected at 6 wks of age with 500xL3 Nb larvae or left uninfected **(Fig. 3.3A)**. After two weeks an adult BALB/c mouse should clear the infection naturally but both infected and uninfected mothers were given Ivermectin in their water bottles to ensure the worm was cleared. Mothers were mated after a week of treatment and their offspring was killed at 2 wks of age. Pup body mass, spleen mass, total splenocytes, proportion and total number of lymphocytes were determined **(Fig. 3.3B)**. Spleen mass and total splenocytes were consistent with previously determined spleen mass of naive pups from naive mothers. Maternal Nb infection did not have an effect on body mass, spleen mass or total splenocytes in 2wk old offspring. However, there was a slight increase in proportions of total splenic lymphocytes from an average of 66.6% to 68.9% with maternal Nb infection but this change was not statistically significant in total number of lymphocytes.

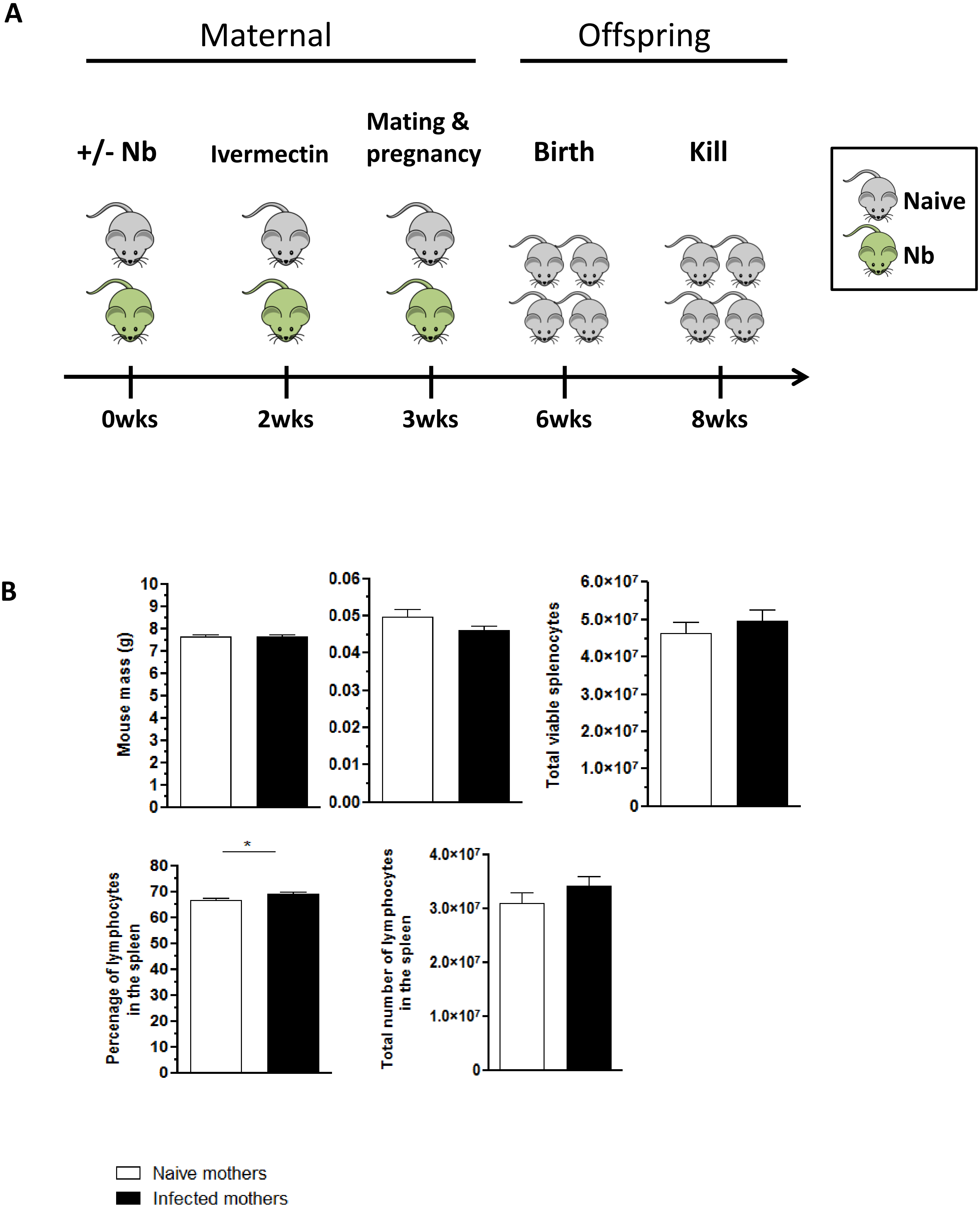


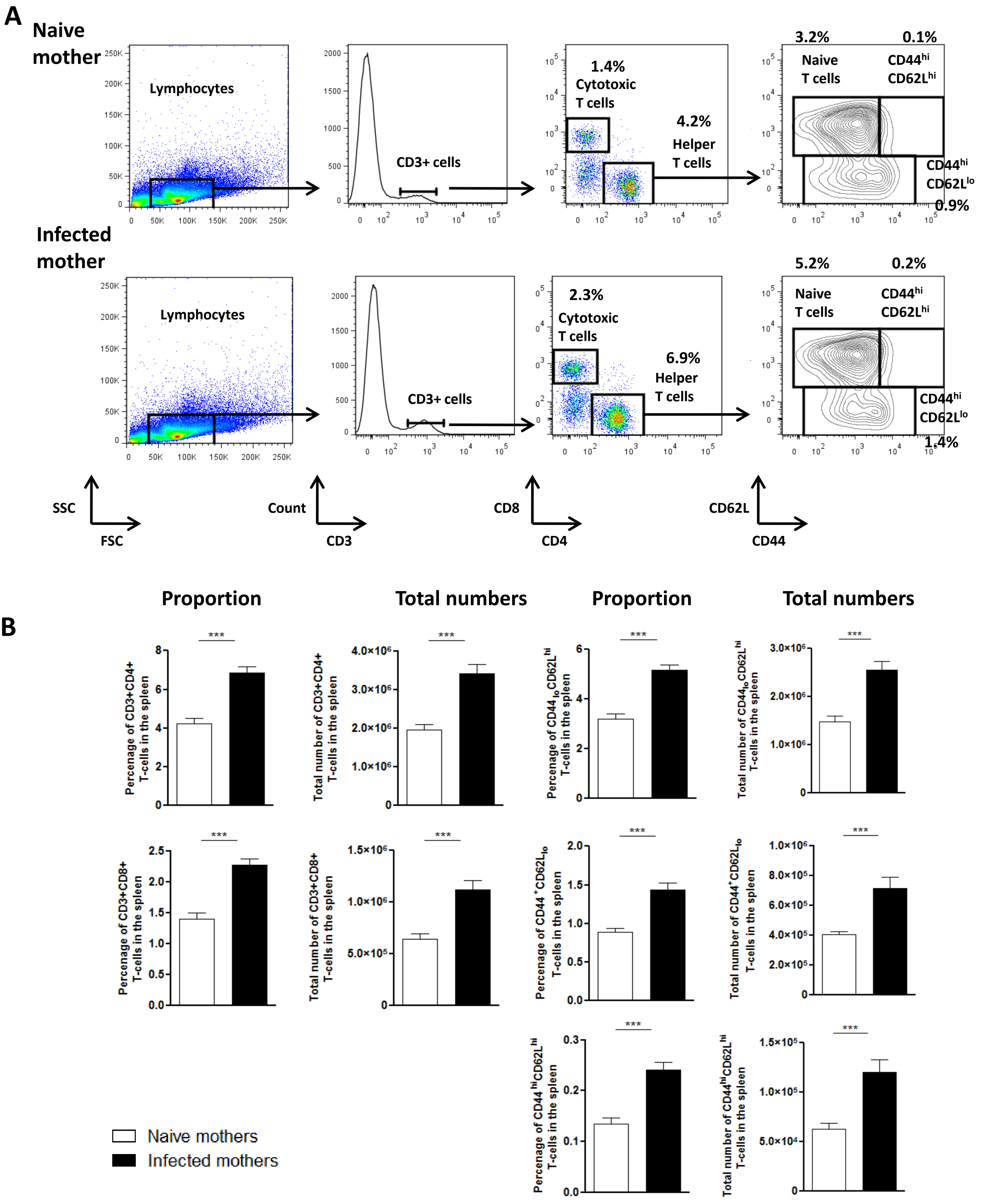
Figure 3.3 **Maternal priming with *N.brasiliensis* infection does not result in changes in mouse body mass, spleen mass or total splenocytes numbers in offspring. (A)** Experiment mouse plan: 6 week old female BALB/c mice were infected with 500xL3 *N.brasiliensis* (Nb) larvae s.c. or left naive, both groups treated with Ivermectin two weeks post infection, mated and their pups were sacrificed two weeks after birth. **(B)** Pup body mass ,spleen mass, total splenocytes, percentage and total number of lymphocytes. *p<0.05. N=6-10. Representative of four independent experiments.

3.2.3 Maternal Nb infection results in an increase in lymphocyte populations in offspring

3.2.3.1 T-cells

Next we assessed whether maternal Nb infection had more subtle effects in offspring that could be seen in changes in lymphocyte populations. Splenic T cell populations in offspring were assessed as previously described **(Fig. 3.4A)**. Populations were quantified as proportions of total lymphocytes and total numbers per spleen **(Fig. 3.4B)**. Maternal Nb infection resulted in a general increase in T cell population proportions and absolute numbers: Total helper T cells and cytotoxic T cells, naive CD4+ T cells, effector and central memory CD4+ T cells.

Proportions of CD3+CD4+ helper T cells increased from an average of 4.2% of total splenic lymphocytes in pups from naive mothers to 6.9% in pups from Nb infected mothers, CD3+CD8+ cytotoxic T cells increased from 1.4% to 2.3% with maternal infection. Naive helper T cells also increased, from an average of 3.2% to 5.2%. Analysis of T cell activation status showed an increase in effector T helper cells (CD3+CD4+CD44^{hi}CD62L$_{lo}$) from 0.9% to 1.4%, and an increase in central memory T cells (CD3+CD4+CD44^{hi}CD62L^{hi}) ($p<0.0001$ for all populations). Total numbers of these T cell populations increased in accordance with their increase in proportions. Thus maternal Nb infection resulted in a general increase in naive and activated T cell populations in the spleen.



<u>**Figure 3.4**</u> **Maternal Nb priming results in an increase in T cell and proportions and total numbers in offspring. (A)** CD4+ and CD8+ T cell populations and CD4+ sub-populations were assessed by flow cytometry in two week old naive pups from Nb infected of naive mothers. **(B)** These populations were quantified as a percentage of total lymphocytes and as total cell numbers. N=8 pups per group.***p<0.001. N=6-10. Representative of four independent experiments.

3.2.3 Maternal Nb infection results in an increase in lymphocyte populations in offspring

3.2.3.2 B-cells

Analysis of B cell maturation with maternal Nb infection **(Fig. 3.5A)** revealed an increase in all B cell population proportions and absolute numbers **(Fig. 3.5B)**: Total B220+CD19+ B cell proportions increased from an average of 36.4% to 43.1% with a corresponding increase in total numbers. FO B cells increased from an average of 13.1% to 15.9%, NF B cells increased from an average of 21.6% to 25.1%, and MZ B cells increased from an average of 0.96% to 1.32%. Activated CD80+ B cells increased from an average of 3.1% to 4.4% with maternal infection.

Generally, proportions and numbers of B cell populations were slightly lower than those determined previously in 2 week old naive pups from naive mothers and resembled those of pups aged between 1 and 2 weeks. While birth time of pups was noted and therefore pups were all age matched, differences in litter sizes may affect intrauterine space and concentration of transferred immune components per pup, as well as access to breastmilk from the mother during suckling. In breeding strategies, litters were kept constant between groups of naive and infected mothers, but litters obtained from the animal unit in the previous study may not have been consistent with this in numbers per mother and per cage and may account for differences in lymphocyte populations between the two experiments.

Since B cell maturation can be examined by a wide range of surface markers, further analysis of B cell maturation using IgM and IgD surface immunoglobulins **(Fig. 3.6A)** revealed less pronounced increases in B cell populations. Pre B cells (IgM-IgD-) increased from an average of 13.1% to 14.9% with maternal infection and immature B cells ($IgD_{lo}IgM^{hi}$) increased from an average of 6.7% to 7.9% **(Fig. 3.6B)**. Mature recirculating B cells ($IgD^{int}IgM_{lo}$) increased relatively more from 6.6% to 8.1% with maternal infection. Transitional B cells ($IgD^{hi}IgM^{hi}$) did not change with maternal infection.

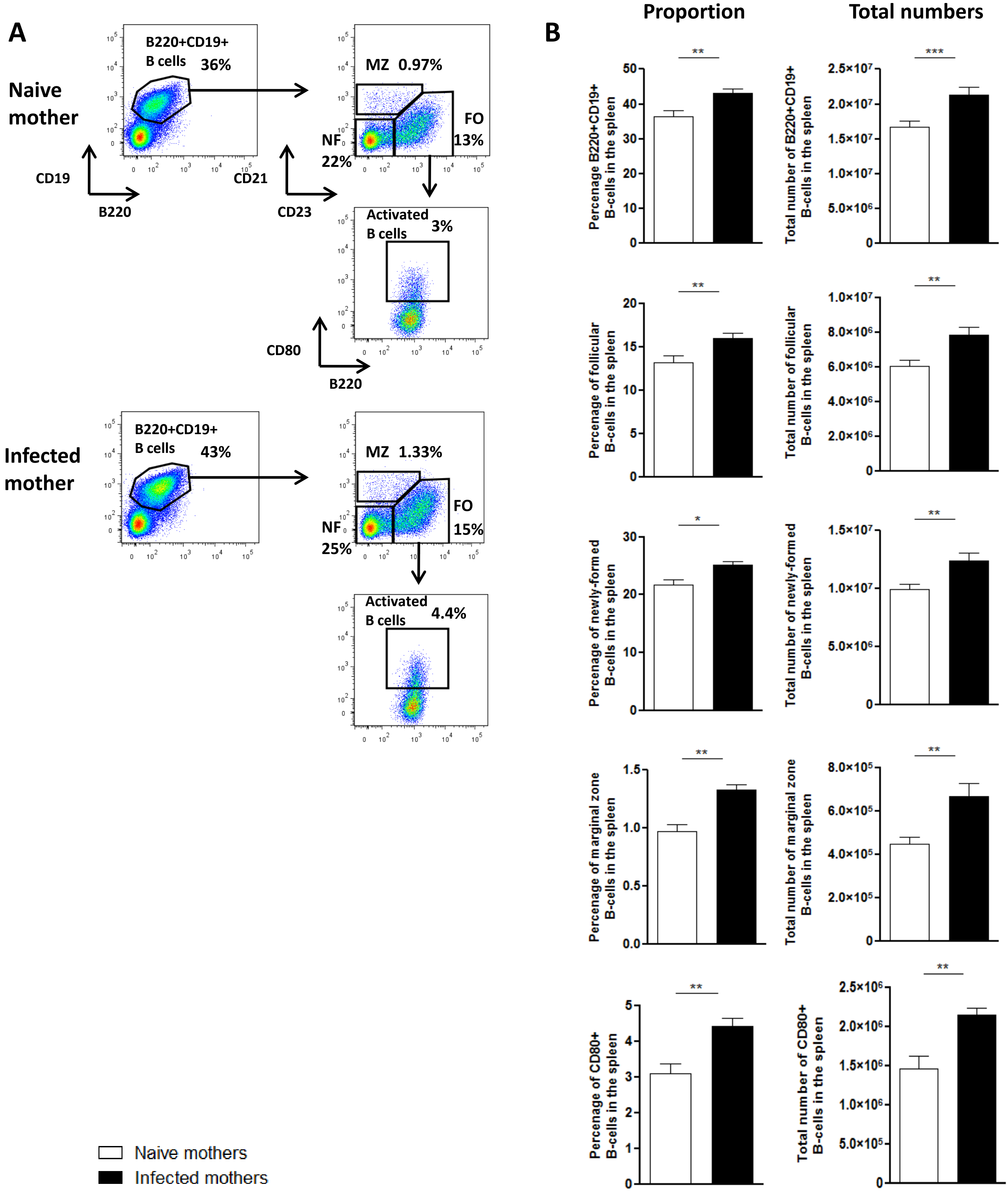


Figure 3.5 Maternal Nb priming results in an increase in B cell proportions and total numbers in offspring. (A) B220+CD19+ B cells and B cell developmental sub-populations as well as activated CD80+ B cells were assessed by flow cytometry in two week old naive pups from Nb infected of naive mothers. **(B)** These populations were quantified as a percentage of total lymphocytes and as total cell numbers. *p<0.05. **p<0.01. **p<0.001. N=6-10. Representative of four independent experiments.

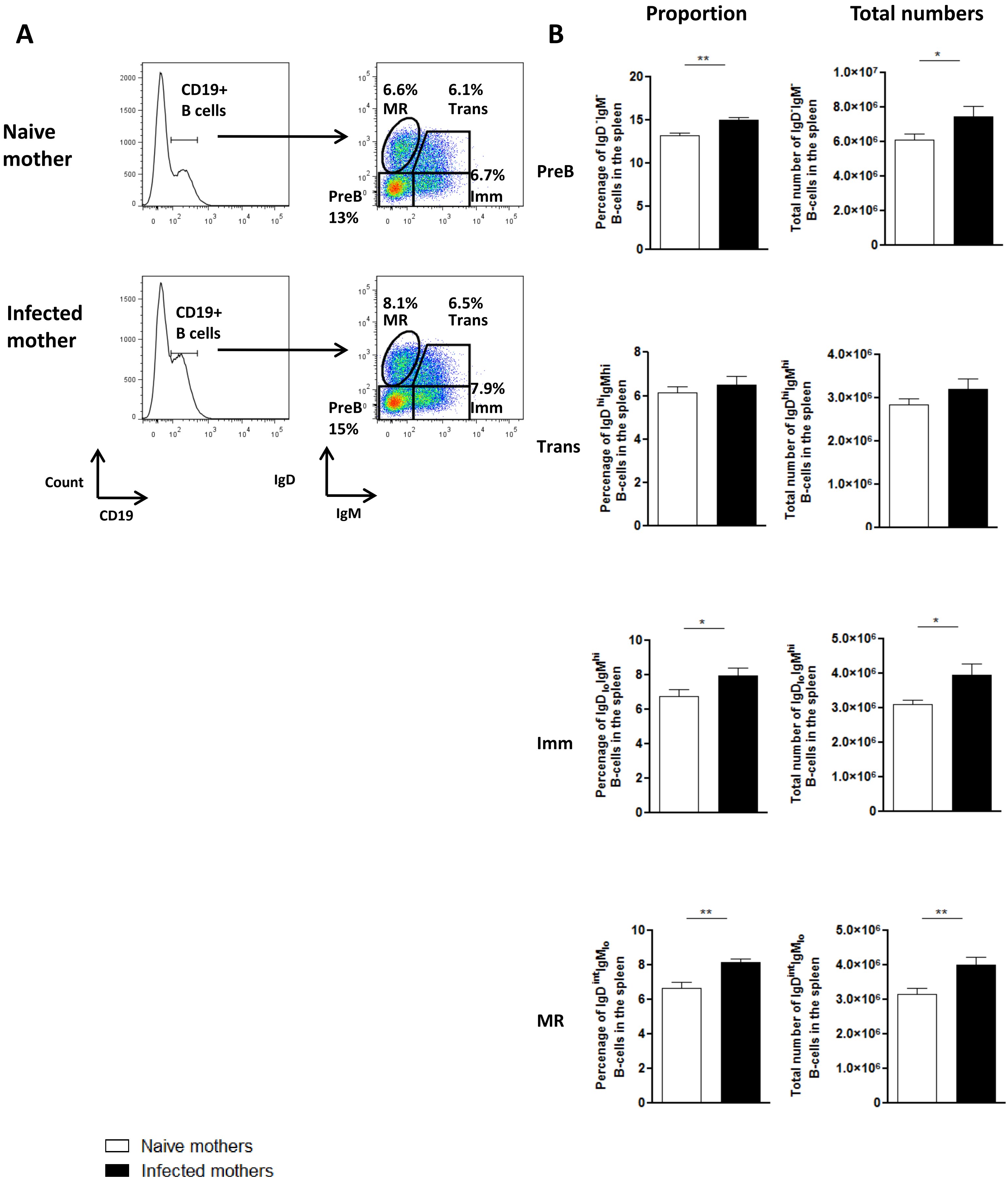


Figure 3.6 Maternal Nb priming results in an increase in B cell and proportions and total numbers in offspring. (A) CD19+ B cells and B cell developmental sub-populations as well as activated were assessed by flow cytometry in two week old naive pups from Nb infected of naive mothers. **PreB: Pre-B cells, Imm: Immature, Trans: Transitional, MR: Mature Recirculating. (B)** These populations were quantified as a percentage of total lymphocytes and as total cell numbers. *p<0.05. **p<0.01. **p<0.001. N=6-10. Representative of four independent experiments.

3.2.4 Offspring from infected mothers have early germinal centre reactions

Immuno-histochemical analysis of spleens revealed organized splenic lymphocyte architecture including T-zones and B cell follicles in 2 week old pups born to naive and Nb infected mothers **(Fig. 3.7A)**. IgM+ B cells were present in marginal zones and in extra-follicular areas in both groups of pups. B1 cells mature with or without antigen to become IgM producing cells, and normal serum IgM concentrations are reached in germ-free mice.[182] Importantly, maternal Nb infection resulted in early formation of germinal centers (GCs) in offspring. GCs were absent in offspring from naive mothers.

Neonates and infants have low IgG responses to immunization, increasing with age, and most IgG found in serum is of maternal origin.[78] We stained spleen sections with IgG1 and IgG2a to see whether maternal Nb infection caused priming of B cells to form IgG1 switched GCs or extra-follicular IgG responses **(Fig. 3.7B)**. While some blue stain was seen with the IgG1 stain, this was due to non-specific staining of non-lymphocyte areas as seen in the wall of an arteriole and did not show staining of IgG1+ cells as seen with IgM+ cells. Therefore maternal Nb infection did not cause early isotype switching to IgG1 or IgG2a in offspring.

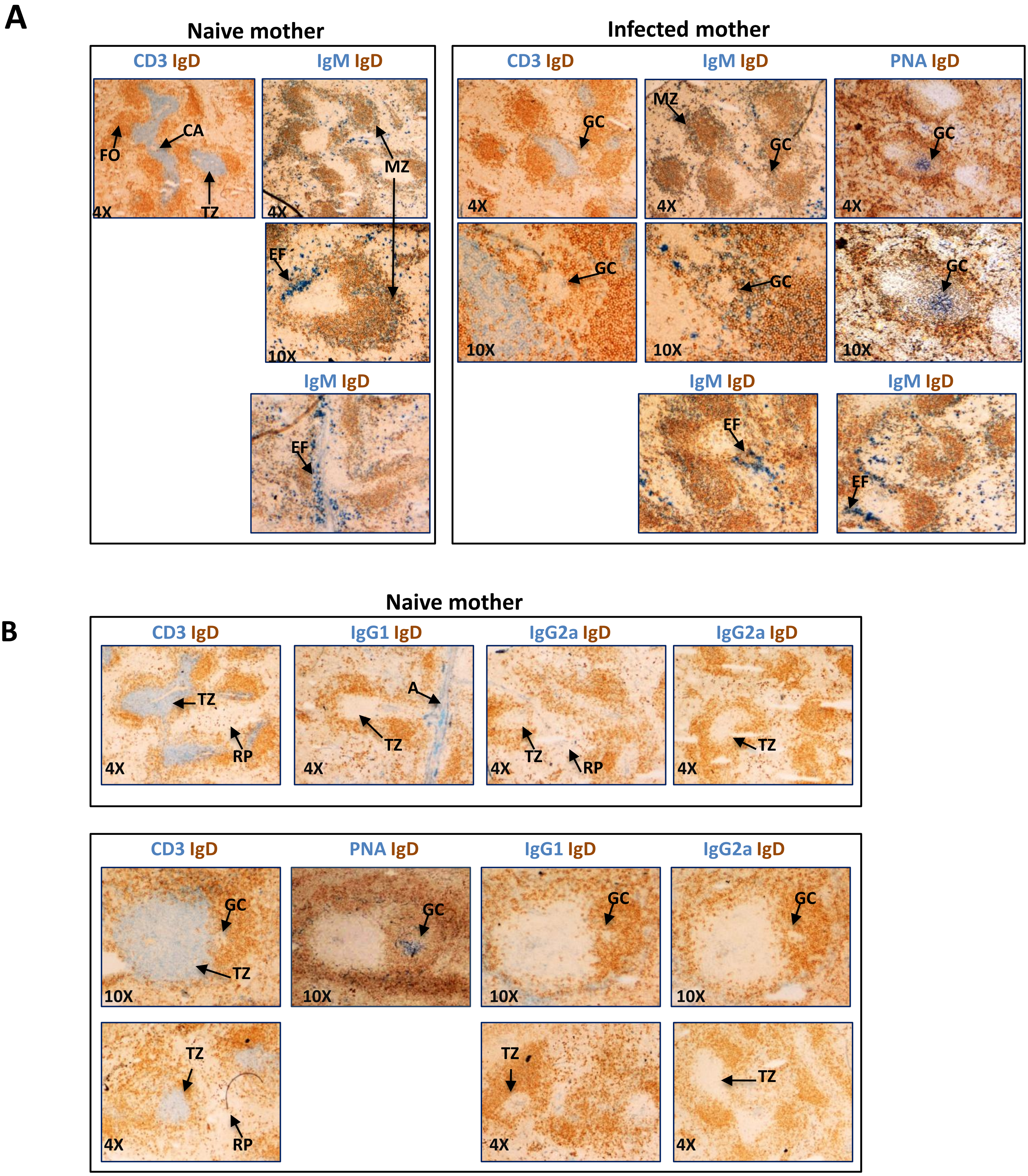
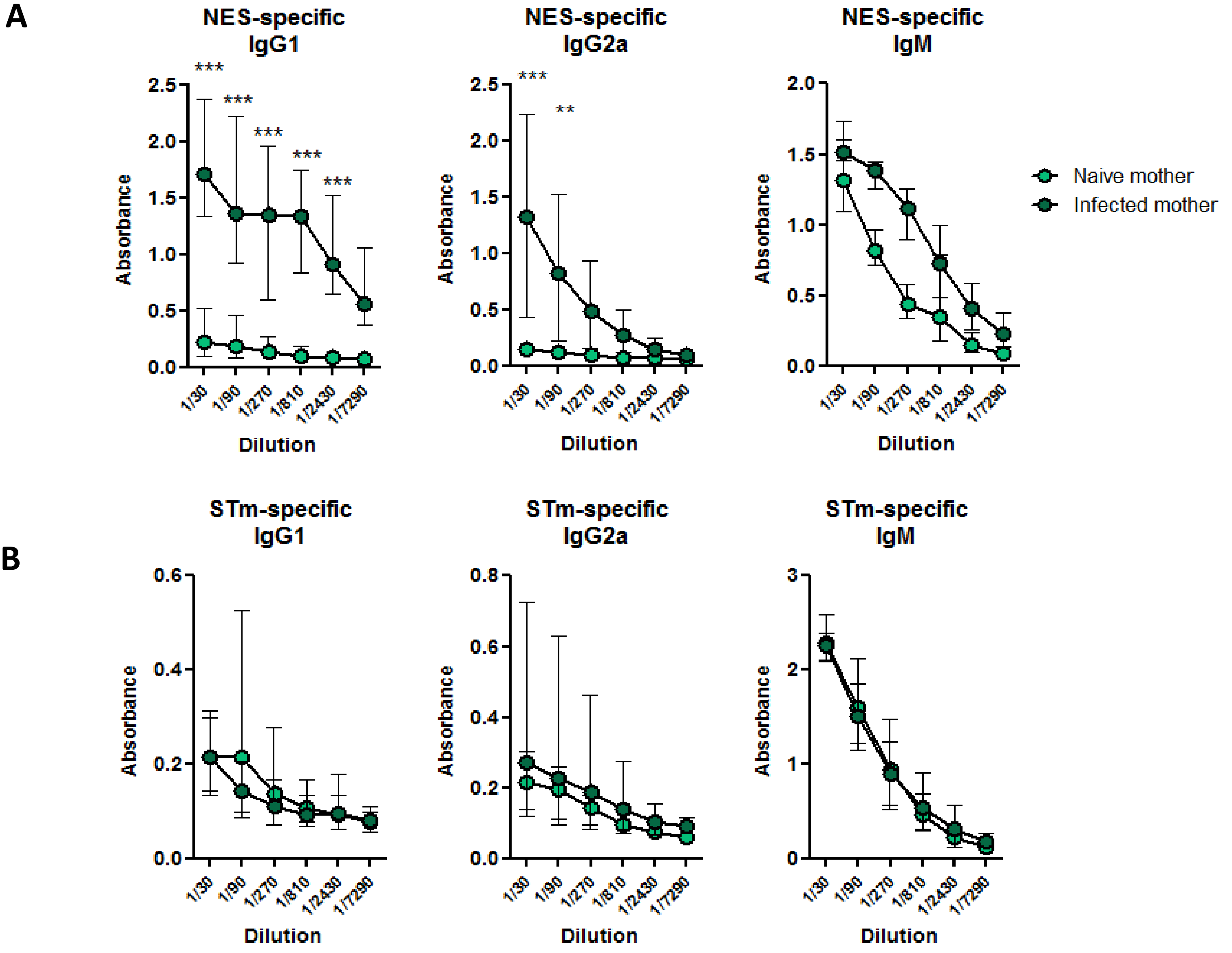


Figure 3.7 Maternal Nb priming results in early germinal center formation in offspring. (A) Frozen spleen sections from 2 week old pups born to Nb infected or naive mothers were sectioned and stained by immunohistochemistry with two dual stains: anti-CD3 (blue) and -IgD (brown) to show T-zones and B cell follicles respectively, and anti-IgM (blue) and -IgD (brown) to show marginal zones and B cell follicles respectively. **(B)** Sections were stained for IgG1 and IgG2a and assessed for B cell isotype switching. No isotype switching was seen. TZ: T-zone, CA: Central arteriole, FO: Follicle, MZ: Marginal zone, GC: Germinal center, EF: Extra-follicular, A: Arteriole. N=6. Experiment was carried out twice.

3.2.5 NES-specific antibodies are still present in the mother at 3 weeks postpartum

Next we looked at the antibody repertoire in mothers as this could be representative immunoglobulins transferred *in utero* and via breastfeeding to offspring. Relative levels of NES-specific IgG1, IgG2a and IgM were determined in mothers at 3 weeks postpartum **(Fig. 3.8A)**. As expected, mothers that were infected had high levels of NES-specific IgG1 but they also had high levels of NES-specific IgG2a, showing that Nb infection induces both Type 1 and Type 2 type antibody responses. While infected mothers showed a trend of slightly increased NES-specific IgM, this was not statistically significant. Type 1 antigen-specific antibodies were assessed as the *Salmonella typhimurium* (STm) antigen OMPS-specific IgG1, IgG2a and IgM **(Fig. 3.8B)**. Both naive and infected mothers had low levels of OMPS-specific IgG1 and IgG2a, and high levels of OMPS-specific IgM with no differences between the groups which may represent naturally occurring IgM antibody.



<u>Figure 3.8</u> Nb specific antibodies are still present in the mother at 3 weeks postpartum whereas naturally occurring Th1 type antibodies are unaffected. (A) The relative levels of Nb-specific antibodies were determined specific to the Nb antigen NES by ELISA: NES-specific IgM, IgG1 and IgG2a in Nb infected and naive mothers 3 weeks postpartum. **(B)** Relative levels of Th1 type antibodies were determined specific to *Salmonella typhimurium* (STm), using purified outer membrane proteins (OMPS) of the bacterium as antigen by ELISA: STm-specific IgG1, IgG2a and IgM. $^{**}p<0.01$. $^{***}p<0.001$. N=6. Experiment was carried out once.

3.2.6 NES-specific antibodies are transferred from infected mothers to offspring

The serum antibody repertoire of offspring was assessed and compared to the MatAb repertoire to determine which antibodies may have been transferred. NES-specific IgG1 and IgG2a was present in pups born to Nb infected mothers and completely absent in pups born to naive mothers, implying that NES-specific IgG1 and IgG2a in offspring were transferred from the mother. In rodents, some IgG is transferred across the placenta via the FcRn receptor and the balance is absorbed from colostrum and breastmilk from the gut into the blood over the first 16-18 days of life until the FcRn receptor is down regulated in the gut.[74] Litter swop experiments from our lab have shown that NES-specific IgG1 is transferred via both routes but predominantly by breastmilk (unpublished results).

IgG1 is the most efficiently transferred IgG and IgG2a is the least, while IgG is transferred preferentially over IgM.[183,184] It is important to note that the ELISA technique does not provide a quantitative measure of antibody in the serum, only a relative one. Therefore it is not possible to comment on absolute levels of antibodies. NES-specific IgM levels were high in both groups, suggesting that this was naturally occurring IgM as seen in extra-follicular IgM+ B cells in the spleen IHC and in mothers. B1 B cells are known to produce natural IgM antibodies in the absence of antigen stimulation, and these antibodies are polyreactive, explaining their cross-reactivity to NES.[185]

Levels of Type 1 type OMPS-specific IgG1 were low and showed no statistically significant differences between the two groups **(Fig. 3.9B)**. OMPS-specific IgG2a levels were low in general, but lower in pups from Nb infected mothers, suggesting a possible interference of the maternal Nb infection in OMPS-specific IgG2a production in offspring. The presence of OMPS-specific IgG2a without STm infection may be due to commensal *Salmonella* in the gut of the young mice. OMPS-specific IgM levels were high but equal between groups.

To determine how long maternal NES-specific antibodies persisted in the serum of offspring, pups from Nb infected and naive mothers were allowed to age. Serum was taken at 8 weeks and levels of NES-specific IgG1 and IgG2a were determined **(Fig. 3.9C)**. Now adult mice

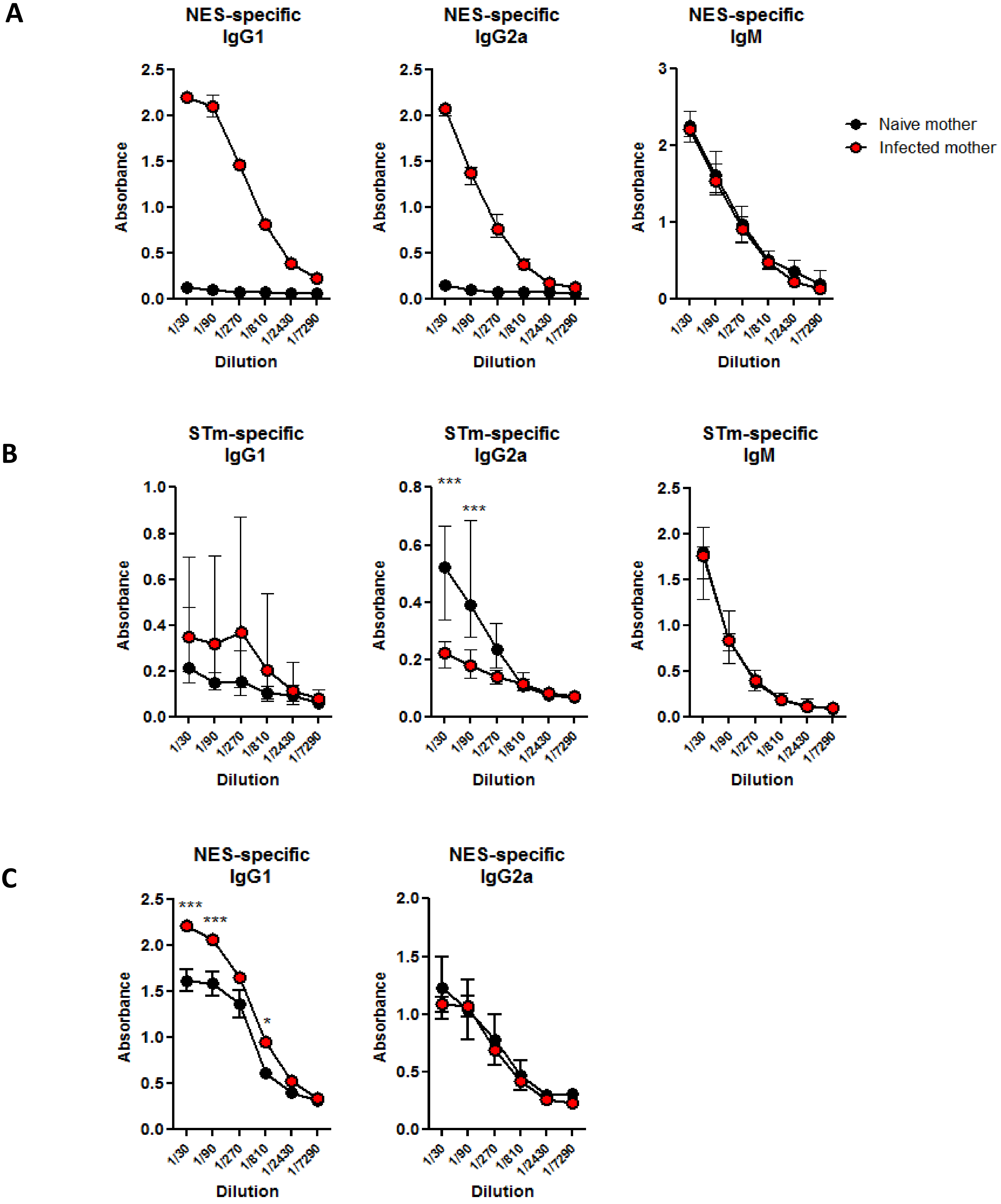


Figure 3.9 **Transfer of maternal NES-specific antibodies in offspring and influence on the presence of naturally occurring Th1 antigen-specific antibody. (A)** NES-specific IgM, IgG1 and IgG2a in naive 2 week old pups from Nb infected or naive mothers. **(B)** STm-specific IgG1, IgG2a and IgM **(B).** NES-specific IgG1 and IgG2a were determined in 8 week old adult mice born to Nb infected and naive mothers. *p<0.05. ***p<0.001. N=6-8. Representative of at least three independent experiments.

born to Nb infected mothers still had higher NES-specific IgG1 than mice born to naive mothers, although these had higher levels than 2 week old pups born to naive mothers and the difference was therefore less pronounced. NES-specific IgG2a levels were the same in both groups. In humans and other primates, maternal IgG is transferred solely via the placenta and levels wane after 3-12 months after birth.[74] Our study shows that in BALB/c mice, maternal Nb-specific IgG1 antibodies are transferred to offspring and persist into adulthood.

3.3 Discussion

This study demonstrates that a maternal helminth infection has significant effects on the development of splenic T and B cell responses in 2 week old naive pups. We initially characterised the splenic lymphocytes populations in mice aged between 1 and 6 weeks. Here we found that T and B cell populations showed distinct changes in composition over time. Splenic CD4+ and CD8+ T cell populations increased with age in number and proportion into adulthood while maintaining their relative proportions. Total splenic B220+CD19+ B cells reach adult proportions faster but continue to differentiate into follicular and marginal zone B cells between 3 weeks of age and adulthood, while B cell numbers rise and fall with total splenic lymphocyte numbers. This is the first study to analyze splenic T and B cell populations in neonatal BALB/c mice in such detail since most studies have looked at peripheral blood in domestic animals and humans. T

he increase in lymphocyte numbers in early life may be a way in which the neonatal immune system attempts to compensate for its naive status.[74] Humans are born with high numbers of T and B cells which decline 3-6 months after birth.[86] The use of newborn mice as models for human neonates has been questioned due to the relatively delayed maturation of the murine immune system, however the primary antibody response elicited in mice aged 1-3 weeks is comparable to that of neonatal humans.[74,78]

This study showed that with maternal Nb infection, 2 week old naive offspring have a general increase in splenic B and T lymphocyte populations without increased maturation or differentiation of activated T cells, follicular or marginal zone B cells. There was however, a more pronounced increase in mature recirculating B cells when compared to Pre B cells and immature B cells. While other studies have shown *in utero* sensitization of the fetus by maternal helminth infections,[111,168–173,186–188] this is the first study to look at the effects of maternal infectious history, i.e. when the parasite has been cleared before pregnancy, on immunity in offspring. There is thus little or no helminth antigen leakage across the placenta. Effects seen in offspring are therefore due to helminth induced sustained immune modulations in the mother being imprinted on the fetus.

The effects of colostrum lymphocyte delivered to the neonate should also be considered. The leukocyte count in human colostrum is extremely high, ranging from 10^5 to 10^7 cells/ml of which 40-50% are macrophages, 40-50% neutrophils, and 5-10% lymphocytes.[189] Of the lymphocytes, about 80% are T cells (CD4+ and CD8+), in which γδ-T cells[190] and memory T cells predominate.[191] Milk macrophages have been shown to contribute to the production of cytokines and growth factors *in vitro* and support antigen mediated proliferation of T cells.[192] Moreover, transepithelial migration of human milk leukocytes from the gut of the offspring and into secondary lymphoid organs through circulation has been shown in rats, baboons and other species.[156,193] Studies in our lab have shown that antibody-independent protection against *N.brasiliensis* is transferred via breastmilk from mother to offspring in BALB/c mice (unpublished results). Thus the effects of breastmilk on systemic immune modulation also need to be considered.

We saw the induction of an early germinal center response with maternal Nb infection. GCs only appear after 3 weeks of age but we observed GCs in 2 week old mice that had Nb infected mothers. These GCs did not produce IgG1 or IgG2a but were surrounded by IgM positive B cells in the marginal zone. The neonate is impaired in its production of IgG isotypes at birth and responds to a restricted antigen repertoire by production of low affinity IgM.[194] Nb infection in the mother resulted in increased GC activation. However, this was not reflected in the serum which is probably due to the early stages of GC development since these GCs were small and present at a low frequency.

NES-specific IgG1 and IgG2a antibodies were present in the serum of offspring from Nb infected mothers but completely absent in pups from naive mothers. Since IgG isotypes were absent in the spleen, any IgG detected in the serum is most likely of maternal origin, passively transferred via the placenta and by breastmilk. However, the possibility of transferred remnant antigen cannot be excluded. The presence of NES-specific IgG1 antibodies in offspring was still detected in adulthood at 8 weeks of age, although levels were much reduced. MatAb are known to decline and disappear from the infant’s serum antibody repertoire by 12 months in humans. In mice, MatAb have a half life of 7 days but remain detectable for 2 months after birth.[195]

The transfer of MatAb is an important effect which may have beneficial and detrimental consequences in offspring. Human neonates and infants are protected by maternal antigen-specific antibodies in serum for the first 3 to 12 months after birth. These MatAb afford protection against specific pathogens but also inhibit offspring's primary antibody responses to vaccinations against and infections with those pathogens in a titre dependent manner.[196] It has also been shown that maternal IgG against the allergens ovalbumin and bee venom phospholipase A_2 (PLA_2) suppresses the development of IgE against the same antigen in offspring, and this effect was maternal IgG titre dependent and independent of maternally derived antigen.[197] Moreover, anti-PLA_2 IgG and IgE recognize non-cross-reactive determinants at the minimal dose used in that study,[198] suggesting that maternal anti-PLA_2 IgG titres do not suppress the offspring anti-PLA_2 IgE response simply by masking immunodominant epitopes on the antigen.

Pre-immune B cell repertoires have been shown to be selected in normal naive mice by environmental antigens and normal serum immunoglobulins.[199] Normal pre-immune maternal IgG transferred to offspring stimulates B cell lineage development in offspring, favouring the accumulation of immature B cells. Offspring had two- to three-fold higher numbers of bone marrow pre-B and B cells and two- to four-fold lower numbers of Ig-secreting plasma cells in the spleen and thymus, as well as six-fold lower serum IgM concentrations.[195] Another study in mice showed that maternal vesicular stomatitis virus (VSV)- specific antibodies can shape the B cell repertoire of offspring, inhibiting accumulation of antigen-specific T2 and MZ B cells and promoting selection into B cell follicles, having functional effects long after the MatAb themselves become untedectable.[47] These documented effects of passively transferred MatAb in offspring suggest that the observed early GC formation in our study may be related to the presence of maternally derived NES-specific IgG which may be priming the neonate's B cell repertoire development and promoting selection into the B cell follicles.

Our study has shown the development of splenic lymphocytes with age from the neonatal to adult BALB/c mouse. We then showed that maternal Nb infection results in increased lymphocyte proportions and absolute numbers in 2 week old naive offspring, early GC formation, transfer of maternal NES-specific IgG, particularly IgG1, which is still present in offspring at adulthood. The next question we asked is whether these factors, especially the presence of high titres of maternal helminth specific antibodies and early GC formation, influence the control of a Th1 type infection with *Salmonella enterica* serovar *Typhimurium* in offspring. We also assessed the efficacy of vaccination with heat-killed *Salmonella* in these offspring and whether any observed effects persist into adulthood.

4. The effect of maternal *N.brasiliensis* infection on control of *S.typhimurium* in offspring

4.1 Introduction

Non-typhoidal *Salmonella* (NTS) presents as self-limiting gastroenteritis in developed countries.[138] In sub-Saharan Africa (SSA), NTS infections can cause progressive bacteraemia in immunodeficient populations: HIV negative children aged between 6 and 24 months[201–203] and HIV positive adults.[204] The period of susceptibility to NTS in children occurs between the loss of passively acquired protective MatAb and before the maturation of the infant's humoral response.[67,205] Virulent strains of NTS are "complement resistant", a trait conferred by bacterial surface lipopolysaccharide (LPS) and the outer membrane protein encoded by the resistance to complement killing (*rck*) gene. This complement resistance is overcome by protective antibodies which mediate complement bactericidal properties of adult serum, and is seen in Malawian children from the age of 24 months.[205]

HIV infection induces permeability of the mucosal surface of the gut, allowing leakage of the highly immunogenic LPS of the STm outer membrane.[206] In adults LPS elicits a strong humoral response, however LPS-specific IgG antibodies are not protective against STm. Rather, by crosslinking the distal portion of LPS called the O-antigen, LPS-specific IgG impedes the access of complement (in humans) and protective antibodies which bind to porins proteins OmpF, C and D on the outer membrane of STm (in humans and mice).[207,208] Therefore, HIV infected individuals have a dysregulated humoral response to STm which inhibits protection against bacteraemia.

Areas with the highest NTS prevalence typically also have a high prevalence of other infections such as malaria[201] and helminths.[209] Children in SSA experience the highest rate of helminth infections.[209] It is therefore likely that high prevalence of NTS may be affected by helminth co-infections. However, little data exists as to how helminth infections may alter immunity to NTS and whether helminth antibodies or antigen may interfere with protective porins-specific antibodies in NTS bacteraemia in children.

Classically, experimental immunology has examined the immune response to single pathogens in isolation. However, helminth polyparasitism and co-infections with helminths and malaria,[209] and helminths and bacteria such as *M.tuberculosis*[210,211](Mtb) are common in SSA and may affect the host's ability to control one or both pathogens, potentially leading to prolonged and exacerbated symptoms. Helminth infections are not only highly prevalent but also highly immunogenic. Infections elicit a strong Th2 response and asymptomatic chronic infection are associated with complex helminth-mediated T-reg and AAM induced immune down regulation of the host inflammatory response.[11] In a co-infection model, helminth influence on host immunity may alter host ability to control other pathogens. While helminth-malaria and helminth-Mtb co-infections are common and studies have shown that helminth infection is associated with increased susceptibility to malaria,[212–215] the effects of helminth infection on control of malaria parasite and Mtb in the host are incongruent. Some studies show impaired control of malaria disease[216–218] and TB[219] and others show improved control of malaria[220,221] and TB.[211] The variation in effects seems to be related to the type of helminth and the time of Mtb infection after the helminth infection.[219,222]

Humans acquire a history of infections over their lifetime, which may influence response to new pathogens, affect immune memory development or vaccine efficacy. In depth mechanistic studies of the host response to co-infections, the interaction of concurrent pathogens, the long-term priming of the immune response by infectious history, and how these factors may influence vaccine efficacy are as yet uncommon. Acquisition of protective immunity to routine vaccines is diminished in developing countries where helminth infections are prevalent.[111] Studies have shown that helminth infections impair protective immunity acquired from oral polio vaccine,[223,224] tetanus toxoid vaccine (TT),[225,226] live oral cholera vaccine,[227,228] BCG,[176] malaria vaccine (GMZ2),[229] and influenza H1N1 vaccine.[230] Moreover, chronic maternal helminth infection is associated with decreased acquisition of protective IgG in children following vaccination with *H.influenza* type b vaccination.[111]

Objectives

Preliminary data in our lab, to be discussed in more detail, showed that priming of the immune system with Nb infection in BALB/c mice resulted in impaired control of subsequent STm infection, and impaired efficacy of the porins STm vaccine.[231] Infectious history with Nb, a complex Th2-inducing and immune modulating parasite, can therefore exacerbate the severity of STm infection, a Th1-inducing bacterium, without the presence of the worm.

Based on this data and the subtle effects of maternal Nb infection on naive offspring lymphocyte populations and transfer of MatAb (described in Chapter 3), we assessed the effect of maternal Nb infectious history or "priming" on control of STm in offspring.

4.2 Results

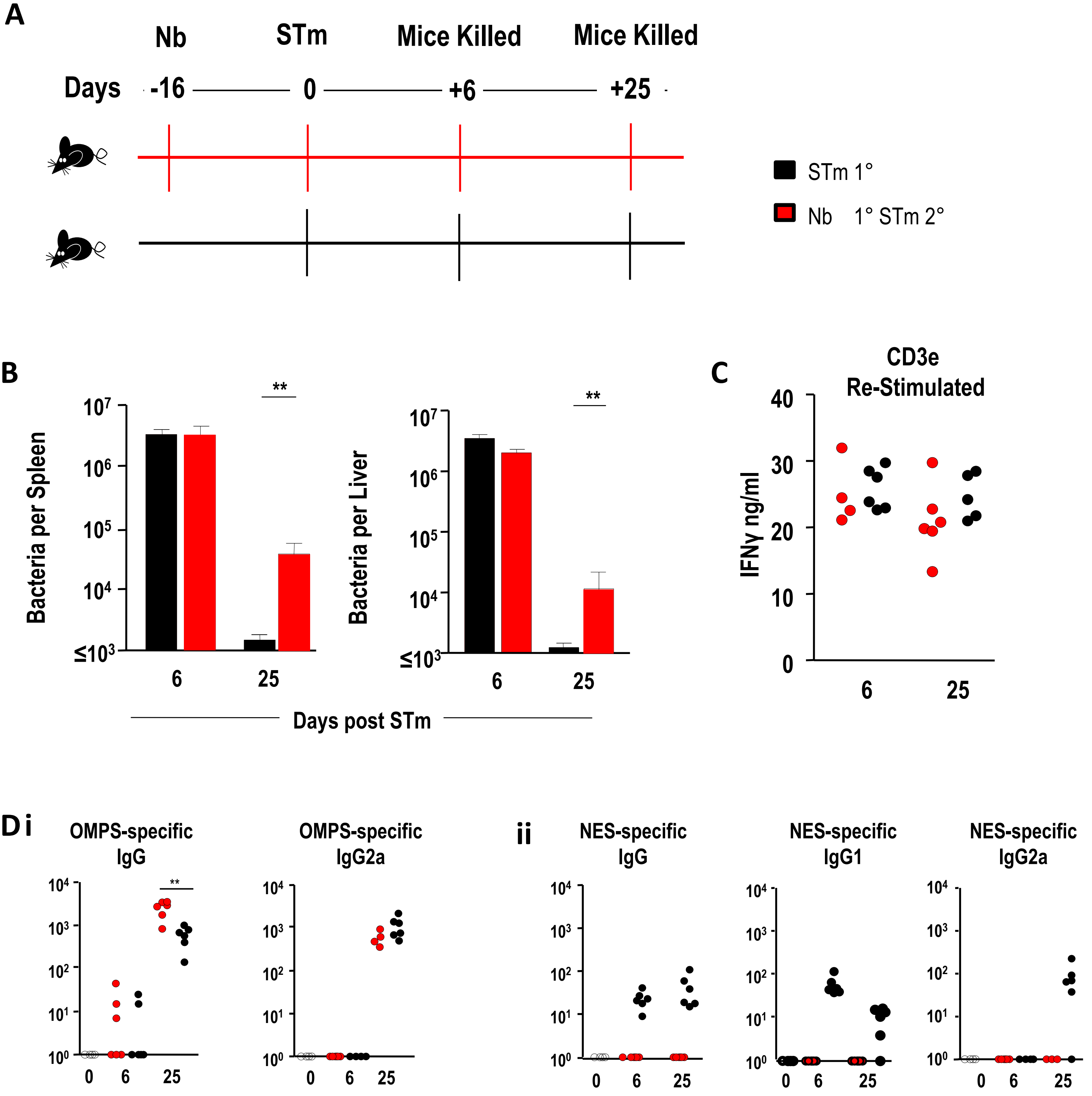
4.2.1 Preliminary results: Primary infection with Nb impairs resolution of subsequent STm infection (S. Bobat & W. Horsnell)

The effect of primary Nb infection on subsequent STm infection was investigated in BALB/c mice by Drs. S. Bobat and W. Horsnell in our lab.[231] This is not a direct co-infection model as the two pathogens are not present in the host at the same time, but examines the effect of infectious *history* with Nb on subsequent STm infection.

Adult BALB/c mice were infected with 500xL3 Nb larvae and at day 16 post infection (6-7 days post worm expulsion) mice were challenged with 5×10^5 CFU *aroA* attenuated strain of STm **(Fig 4.1A)**. STm CFU counts were determined in the spleen and liver at day 6 and 25 post-STm challenge **(Fig 4.1B)**. CFU counts were unaffected by Nb priming at day 6 post STm infection. By day 25 post STm, when the adaptive response to STm comes into play,[151,231,232] CFU counts increased as expected and Nb-primed mice had significantly increased bacterial burden in both the spleen and liver. This suggested that primary infection with Nb impaired subsequent control of STm.

There was a marginal decrease in CD4+ helper T cells in proportion to total lymphocytes in Nb primed mice, and a marginal increase in effector CD4+CD62L^{lo} T cells. This was not reflected in absolute numbers of these cells (data not shown). This effect was not related to changes in IFNγ levels from anti-CD3 restimulated splenocytes at either day 6 or day 25 **(Fig 4.1C)**, although NES- and HK-STm-restimulated splenocytes had increased IFNγ levels at day 6 post STm but not at day 25 (data not shown).

Assessment of antibody specificity **(Fig 4.1D)** revealed that OMPS-specific IgG levels were equivalent at day 6 post STm infection, but were significantly lower in Nb-primed mice at



<u>Figure 4.1</u> Primary infection with Nb impairs resolution of subsequent STm infection (S. Bobat & W. Horsnell). (A) BALB/c mice were infected with 500xL3 Nb s.c. alongside naive mice and challenged on day 16 with $5x10^5$ STm i.p. and responses were assessed at day 6 and 25 post STm challenge. **(B)** Splenic and liver CFU counts were determined. **(C)** INFγ levels were determined by ELISA from *in vitro* anti-CD3 restimulated splenocytes. **(Di)** Serum OMPS-specific IgG and IgG2a, **(Dii)** and NES-specific IgG, IgG1 and IgG2a titres were determined by ELISA. Antibody graphs represent relative levels. **p<0.01. Dots represent individual mice. Experiment was carried out once.

day 25 p.i. However, the protective OMPS-specific IgG2a levels were equivalent **(Fig 4.1Di)**. OMPS-specific IgG1 levels did not increase beyond background levels in either group (data not shown). As expected, NES-specific IgG, IgG1 and IgG2a levels were high in Nb-primed mice only **(Fig 4.1Dii)**.

This data demonstrates that infectious history with Nb disrupts the host’s ability to control subsequent STm infection, an effect that is correlated to the time when the adaptive response is required to control the later stages of STm infection. No obvious changes in T cell populations point to a mechanism, but this does not exclude the role of migration and/or communication of immune effectors. The initial adaptive response to STm appears unaffected by priming with Nb. Although B cells and antibody are not essential to control of primary STm infection, a decrease in STm-specific IgG may have consequences for vaccine efficacy and secondary response.

4.2.2 Preliminary results: Priming with Nb impairs efficacy of Porin vaccine after subsequent immunisation with STm

(S. Bobat, W. Horsnell & D. Mrdjen)

As described in chapter 2, vaccination with STm porins induces early control of STm infection and is dependent on OmpD-specific antibody.[208] Since Nb priming could disrupt the control of subsequent STm infection in the spleen and liver, and this correlated to a decreased OMPS-specific IgG titre, we assessed whether Nb priming could also disrupt the protection acquired from vaccination with porins. OMPS contain a mixture of total outer membrane proteins of STm and LPS, while porins contain only highly purified porins OmpC, OmpD and trace amounts of OmpD, the three major porins of STm.[208,231]

BALB/c mice were infected with 500xL3 Nb larva for 19 days before vaccination with porins for 11 days prior to challenge with 5×10^6 STm for 5 days **(Fig 4.2A)**. As a control, mice were either infected or immunised with Nb only or porins only for 11 days prior to challenge with STm. CFU counts in the spleen revealed that unvaccinated Nb-primed mice had a similar bacterial load as non-primed unvaccinated mice, an expected outcome since the effects of Nb are only seen at day 25 post STm infection **(Fig 4.2B)**. Porins vaccination did indeed confer protection against STm infection, as reflected in the almost 10^4-fold decrease in bacterial burden in the vaccinated unprimed group. However, priming with Nb resulted in a 30-fold greater bacterial burden compared to unprimed mice. Nb infectious history therefore disrupted the protection against subsequent STm infection conferred by porins vaccination.

Assessment of the porins-specific antibody profile prior to STm infection **(Fig 4.2C)** revealed that priming with Nb had no effect on porins-specific IgM but did result in a 6-fold decrease in porins-specific IgG, a 12-fold decrease in porins-specific IgG2a and an 8-fold decrease in porins-specific IgG1. This suggested that, since protection conferred by porins vaccination is OmpD-specific antibody dependent, disrupted protection by Nb priming may be related to disruption of the development of OmpD-specific antibody. However, upon assessment of the antibody profile after STm infection, porins-specific IgM and IgG titres

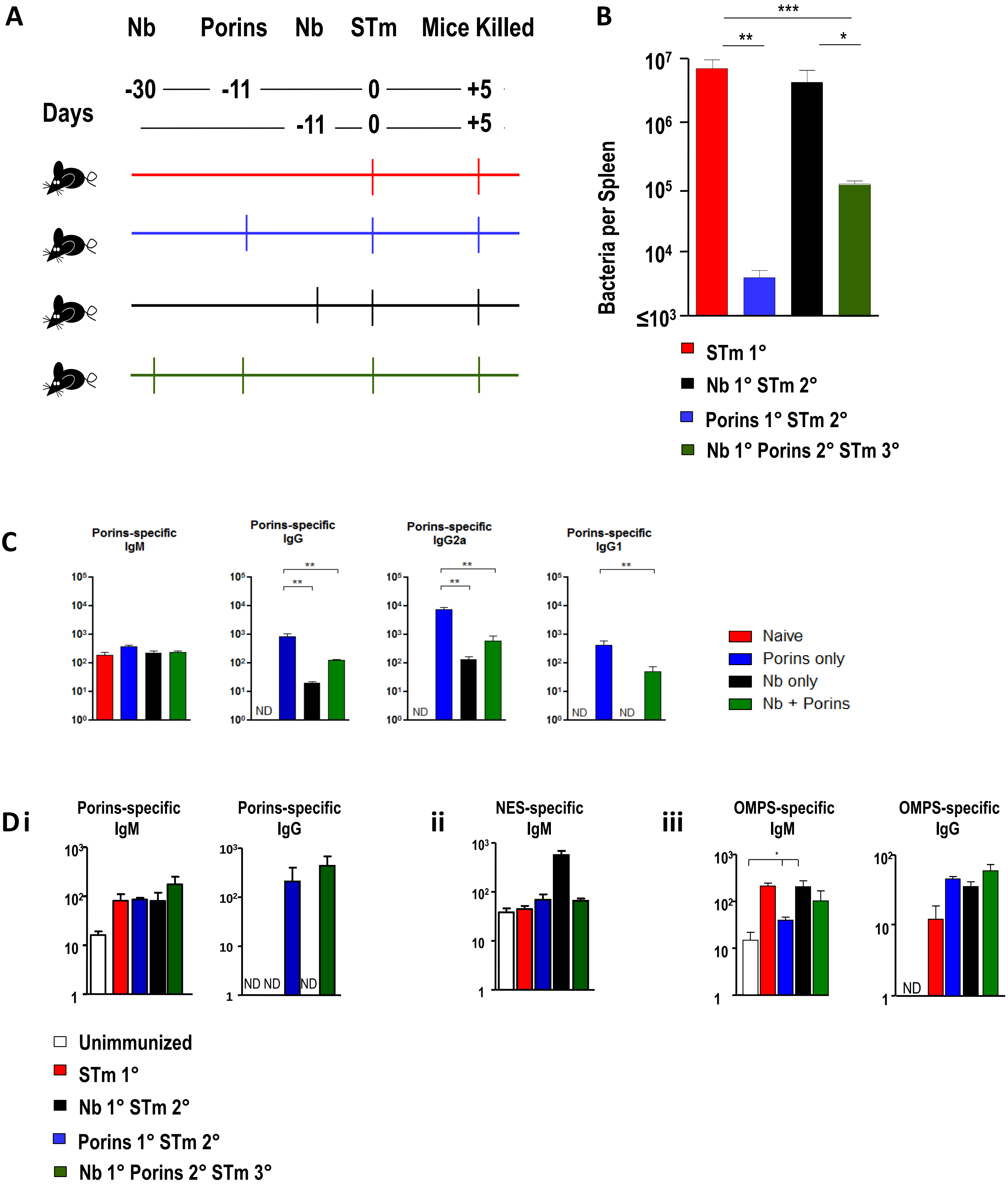


Figure 4.2 Priming with Nb impairs efficacy of Porin vaccine after subsequent immunisation with STm (S. Bobat, W. Horsnell & D. Mrdjen). (A) BALB/c mice were infected with 500 x L3 Nb s.c. and/or immunised with 20µg Porins i.p., prior to challenge with 5x10^5 STm i.p. **(B)** Splenic CFU were quantified at day 5 post STm infection. **(C)** Porins-specific IgM, IgG, IgG1 and IgG2a titres were determined by ELISA prior to STm infection. **(Di)** Porins-specific IgM and IgG titres, **(Dii)** NES-specific IgM titres and **(Diii)** OMPS-specific IgM and IgG titres were determined post STm infection. Antibody graphs represent relative levels.$^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. N=6. Experiment was carried out once.

were similar between all groups **(Fig 4.1Di)**, and NES-specific IgM was high in Nb-primed mice only **(Fig 4.1Dii)**. Surprisingly, OMPS-specific IgM and IgG responses were broadly equivalent between the groups. Unprimed but porin vaccinated mice had a slightly lower OMPS-specific IgM response and yet had the greatest level of protection **(Fig 4.2Diii)**. This suggests that since there is antibody present but less protection in the Nb-primed porin vaccinated mice, the difference is likely to involve a disrupted killing of STm by macrophages rather than directly by antibody, possibly due to inappropriate or inefficient opsonisation of the bacteria by opsonizing antibody. This remains to be shown by opsonisation experiments with various sera in naive or Nb primed mice.

Discussion

These results show that infectious history with Nb impairs control of subsequent STm infection 40 days after clearance of the helminth from the host. This disruption is not correlated to any obvious changes in T cell or cytokine responses and is more likely due to impaired killing of bacteria by macrophages. Moreover, disruption occurs without continual infection with the helminth. Since large amounts of antigen are deposited by the moulting and secretion cycle of the helminth,[233] it is possible that Nb antigen persists long after expulsion of the worms, sustaining the effects on macrophages and/or Th2 polarization. While antibody is dispensable in to resolution of primary STm infection and B cell deficient mice do not succumb to infection,[153] antibody can increase the efficiency of bacterial clearance in a secondary infection[234] and is required along with T cells for recall immunity to oral challenge with mouse-virulent STm C5.[235] Furthermore, antibody induced during primary infection can help restrict bacteraemia.[150] Nb priming also impairs efficacy of the porin vaccine, a secondary response to STm infection after vaccination. This is not related to changes in OMPS-specific serum IgG, but may be related to the decrease seen in porins-specific IgG present in circulation before STm challenge and macrophage killing of opsonized STm.

These data combined with the high prevalence in SSA of helminth infections in pregnant women and NTS infections in children, as well as impaired responses to routine childhood vaccinations in these areas, leads to the question of whether the adverse effects of Nb-STm responses seen in the same host could also be seen in a mother-child model. Here the effects of a maternal Nb infection may be transferred to the child and affect control of STm and vaccine efficacy in the child. In this chapter we investigated this in a mouse model by “priming” female mice with an Nb infection prior to pregnancy (as in the previous chapter), and challenging offspring with an STm infection.

4.2.3 Maternal Nb infection in BALB/c mice does not impair resolution of splenic bacterial burden in young STm infected offspring

As in the previous chapter, female BALB/c mice were infected with 500xL3 Nb s.c., infection was cleared with Ivermectin at 2 weeks p.i. (control groups were also treated) and females were mated. In this instance, offspring were infected with 1x10^5 CFU STm at two weeks of age and killed at 7, 18, 27 or 35 days post STm infection **(Fig 4.3A)**. STm CFU counts were determined in the spleen as CFU per spleen and per gram of spleen (**Fig 4.3 B)**. The STm infection followed the expected progression of bacterial burden seen in adults, starting with approximately 10^6 CFU at day 7 p.i. and clearing the bacteria to approximately 10^3 CFU at day 35 p.i., although adults did clear the infection further to approximately 10^2 CFU at day 35 p.i.[150] However, there were no differences in CFU counts between pups born to naive or Nb infected mothers at any stage in the STm infection.

Spleen mass and total splenocyte numbers **(Fig 4.3 C)** also followed a similar progression as seen in adults, though generally reduced in both parameters since mice were younger. Spleen mass reached the peak of splenomegaly at day 18 p.i. after which it declined again until day 35 p.i., and total splenocytes were generally high from day 7 until declining at day 35 p.i. Once again, no differences were seen in spleen mass or total splenocyte numbers between pups born to naive or Nb infected mothers. This, however, was also the case for spleen mass and total splenocyte numbers in Nb-primed STm infected adult mice in work done by Drs S. Bobat and W. Horsnell.[231]

From this data we can conclude that in BALB/c mice, maternal Nb infection does not alter the ability of offspring to control STm infection as seen in splenic bacterial burden. In the tissue, primary infection with STm is cleared by a strong Th1 response while bacteraemia and secondary infection is controlled by a humoral response against OmpD.[149,208] No obvious changes in splenic T cell populations were seen in naive pups born to Nb infected mothers while a there was a definite and sustained presence of maternal NES-specific IgG1, and a marginal decrease in naturally occurring OMPS-specific IgG2a. If MatAb do interfere with control of STm in offspring, and these antibodies are present in the serum of offspring, perhaps maternal Nb infection impacts on bacteraemia in offspring and a better place to look would be in the blood of STm infected offspring.

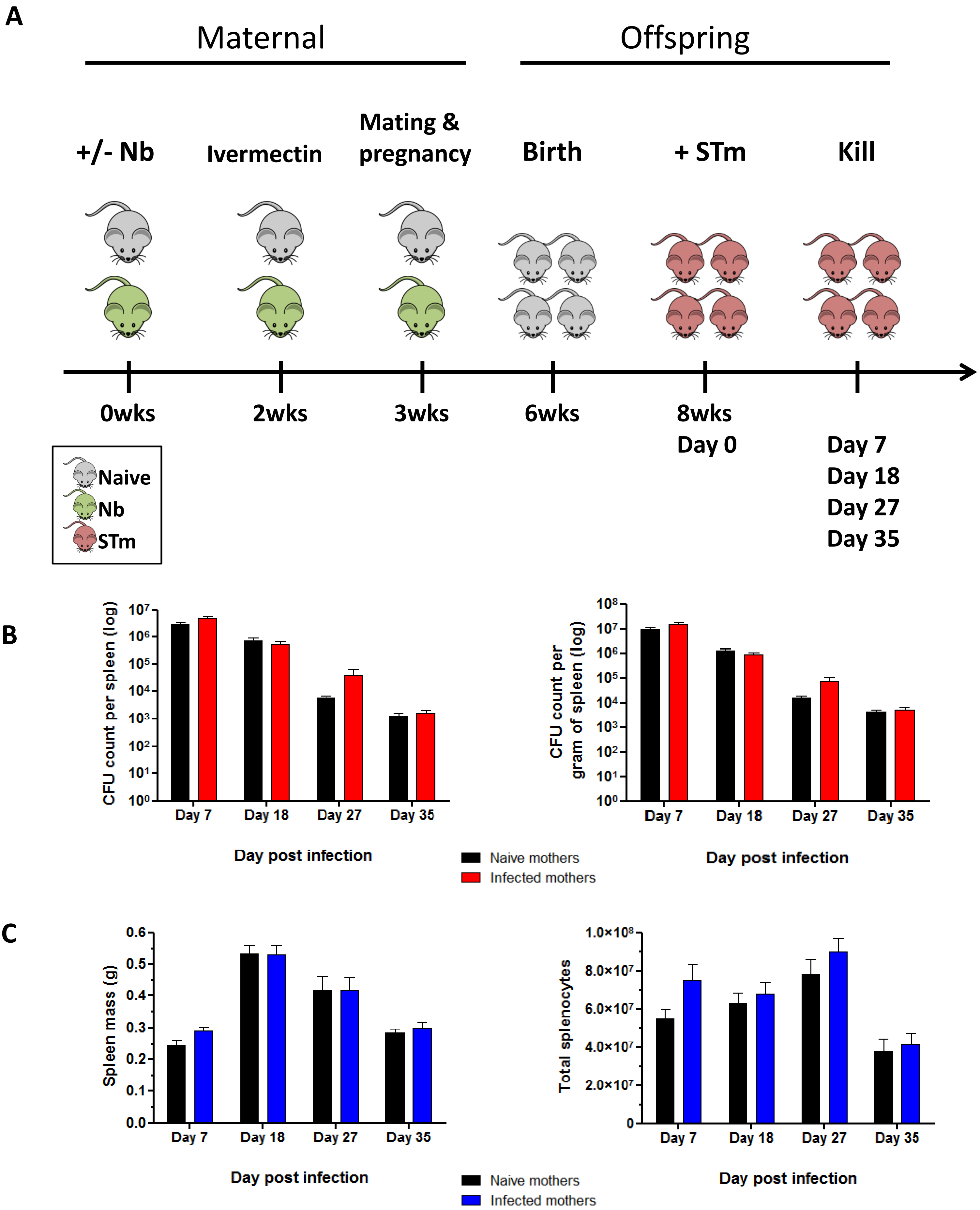


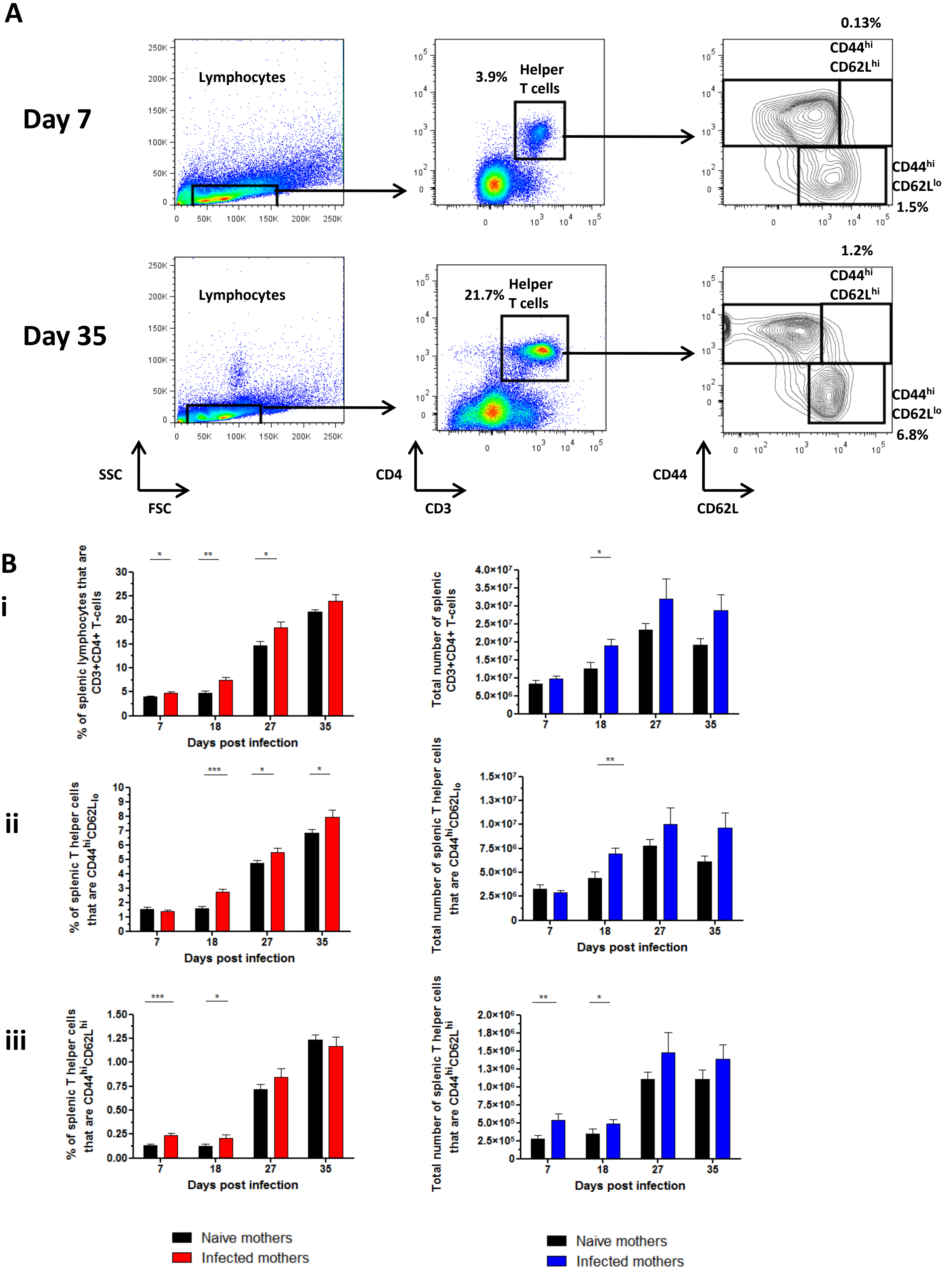
Figure 4.3 Maternal Nb infection in BALB/c mice does not impair resolution of splenic bacterial burden in young STM infected offspring. (A) Experiment plan: Female BALB/c mice were infected with 500xL3 Nb s.c., infection was cleared with Ivermectin at 2 weeks p.i., females were mated and offspring were infected with $1x10^5$CFU STm i.p. at two weeks of age and killed at 7, 18, 27 or 35 days post STm infection. **(B)** STm CFU count per spleen and per gram of spleen. N=6-15. 3-4 pooled experiments. No statistical significance.

4.2.4 Maternal Nb infection alters T cell populations in offspring

Th1 CD4+ T cells are essential in clearance of STm infection and come into play in the third week of infection.[149,151] Total splenic CD4+ helper T cells and their activation status were analysed throughout the course of the STm infection, as previously described, and a representative gating strategy is depicted from day 7 and day 35 p.i. **(Fig 4.4A)**. Subtle increases were seen with maternal Nb infection, as seen in naive pups. A progressive increase in proportions of CD4+ helper T cells as well as effector and central memory CD4+ T cells in proportion to total splenic lymphocytes was seen with age of pups in both groups, as expected.

Proportions of total CD4+ helper T cells as a percentage of total splenic lymphocytes were increased in pups born to Nb infected mothers at day 7 from 4% to 4.9%, at day 18 from 4.7% to 7.4%, at day 27 from 14.7% to 18.4% while no statistical significance was seen at day 35 **(Fig 4.4 Bi left)**. Differences in total numbers of splenic CD4+ helper T cells were only seen at day 27, with an increase from 1.3×10^7 cells to 1.9×10^7 cells with maternal Nb infection **(Fig 4.4 Bi right)**. Proportions of effector T helper cells ($CD3^+CD4^+CD44^{hi}CD62L_{lo}$) were increased at days 18, 27 and 35 **(Fig 4.4 Bii left)** while numbers were increased at day 18 only **(Fig 4.4 Bii right)**. Proportions of splenic central memory T cells ($CD3^+CD4^+CD44^{hi}CD62L^{hi}$) were increased at days 7 and 18 only **(Fig 4.4 Biii left)**, and this was also seen in total numbers **(Fig 4.4 Biii right)**.

While not making detailed remarks on the effects of STm infection on T cell populations compared to naive mice, total splenic CD4+ T cell proportions were slightly reduced for the age of the mice compared to naive mice born to naive mothers **(see Fig 3.1 C)** while total numbers of these cells were generally increased in age-corresponding naive offspring mice (day 7 post STm infected mice were 3 weeks of age, and day 27 post STm infected mice were 6 weeks of age). However, effector T helper cells were increased in proportions as well as numbers compared to naive mice of corresponding age, and central memory T cells were greatly increased in proportion and number. This is in correspondence to the expected expansion of STm specific CD4+ T cells and rapid acquisition of Th1 effector function during STm infection.[236]



<u>Figure 4.4 </u>**Maternal Nb infection increases T cell populations in offspring. (A)** Analysis of helper T cell populations and activation status, representative examples from offspring born to naive mothers and killed at 7 and 35 days post STm infection. **(B)** Proportions and total numbers of of **(i)** CD3+CD4+ T-helper cells, **(ii)** CD3+CD4+CD44+CD62L^{lo} effector memory T-cells, **(iii)** CD3+CD4+CD44+CD62L^{hi} central memory T-cells. *p<0.05. **p<0.01. ***p<0.001. N=15. Representative of at least three experiments.

4.2.5 Maternal Nb alters B cell populations in young offspring

B cells are dispensable for clearance of primary infection with attenuated STm.[237] Total splenic B220+CD19+ B cell populations and their maturation and activation status were analysed through the course of the STm infection in pups, as previously described, and representative gating strategy is depicted from day 7 and day 35 p.i. **(Fig 4.5A)**. Again, subtle changes were seen between pups from naive and infected mothers.

Proportions of total splenic B220+CD19+ B cells were increased slightly from 9.2% to 12.7% as a percentage of total splenic lymphocytes with maternal Nb infection at day 7 post STm in pups, at day 27 p.i. from 25.6% to 33.4% **(Fig 4.5 Bi left)**. In total numbers, splenic B220+CD19+ B cells were increased only at day 7 p.i. **(Fig 4.5 Bi right)**. Activated CD80+ B cells were increased at day 7 p.i. from 0.15% to 0.30%, but decreased from 2.4% to 1.8% at day 27 p.i. and from 1.7% to 1.2% at day 35 p.i. **(Fig 4.5 Bii left)**, however only the increase at day 7 p.i. was seen in total number of activated CD80+ B cells **(Fig 4.5 Bii right)**. FO B cell proportions were increased at days 7, 18 and 27 p.i. **(Fig 4.5 Biii left)**, and numbers at days 7 and 18 **(Fig 4.5 Biii right)**. NF B cell proportions were increased at days 7 and 27 **(Fig 4.5 Biv left)**, and numbers at days 7 and 18 **(Fig 4.5 Biv right)**. MZ B cells were increased at day 7 p.i. but decreased from 0.64% to 0.38% at day 18 p.i. **(Fig 4.5 Bv left)**, and this pattern was also seen in total number of MZ B cells **(Fig 4.5 v right)**. MatAb have been shown to influence the development of the B cell repertoire of offspring, preventing the accumulation of antigen-specific T2 and MZ B cells and promoting their selection into B cell follicles. This effect lasted up to adulthood.

Briefly, compared to naive mice from naive mothers in Chapter 3, splenic B cells were lower in proportion to total splenic lymphocytes, where naive mice had almost 50% B cells at 3 and 6 weeks of age, STm infected mice had only 9.2% at 3 weeks and 25.6% at day 27 p.i. **(see Fig 3.1 B & C)**. This may be due to the requirement of a T cell response rather than

B cell response in clearance of primary STm infection, since effective clearance is completely independent of B cells as mice lacking in B cells do not succumb to infection.[234,237,238]

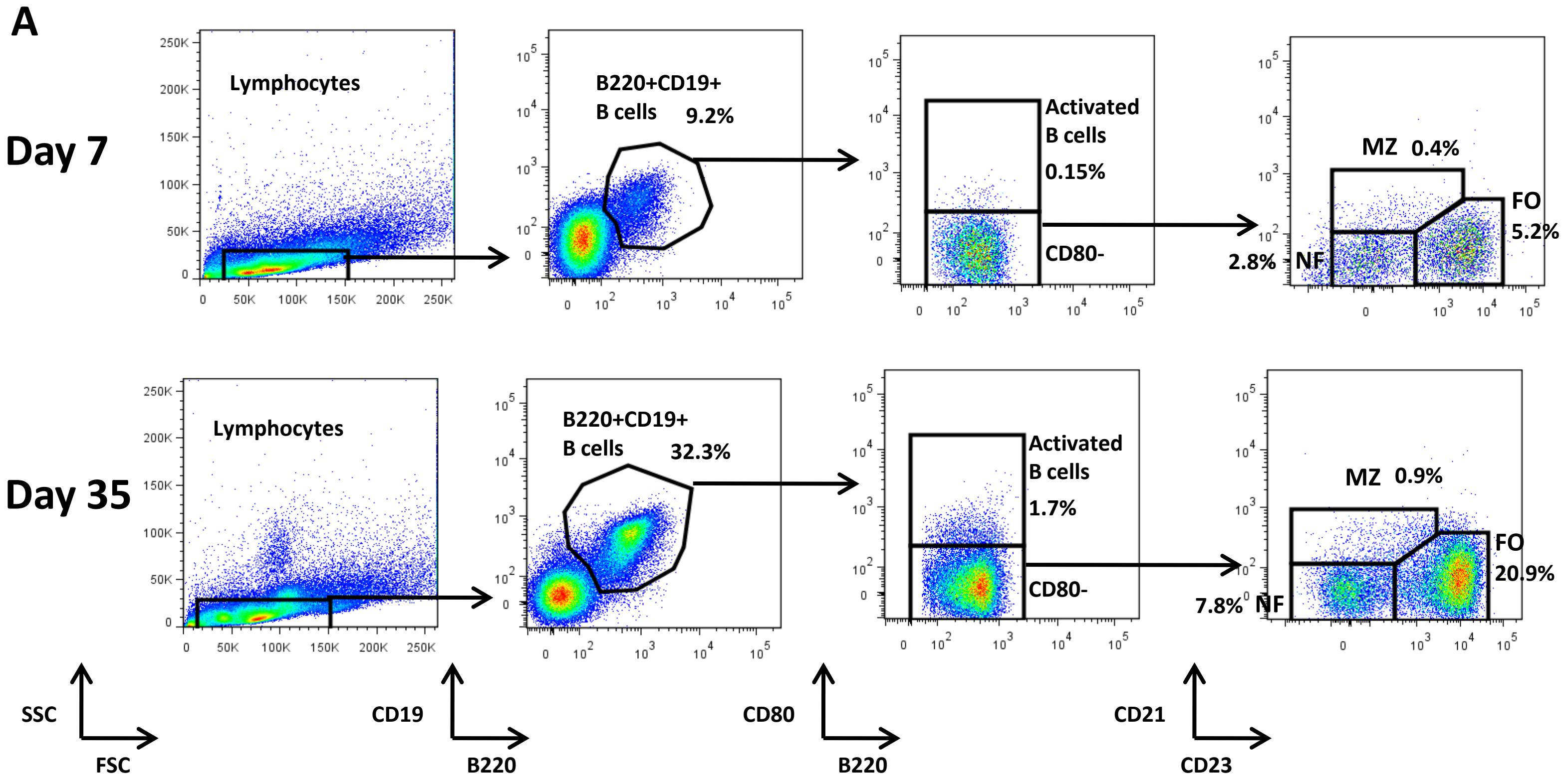


Figure 4.5 Maternal Nb alters B cell populations in young offspring. (A) Analysis of B cell populations and activation status, representative examples from offspring born to naive mothers and killed at 7 and 35 days post STm infection. **(B)** Proportions and total numbers of (i) B220+CD19+ B cells, (ii) activated CD80+ B cells, (iii) CD23^{hi}CD21$_{lo}$ follicular B cells, (iv) CD23$_{lo}$CD21^{-} newly formed B cells, and (v) CD21^{hi}CD23^{int-lo} marginal zone B cells. N=15. Representative of at least three experiments.

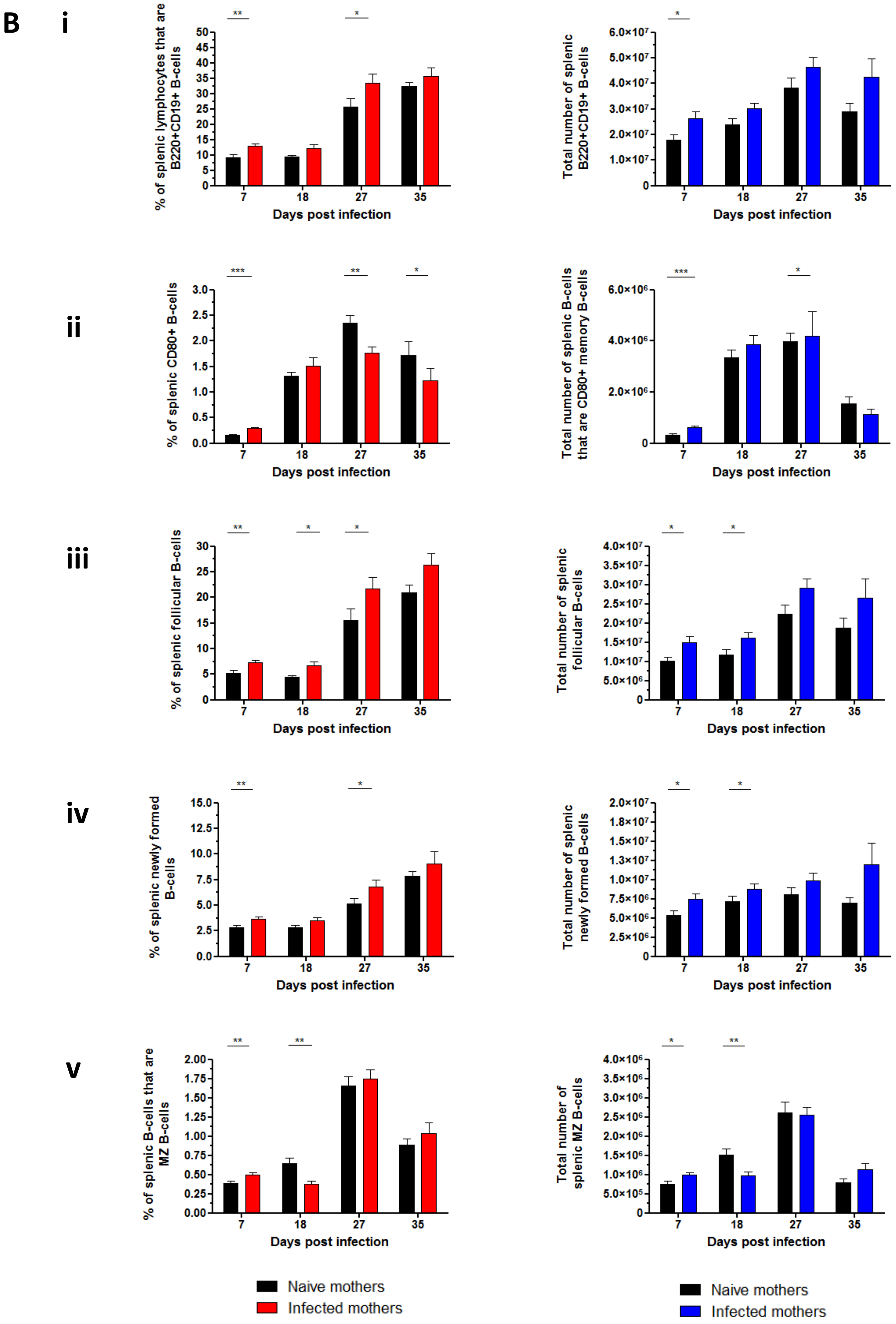


Figure 4.5 Maternal Nb alters B cell populations in young offspring. (A) Analysis of B cell populations and activation status, representative examples from offspring born to naive mothers and killed at 7 and 35 days post STm infection. **(B)** Proportions and total numbers of (i) B220+CD19+ B cells, (ii) activated CD80+ B cells, (iii) CD23^{hi}CD21$_{lo}$ follicular B cells, (iv) CD23$_{lo}$CD21^{-} newly formed B cells, and (v) CD21^{hi}CD23$^{int\text{-}lo}$ marginal zone B cells. *p<0.05. **p<0.01. ***p<0.001. N=15. Representative of at least three experiments.

4.2.6 The effect of maternal Nb infection on antibody repertoire and levels of IFNγ in young STm infected offspring

We have already shown the pronounced presence of NES-specific IgG1 in 2 week old naive pups from Nb infected mothers, assumed to be transferred from the mother *in utero* and via breastmilk, and that the presence of these MatAb is still detectable at 8 weeks of age in offspring. We now assessed the effect of STm infection in offspring on the persistence of what we assume to be maternally derived NES-specific IgG1.

NES-specific IgG1 was pronounced before STm infection in 2 week old naive pups (day 0 p.i.), with a 17-fold relative increase compared to pups from naive mothers **(Fig 4.6 Ai)**. Antibody levels declined and were 4.2-fold increased in 3 week old STm infected pups at day 7 p.i. but then increased to 8-fold at day 18 p.i. after which levels were equivalent between offspring born to naive and Nb infected mothers. This increase in NES-specific IgG1 between days 7 and 18 post STm infection in pups born to Nb infected mothers cannot have been due to continued passive transfer from breastmilk as pups are weaned at 3 weeks of age (and at day 18p.i. pups are over a month old). Therefore, increased NES-specific IgG1 between day 7 and 18 post STm may be due to its production by the pup itself. This time point corresponds with the peak of immune activation and recruitment of T cells during STm infection. MatAb can also prime offspring B cell repertoire, selecting an activated antigen-specific phenotype.

While OMPS-specific IgG2a was lower in naive pups from Nb infected mothers, relative levels were equivalent between groups until day 35 post STm infection where levels were 2-fold increased in offspring from Nb infected mothers **(Fig 4.6 Aii)**.

STm induces an early extrafollicular switched Type 1 humoral response which is T-dependent but GCs only form after the first month of infection, corresponding to elevated levels of high-affinity IgG2a.[150] This is in sharp contrast to the early GC formation of a typical Type 2 humoral response where GCs have formed by day 7 p.i. and high affinity antibody

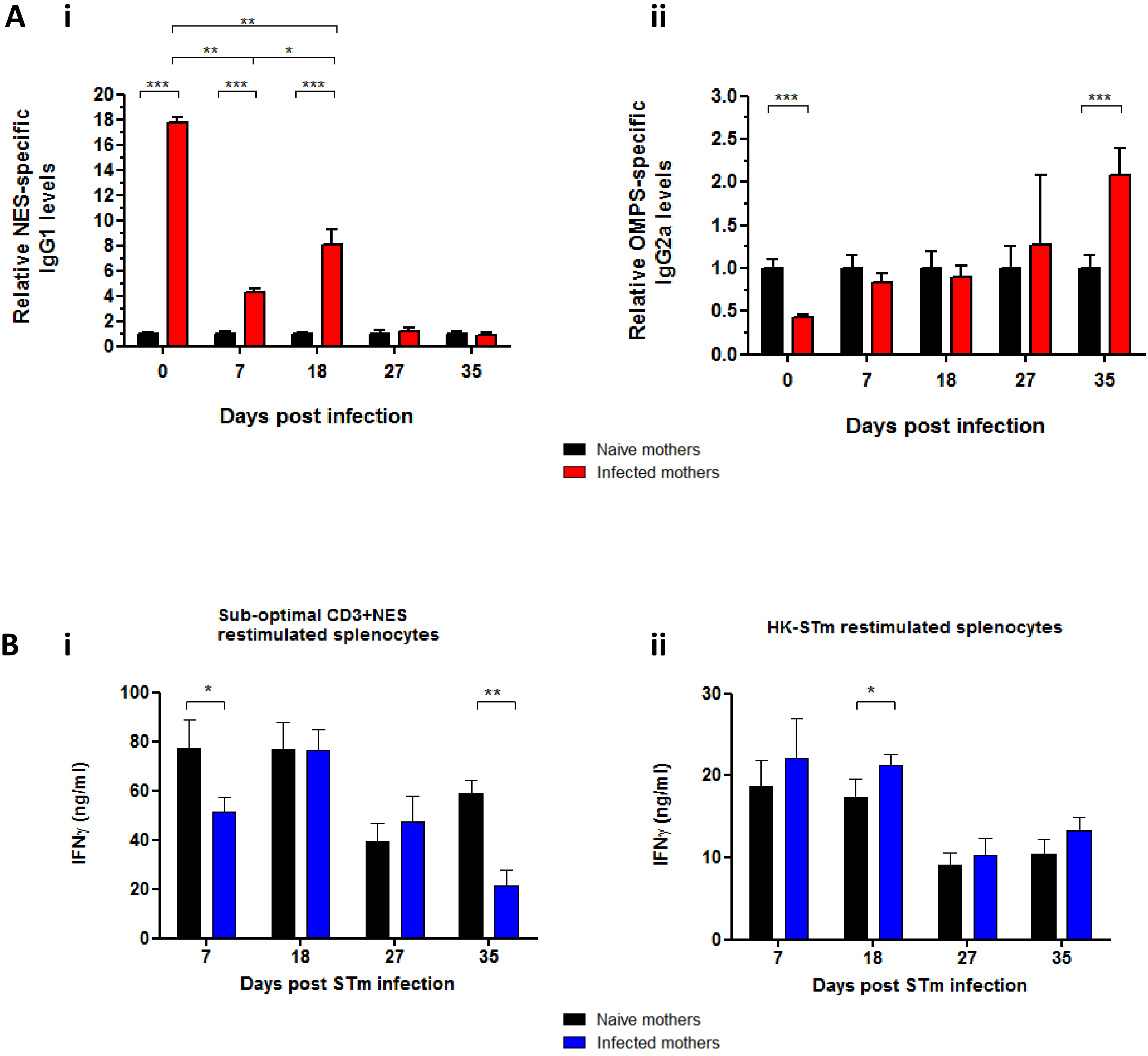


Figure 4.6 The effect of maternal Nb infection on antibody repertoire and levels of IFNγ in young STm infected offspring. (Ai) Relative change in levels of NES-specific IgG1 and **(Aii)** OMPS-specific IgG2a in STm infected offspring with Nb infection in the mother throughout the course of STm infection. Levels were normalized to a value of 1 in pups from naive mothers. Absolute levels of IFNγ secreted by splenocytes restimulated with a **(Bi)** sub-optimal concentration of anti-CD3 (1µg/ml) and NES (10 µg/ml) and **(Bii)** HK-STm (10µg/ml) from STm infected offspring born to naive and Nb infected mothers. *p<0.05, **p<0.01, ***p<0.001. N=6. Antibody analysis is from 3 pooled experiments. Cytokine analysis was carried out on one experiment.

are detected after 2 weeks.[239] As we saw early formation of GCs in naive pups born to Nb infected mothers, it may be possible that the presence of NES-specific IgG1 or maternally derived NES antigen/immune complexes enhances the late phase of high affinity antibody production by GCs with STm infection. Immunohistochemical analysis of spleens should be carried out in these mice to see whether the early GC response is present with STm infection, as well as detection of follicle-residing Nb immune complexes which may be priming the GC response.

Levels of the Type 1 cytokine IFNγ that acts in clearance of STm infection were quantified. Interestingly, levels of IFNγ secreted by splenocytes restimulated with a sub-optimal concentration of anti-CD3 (1µg/ml) and NES (10 µg/ml) were decreased at day 7 p.i. from 77ng/ml to 55.5ng/ml with maternal Nb infection **(Fig 4.6Bi)**, and at day 35 p.i. from 58.9ng/ml to 22.3ng/ml. Conversely, splenocytes restimulated with HK-STm (10µg/ml) secreted higher levels of IFNγ at day 18 p.i. in offspring from Nb infected mothers **(Fig 4.6Bii)**. Restimulation by NES mixed with sub-optimal anti-CD3 may have been too high and may have overridden the NES-specific cytokine response. A better concentration of anti-CD3 to use may have been 0.1µg/ml. Thus the results may reflect IFNγ production from anti-CD3 responding splenocytes. T cells are important at day 18 for clearance of bacterial burden during STm infection,[151] corresponding to the time point when HK-STm-specific IFNγ levels were increased in offspring from Nb infected mothers, however no differences were seen in bacterial burden at this or any other time point.

4.2.7 Maternal Nb infection in BALB/c mice does not impair resolution of STm infection in adult offspring

To determine whether maternal Nb infection had any effects on control of STm in offspring at a later stage in life, young adult offspring from naive of Nb infected mothers were infected with 5x10^5 STm i.p. at 6 weeks of age instead of 2 weeks and sacrificed at 7, 16, 18 and 35 days post STm infection **(Fig 4.7 A)**. However, no differences were seen in bacterial burden throughout the infection time course **(Fig 4.7 B)**. Spleen mass was increased from an approximately 0.7g to approximately 1.1g in offspring from Nb infected mothers **(Fig 4.7 C left)**. There were no differences between the two groups in total splenocytes **(Fig 4.7 C right)**.

As expected, spleens in adults were larger and had more cells than young offspring, but bacterial burdens were largely equivalent between adults and pups **(see Fig 4.3)**.

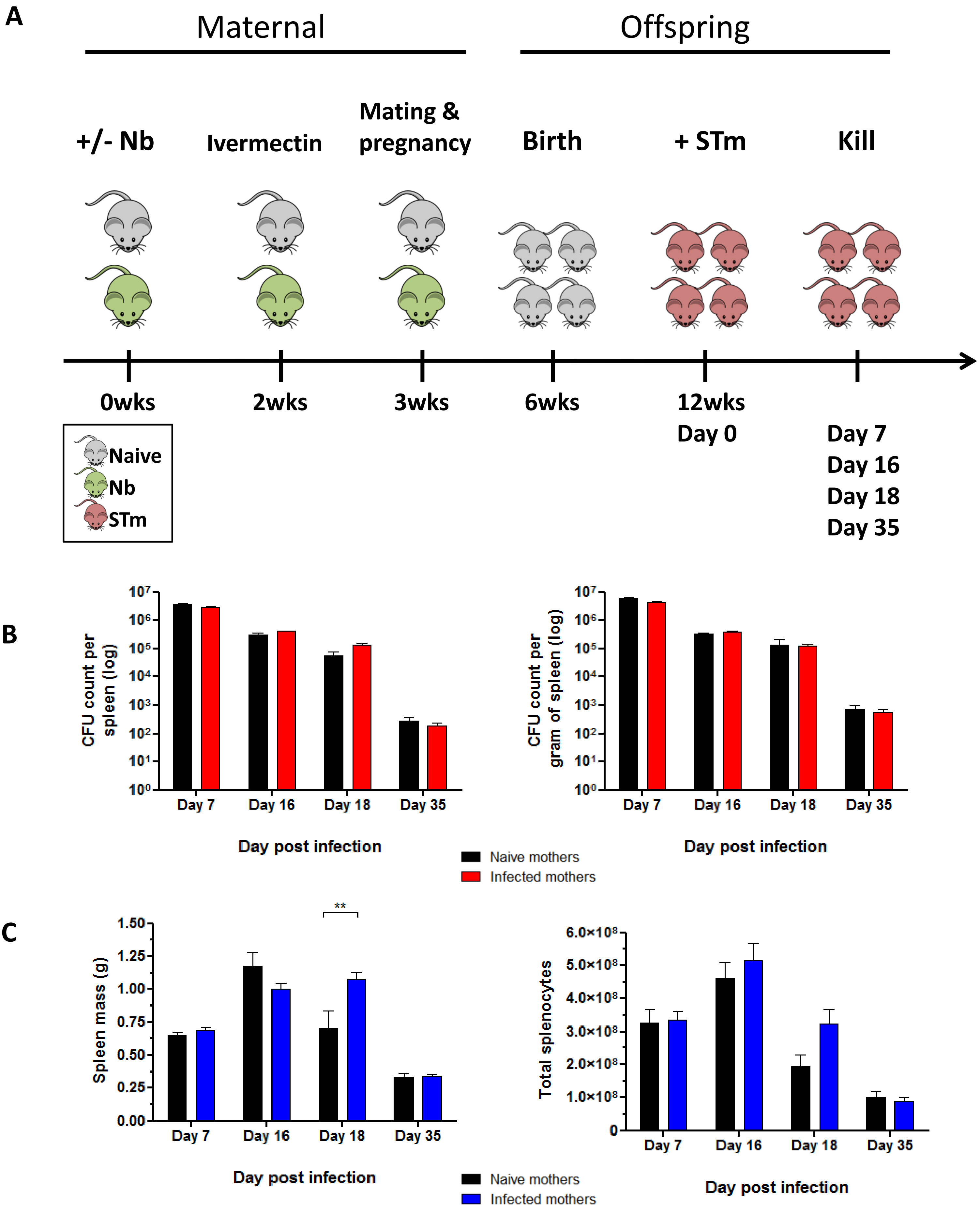


Figure 4.7 Maternal Nb infection in BALB/c mice does not impair resolution of STm infection in adult offspring. (A) Experiment plan: Female BALB/c mice were infected with 500xL3 Nb s.c., infection was cleared with Ivermectin at 2 weeks p.i., females were mated and offspring were infected with 5x10^{5}CFU STm i.p. at 6weeks of age and killed at 7, 16 or 35 days post STm infection. **(B)** STm CFU count per spleen and per gram of spleen. N=6-15. **p<0.01.Experiment was carried out once.

4.2.8 The effect of maternal Nb infection on antibody repertoire in adult STm infected offspring

Assessment of the antibody repertoire of adult STm infected offspring revealed that NES-specific IgG1 was still present at day 7 post STm infection (in now 7 week old mice), as seen in naive 8 week old mice (day 0 p.i. in figure) **(Fig 4.8 Ai)**. Moreover, NES-specific IgG1 levels increased further to 2-fold the levels in offspring from naive mothers at day 18 p.i., as seen in young offspring at the same time point during the STm infection **(see Fig 4.6 Ai)**. This supports the theory that general immune activation and involvement of T cells at this time point in the STm infection kinetic may promote production of NES-specific IgG1 from MatAb-primed offspring B cells by facilitating T cell help.

Analysis of OMPS-specific IgG2a levels in adult naive offspring showed a marginal decrease in relative levels in offspring from Nb infected mothers, corresponding to a decrease in young offspring, but this was not statistically significant in this case **(Fig 4.6 Aii)**. OMPS-specific IgG2a levels were elevated at day 7 post STm in adult offspring (7 weeks of age) from Nb infected mothers, but were equivalent between groups at subsequent time points. In young offspring, elevated levels of OMPS-specific IgG2a were seen at day 35 p.i., when mice were 7 weeks of age. The significance of this is unclear.

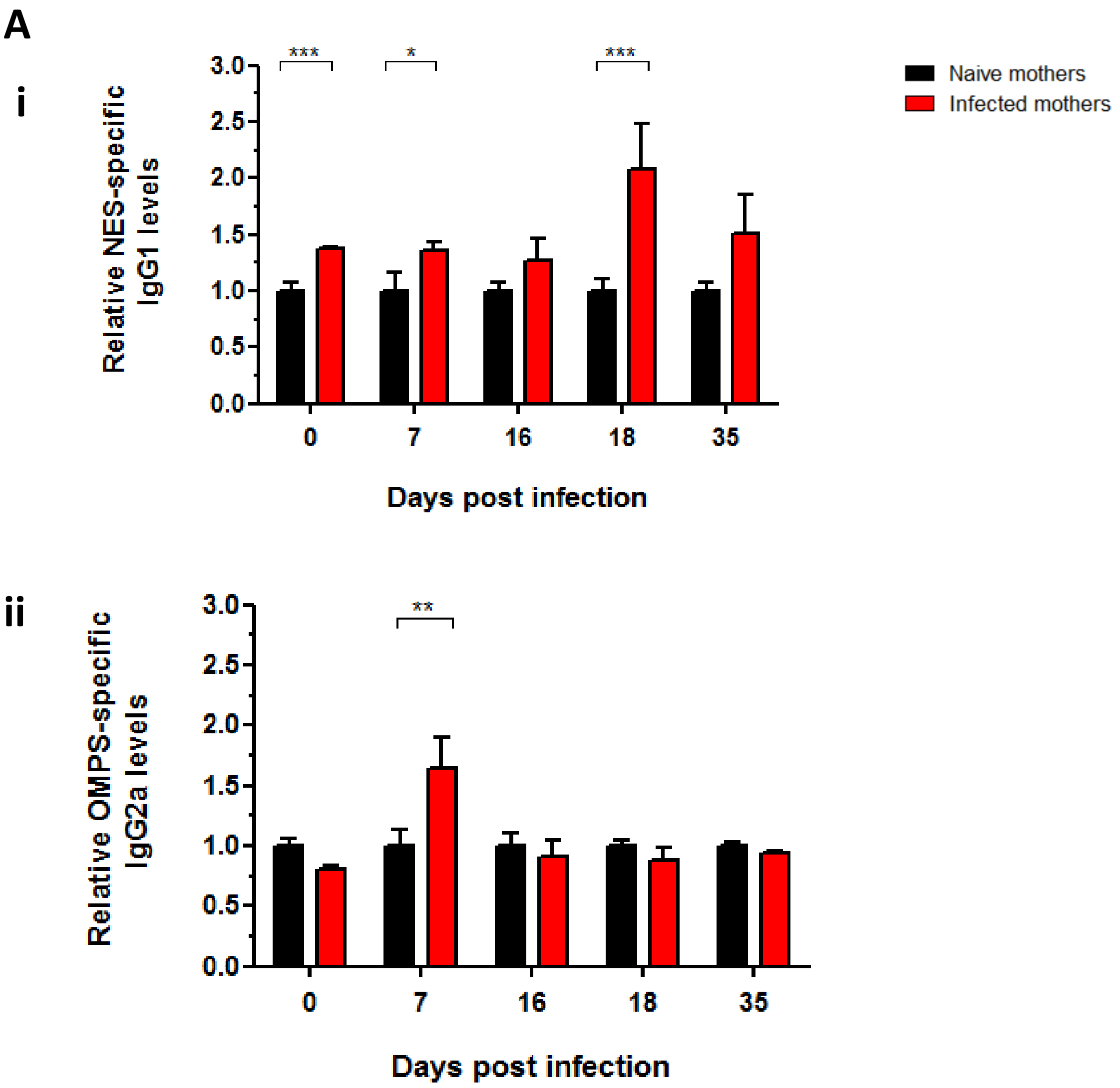


Figure 4.8 The effect of maternal Nb infection on antibody repertoire in adult STm infected offspring. Relative change in levels of **(Ai)** NES-specific IgG1 and **(Aii)** OMPS-specific IgG2a in STm infected adult offspring with Nb infection in the mother throughout the course of STm infection. Levels were normalized to a value of 1 in pups from naive mothers. $*p<0.05$, $**p<0.01$, $***p<0.001$. N=6. Analysis was carried out on one experiment.

4.2.9 Breastfeeding by Nb infected mothers has no impact on control of splenic STm bacterial burden in foster pups

To determine the effect of maternal Nb infectious history on the influence of breastfeeding and the transfer of immune components through breastmilk, we carried out litter swop experiments. Once again, female BALB/c mice were infected with 500xL3 Nb s.c., infection was cleared with Ivermectin at 2 weeks p.i. (controls were also treated), and females were mated. Offspring born to these mothers was then replaced with 5-day old naive foster pups from separate naive mothers and these pups were infected with 1x10^{5}CFU STm at 2 weeks of age and killed at 18 days post STm infection **(Fig 4.9 A)**. There were no differences in splenic bacterial burden in foster pups at day 18 post STm infection **(Fig 4.9 B)**, and no effect was seen on spleen mass and total splenocyte numbers **(Fig 4.9 C)**. Therefore, breastmilk from Nb infected mothers does not influence offspring to such an extent that it modifies control of another infection. However, we now wanted to dissect the effect of maternal breastmilk on immune components in offspring.

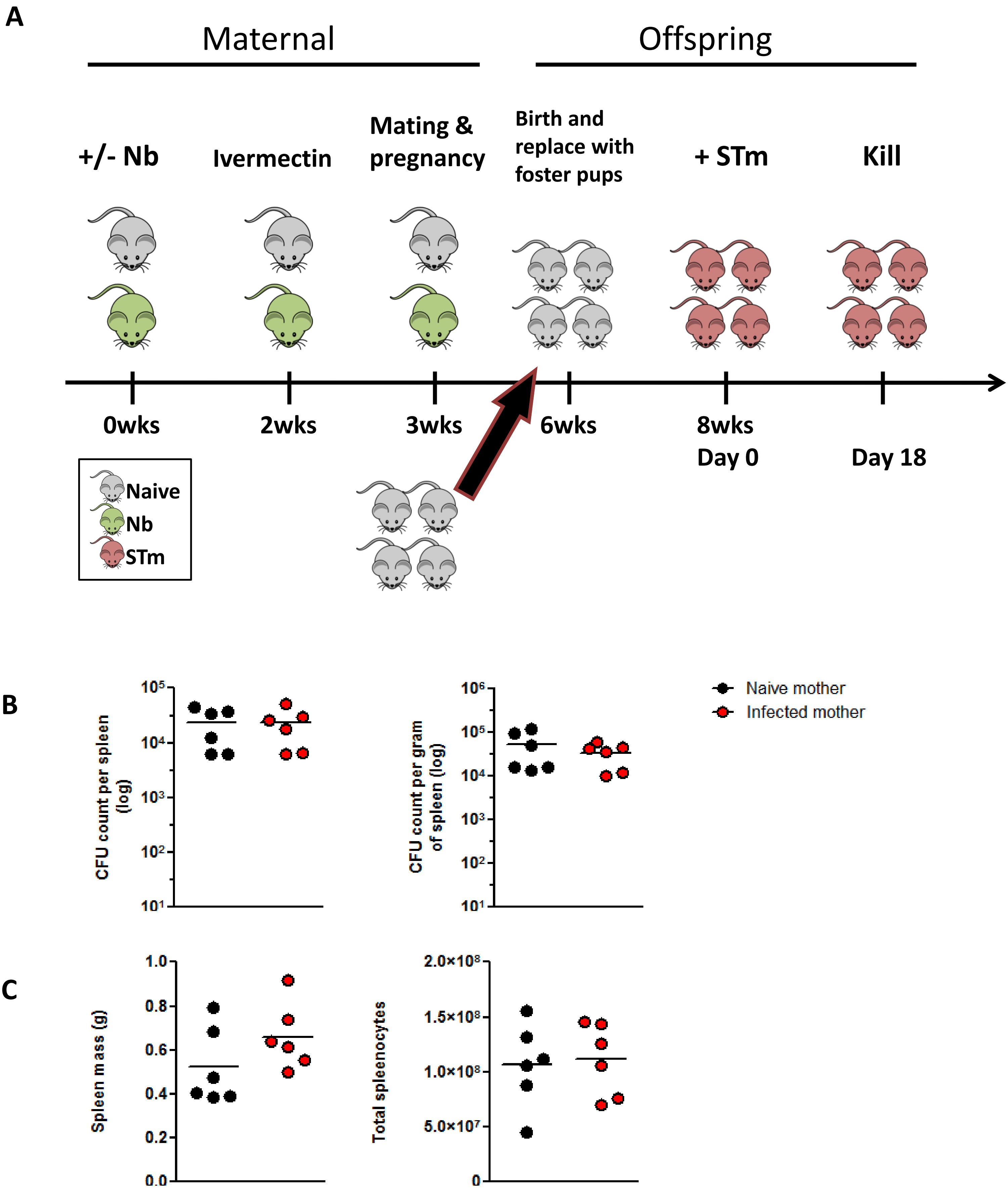


__Figure 4.9__ **Breastfeeding by Nb infected mothers has no impact on control of splenic STm bacterial burden in foster pups. (A)** Experiment plan: Female BALB/c mice were infected with 500xL3 Nb s.c., infection was cleared with Ivermectin at 2 weeks p.i., females were mated and their offspring was replaced with 5-day old naive foster pups from naive mothers and these pups were infected with $1x10^5$CFU STm i.p. at 2 weeks of age and killed at 18 days post STm infection. **(B)** STm CFU count per spleen and per gram of spleen. **(C)** Spleen mass and total splenocytes. Dots represent individual mice. Experiment was carried out once.

4.2.10 Cytokine response in STm infected foster pups breastfed by Nb infected mothers

The effect of breastmilk from Nb infected mothers on Type 1 and Type 2 cytokine levels in foster pups was assessed by ELISA. Levels of IFNγ were quantified from splenocytes restimulated with optimal anti-CD3 (10µg/ml), HK-STm (10µg/ml) and NES (10µg/ml) alone **(Fig 4.10 A)**. Pups fostered by Nb infected mothers showed a decline in IFNγ from an average of 915ng/ml to an average of 555ng/ml from anti-CD3 restimulated splenocytes, but this was not statistically significant. HK-STm restimulated splenocytes produced approximately 3 times more IFNγ in pups fostered by Nb infected mothers and NES restimulated splenocytes produced approximately 15 times more IFNγ in pups fostered by Nb infected mothers. Similar cytokine profiles were seen in experiments performed by Drs S. Bobat and W. Horsnell: Nb primed STm infected adult mice also showed elevated IFNγ levels from NES- and HK-STm-restimulated splenocytes and equivalent levels from anti-CD3 restimulated splenocytes, however this was at day 6 post STm infection while our results are form day 18 post STm.[231]

Levels of IL-13 were quantified from splenocytes restimulated with optimal anti-CD3 (10µg/ml), HK-STm (10µg/ml) and NES (10µg/ml) alone **(Fig 4.10 B)**. IL-13 is required for clearance of Nb through IL-4Rα signalling[240,241] but is not involved in clearance of STm. IL-13 levels were increased in foster pups breastfed by Nb infected mothers when splenocytes were restimulated with anti-CD3, where pups fostered by naive mothers had an average of 2.2ng/ml, pups fostered by Nb infected mothers had an average of 6.8ng/ml. There was no IL-13 production by splenocytes restimulated by NES from pups fostered by naive mothers; however pups fostered by Nb infected mothers had an average of 1ng/ml IL-13. HK-STm restimulation also showed an increase in IL-13 production but this was not statistically significant.

IFNγ is the predominant cytokine transferred in breastmilk and levels of other cytokines are normally low in human breastmilk.[242] However, human milk mononuclear cells have been shown to produce other cytokines by *in vitro* stimulation.[192] Antibody and antigen is also transferred through breastmilk.[243] However, we find that splenocytes from offspring appear primed to produce higher levels of cytokines not only specific to NES but also to HK-STm and by general activation of T cells with anti-CD3. Therefore, this cytokine production may occur by a generalized activation of offspring splenocytes by breastmilk transferred immune components like NES-specific IgG1 and cytokines, possibly in combination with transferred NES/immune complexes, which activate the cells of offspring.

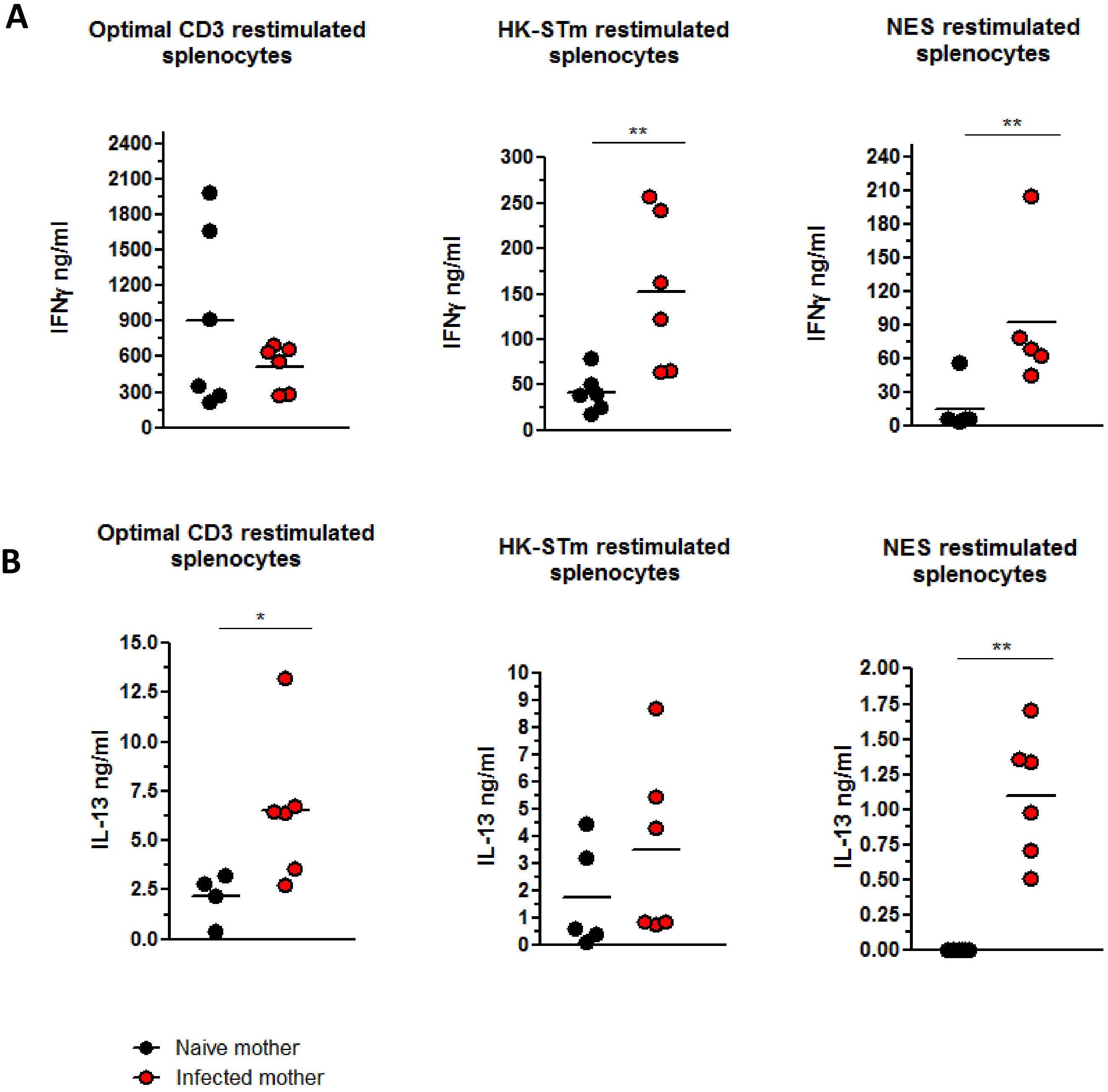


__Figure 4.10__ **Cytokine response in STm infected foster pups breastfed by Nb infected mothers. (A)** Levels of IFNγ were quantified from splenocytes restimulated with optimal anti-CD3 (10µg/ml), HK-STm (10µg/ml) and NES (10µg/ml) alone. **(B)** Levels of IL-13 were quantified from splenocytes restimulated with optimal anti-CD3 (10µg/ml), HK-STm (10µg/ml) and NES (10µg/ml) alone. *p<0.05. **p<0.01. Dots represent individual mice. Analysis was carried out on one experiment.

4.2.11 Transfer of NES-specific maternal antibodies from Nb infected foster mothers and the influence on OMPS-specific antibody in STm infected foster pups

Assessment of the antibody repertoire of STm infected foster pups revealed the presence of NES-specific IgG1 in those breastfed by Nb infected mothers, which was absent in pups fostered by naive mothers **(Fig 4.11 A)**. This shows that MatAb is transferred via breastmilk. In Chapter 3 we showed that NES-specific IgG1 is found in the serum and not in the spleen in naive 2 week old pups born to Nb infected mothers, and therefore must be passively transferred via breastmilk or *in utero*. In this experiment the pups were born to naive mothers but breastfed by Nb infected mothers, are now 4.5 weeks of age, and were weaned at 3 weeks. MatAb is still detectable in naive mice until 2 months of age.[195] The presence of NES-specific IgG1 is likely the remnant of antibody transferred through breastmilk. The source may also be the humoral response of the pup itself responding to activation by MatAb. Trance amounts of transferred NES immune complexes may also contribute. The early GC formation seen in 2 week old naive pups supports the hypothesis that offspring B cell repertoires are influenced and selected for an activated phenotype, and that this may prime the pup's later humoral response.

OMPS-specific antibody levels were determined **(Fig 4.11 B)**. OMPS-specific IgM, IgG and IgG1 were found to be decreased in pups fostered by Nb infected mothers; however levels of OMPS-specific IgG2a, the Type 1 antibody important in later control of STm infection, were equivalent between groups of foster pups. In previous experiments equivalent levels of OMPS-specific IgG2a were also seen between groups of pups but other OMPS-specific Ig isotypes were not assessed. Here we see that they are decreased when the mother has been infected with Nb and this may again be due to priming by MatAb and trace NES immune complexes.

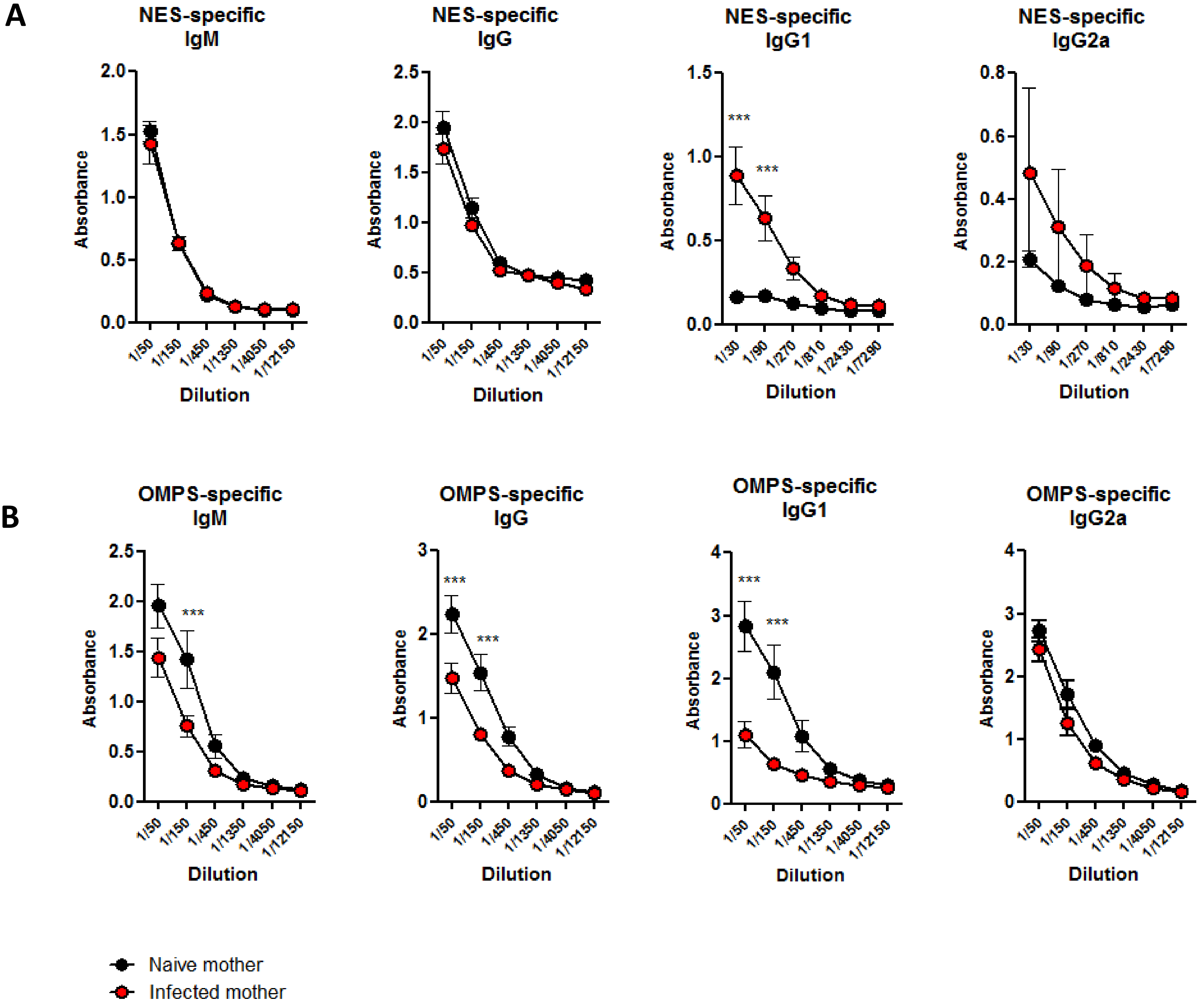
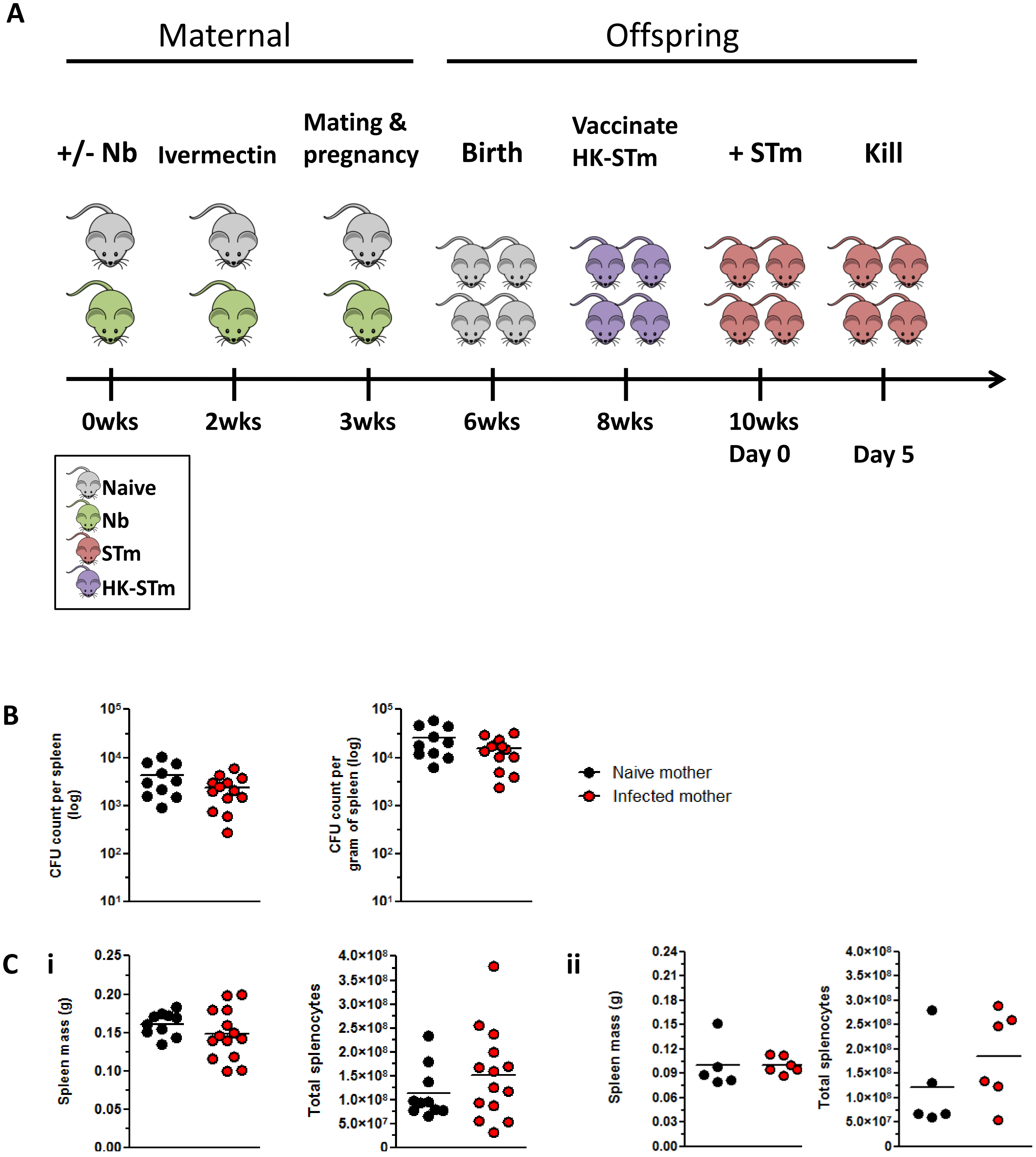


Figure 4.11 Transfer of NES-specific maternal antibodies from Nb infected foster mothers and the influence on OMPS-specific antibody in STM infected foster pups. (A) NES-specific IgM, IgG, IgG1 and IgG2a levels and **(B)** OMPS-specific IgM, IgG, IgG1 and IgG2a were determined by ELISA in foster pups breastfed by naive and Nb infected mothers. ***p<0.001. N=6. Analysis was carried out on one experiment.

4.2.12 Maternal Nb priming does not affect control of splenic bacterial burden in offspring infected with STm after vaccination with HK-STm

Although no effects of maternal Nb priming were seen on control of splenic bacterial burden in a primary STm infection in offspring, we tested whether maternal Nb priming had an effect on secondary response to STm in offspring. Once again, Nb infected mothers were mated after clearance of the worms. At 2 weeks of age offspring were vaccinated with 20µg HK-STm, infected with 1x10^{5} CFU STm i.p. 2 weeks post vaccination and killed at 5 days later **(Fig 4.12 A)**. Splenic bacterial burdens were quantified per spleen and per gram of spleen **(Fig 4.12 B)**. Although unvaccinated controls were not included, and will be for future publications, splenic CFU counts were greatly reduced in both groups compared to previous unvaccinated groups (see day 7 p.i., Fig 4.3 B) where counts were over 10^{6} CFU per spleen at day 7 post STm. However, no differences in CFU counts were seen between pups born to naive and Nb infected mothers. Also, there was no change in spleen mass or total splenocytes in STm infected and HK-STm vaccinated pups **(Fig 4.12 Ci)**, or uninfected but vaccinated pups born to naive or Nb infected mothers **(Fig 4.12 Cii)**.

Heat killed *Salmonella* is an approved vaccine against *Salmonella serovar Typhi* (ST) and not STm.[244,245] The porins vaccine has been shown to protect against STm[208] but this was not available at the time of the experiment. The difference between HK-STm and porins is that during heat inactivation, the antibody inducing porins are denatured in HK-STm and lose their ability to bind to antigen specific BCRs and induce the protective antibody response. Thus if maternal antibodies play a role here, an effect due to maternal Nb priming may be seen in offspring vaccinated with the porins vaccine.



<u>Figure 4.12</u> Maternal Nb infection does not affect control of splenic bacterial burden in offspring infected with STm after vaccination with HK-STm. (A) Experiment plan: Female BALB/c mice were infected with 500xL3 Nb s.c., infection was cleared with Ivermectin at 2 weeks p.i., females were mated and offspring was vaccinated with 20µg HK-STm at 2 weeks of age. Two weeks post vaccination, offspring was infected with $1x10^5$CFU STm i.p. and killed at 5 days later. **(B)** STm CFU count per spleen and per gram of spleen. **(Ci)** Spleen mass and total splenocytes from vaccinated and STm infected pups and **(Cii)** vaccinated but uninfected pups. Dots represent individual mice. STm infected experiment was carried out twice. Uninfected vaccination experiment was carried out once.

4.2.13 Maternal Nb infection in C57BL/6 mice impairs resolution of splenic bacterial burden in young STm infected offspring

The different genetic background of laboratory mice accounts for varied Th1/Th2 balance in response to infection, a critical aspect in the control of many diseases.[246] BALB/c mice are a Th2 dominant strain, i.e. they control Th2-inducing pathogens like Nb more efficiently than Th1-inducing pathogens like STm, while C57CBL/6 mice are a Th1 dominant strain, i.e. they control Th1-inducing pathogens like STm more efficiently than Th2-inducing pathogens like Nb.[247] No effects of Nb priming in BALB/c mothers were seen on control of splenic bacterial burden in offspring. We now tested whether there were any effects in C57BL/6 mice.

The same experimental procedure was followed. Female C57BL/6 mice were infected with 500xL3 Nb s.c., infection was cleared with Ivermectin at 2 weeks p.i. (controls were also treated), females were mated and offspring were infected with 1x10^{5}CFU STm i.p. at two weeks of age and killed at 7 and 27 days post STm infection **(Fig 4.13 A)**. This time, Nb priming in the mother impaired control of splenic bacterial burden in offspring. This was seen in a marginal increase in CFU per spleen at day 7 post STm, from 7.5x10^6 CFU in pups from naive mothers to 1.1x10^7 CFU in pups from Nb infected mothers, and more dramatically at day 27 post STm, from 4.3x10^5 CFU per spleen to 3.1x106 CFU with maternal Nb priming, a time point when the adaptive response to STm becomes important **(Fig 4.13 Bi)**. While the expected increase in splenomegaly between day 7 and 27 was seen in both groups of pups, there were no differences in spleen mass between the groups **(Fig 4.13 Bii)**. Unfortunately, due to time constraints, further analysis of cell populations, antibody repertoire, cytokine responses in these pups was not possible.

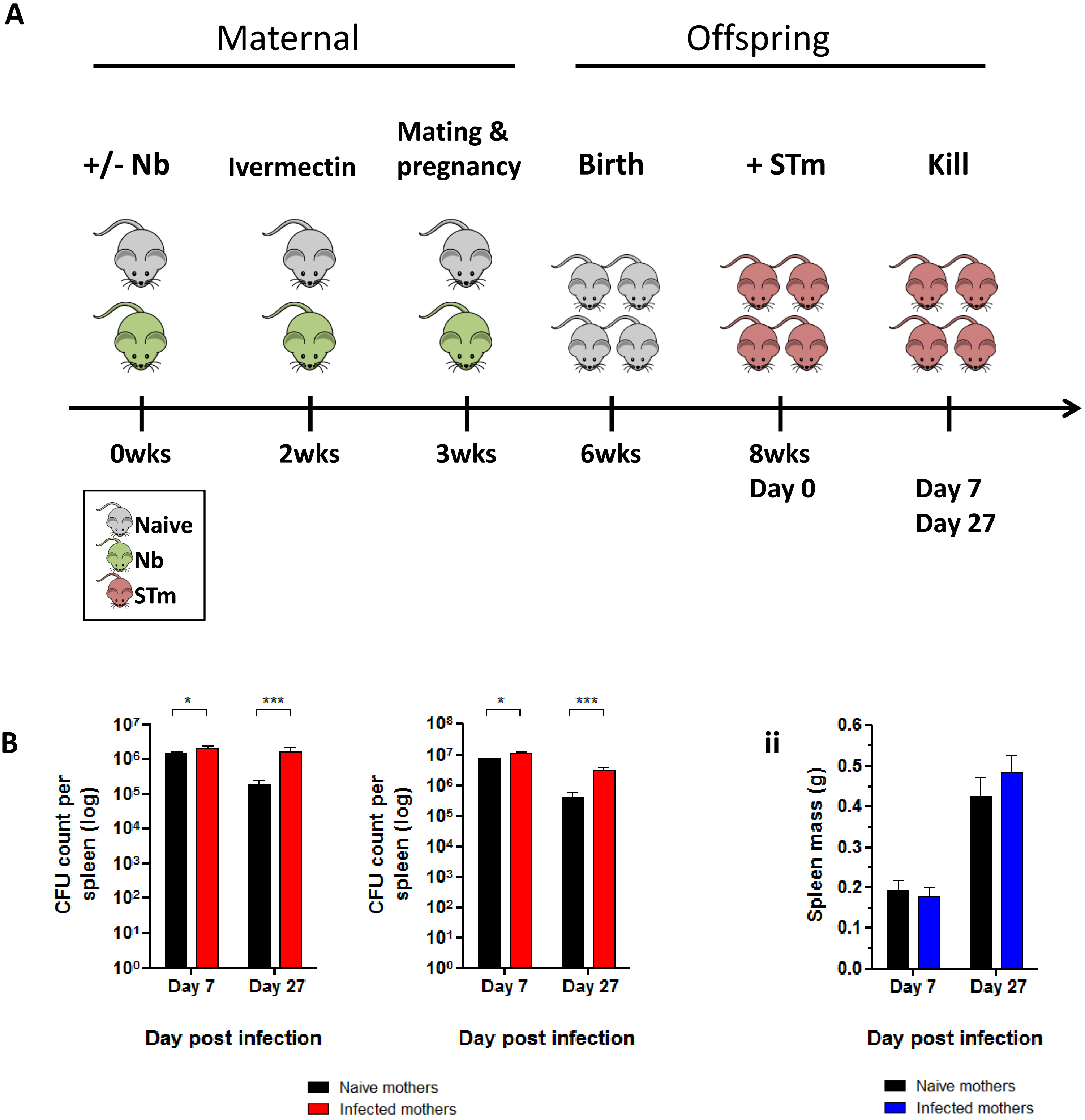


Figure 4.13 Maternal Nb infection in C57BL/6 mice impairs resolution of splenic bacterial burden in young STM infected offspring. (A) Experiment plan: Female C57BL/6 mice were infected with 500xL3 Nb s.c., infection was cleared with Ivermectin at 2 weeks p.i., females were mated and offspring were infected with $1x10^5$CFU STm i.p. at two weeks of age and killed at 7 and 27 days post STm infection. **(Bi)** STm CFU count per spleen and per gram of spleen and **(Bii)** spleen mass. N=8-15. $*p<0.05$.$***p<0.001$. Experiment was carried out once.

4.3 Discussion

In BALB/c mice, maternal priming with Nb did not affect control of splenic bacterial burden in young or adult offspring after STm infection at any time point throughout the infection. However, in young offspring, maternal Nb infection was linked to subtle changes in splenic T and B cell populations. Subtle changes were also seen in naive pups born Nb infected mothers and this may reflect the influence of maternal inflammation in the form of transferred MatAb, cytokines and/or NES immune complexes, on the young developing immune system.

Most notably, the presence of NES-specific IgG1 was still seen at day 7 and 18 post STm infection. This is most likely due to continued transfer of MatAb via breastmilk until 3 weeks of age. MatAb is still detected in naive mice up to 2 months of age.[195] As mentioned in Chapter 3, maternal IgG can prime offspring B cells and influence B cell repertoire development.[200] This may explain the increase that we see here in activated CD80+ B cells at day 7 post STm and FO B cells at days 7, 18 and 27 post STm, in pups whose mothers were infected with Nb. Furthermore, NES-specific IgG1 levels increase between day 7 and 18 post STm and this may reflect 1) the peak of immune activation and CD4+ T cell recruitment during the STm infection, promoting any GC reactions which may have been primed by the maternal Nb infection, and/or 2) B cell priming by MatAb to produce more NES-specific IgG1. In adult offspring, NES-specific IgG1 was present in naive adult mice born to Nb infected mothers, was lower at day 7 post STm and levels also increased at day 18.

The effects of maternally derived NES antigen should also be taken into account, however antigen transferred with antigen-specific antibody would be quickly removed by phagocytes. FDCs can capture and retain antigen, unprocessed in the form of immune complexes, in B cell follicles for up to a year.[60] NES may be retained in this was by FDCs and may stimulate NES-specific IgG1 production through GC reactions in the pups. Whether NES is actually transferred from mothers that have been treated with Ivermectin should be assessed, and whether amounts of NES transferred are sufficient to provide this level of antigenic stimulation in offspring may be questionable.

The elevated levels of OMPS-specific IgG2a, the antibody that helps control bacteraemia in STm infection, seen at day 35 p.i. corresponds to the time point when high affinity IgG2a produced by GC reactions is present during the STm infection time course.[150] The fact that levels of this antibody are elevated may be due to a generally more activated immune system due to priming with MatAb.

NES-specific IgG1 was present in foster pups breastfed by Nb infected mothers and associated with lower levels of OMPS-specific IgM and IgG1 but not IgG2a. Breastmilk associated increase in IFNγ levels from NES and HK-STm restimulated splenocytes and IL-13 from anti-CD3 and NES restimulated splenocytes in young offspring fostered by Nb infected mothers suggests that mothers may transfer cytokines and/or NES antigen through breastmilk which cross the epithelial layer in the gut of their pups and enter the spleen through the circulation. What exactly is transferred through breastmilk and *in utero* and accounts for the changes in serum antibody, splenic cytokine levels and cell populations remains to be elucidated.

Interestingly, when the experiment model was repeated in C57BL/6 mice, an impairment in the control of splenic bacterial burden was seen in STm infected offspring whose mothers had been Nb infected. This change was marginal at day 7 p.i. and pronounced at day 27 p.i. (by one log difference). Different commonly used mouse strains have varied levels of susceptibility to intracellular pathogens like STm, a trait associated with a macrophage divalent cation transporter called Slc11a1 (also known as Nramp1).[248] A single mutation in Slc11a1 determines resistance (Gly169) or susceptibility (Asp169).[249] Paradoxically to this study, both BALB/c and C57BL/6 mouse strains are innately susceptible to infection with virulent strains of STm compared to, for example, A/J or CBA mice, but do clear live attenuated STm which we used in our study.[250,251] Therefore Slc11a1 mutations may not be responsible for the differences we see here. However, genetic background in mice is also important for the Th1/Th2 balance in response to infection: BALB/c mice are Th2 dominant and C57BL/6 mice are Th1 dominant. In addition to T cell responses, the Th1/Th2 function of innate cells also differs between these two mouse strains. Macrophages from BALB/c mice show impaired bactericidal activity and reduced IFNγ production relative to those from C57BL/6 mice.[247,252] Therefore, when studying the effects of two pathogens that induce opposing Th responses, like Nb and STm, it is important to consider the possible effects of the genetic background of the mouse strain used.

Since C57BL/6 mice are better than BALB/c mice at clearing intracellular pathogens, the effect on control of STm may be due to the different Th2 response from the maternal Nb infection between the two mouse strains. Although it is an attractive idea to propose that the Th1 bias of C57BL/6 mice may result in a more severe Nb infection in the mothers, resulting in more pronounced effects on their offspring and accounting for the impaired control of STm that we see here, BALB/c and C57BL/6 mice also clear Nb infections at the same rate.[15] Further experiments need to be conducted to determine why we see this effect in C57BL/6 mice and not in BALB/c mice. Based on analysis done in the BALB/c model it is possible to speculate that in C57BL/6 mice maternal NES-specific antibodies and cytokines as well as NES immune complexes are transferred, and that offspring have early germinal centre formation which may be Th2 biased. These factors may impair a Th1 response to STm, including a down regulation of CAMs and killing of bacteria and an upregulation of AAMs, a characteristic of helminth infections.

While antibody is dispensable for the control of primary STm infection, it does help to control bacteraemia and increases efficiency of bacterial clearance during secondary infection.[150,234] MatAb are known to interfere with the development of the infant's own humoral response.[82] Although these interfering antibodies are specific to the same antigen, it is plausible to suggest that maternal NES-specific IgG1 interferes with control of STm infection by the presence of a dysregulated humoral response which inhibits the protective humoral response, similar to that seen in HIV positive adults also infected with STm.[207] This study did not look at STm CFU counts in the blood and therefore we cannot say whether this is true in mice. Maternal PLA_2-specific IgG was shown to inhibit production of PLA_2-specific IgE in offspring.[197] Similarly, maternal NES-specific IgG1 may inhibit production of OMPS-specific IgG2a in offspring.

The effect of maternal Nb infection on the humoral response of offspring to STm is an important point to consider in human infants infected with virulent strains of STm which require protective OMPS-specific antibodies to control bacteraemia. Weak neonatal and infant humoral responses may be further disadvantaged if their mothers were infected with one or even multiple helminths and passed on helminth specific IgG1 to their children.

5. The effect of maternal *N.brasiliensis* infection on serum protection against *S.typhimurium*

5.1 Introduction

As mentioned, antibody is not essential in the clearance of a primary STm infection where CD4+ T cells are the main effectors of infection clearance.[149] Indeed, the effector antibody, STm-specific IgG2a, is only elicited late in the infection timeline (after day 20 p.i.) when the splenic bacterial burden is already low.[150] However, antibody is very important in increasing the efficiency of bacterial clearance during a secondary response.[238] Antibody induced during a primary infection can help restrict bacteremia,[150] and B1b cell derived antibody is required for the protection seen with the porins vaccine.[208] Moreover, simultaneous adoptive transfer of both opsonizing immune serum with CD4+ T cells drives protection of BALB/c mice against the virulent strain *S.typhimurium* C5.[235] Thus both T cells and antibody are involved in optimal *resistance* to STm challenge.[153,208,235,237]

Antibodies can protect in a cell-independent manner through complement-dependent bactericidal activity. Antibodies can also protect in a cell-dependent manner through the uptake and killing by phagocytes of antibody opsonized bacteria (antibody dependent cell mediated cytotoxicity, ADCC). In humans, cell-independent complement mediated killing of STm is activated by IgM and IgG and occurs by formation of the membrane attack complex.[253] However, virulent NTS strains are commonly complement-resistant due to the large amount of LPS-specific antibodies that are generated which crosslink outer membrane O-antigens on LPS molecules and impede access of complement to the outer membrane (as mentioned in Chapter 4). This suggests that ADCC by macrophages is likely more important *in vivo*.[254] Mouse serum is not effective at cell-independent complement mediated activity and membrane attack complex formation against NTS. Therefore mice rely solely on phagocytosis by ADCC for protection in the blood.[255]

Objectives

We have shown that maternal Nb infection in mice results in the presence of what we assume to be *in utero* and breastmilk maternally transferred NES-specific IgG1 antibody in pups which persists into adulthood. We have also shown that pups born to Nb infected mothers have early formation of splenic GCs which may mean that offspring B cell repertoire is influenced by MatAb. The maternal infection does not, however, result in an impaired ability to control STm in neonatal or adult pups in BALB/c mice. We now wanted to test in a vaccination model system whether the altered antibody repertoire and early germinal centre formation of naive pups born to Nb infected mothers affects serum-opsonized bacterial clearance (by ADCC) of STm in adult naive BALB/c mice.

5.2 Results

5.2.1 Opsonization experiment plan

To assess the effects of maternal Nb infection solely on the protective ability of serum from naive pups against STm infection, we conducted an opsonization experiment using serum from pup and mother donors and infected naive adult BALB/c mice with serum-opsonized STm.

As before, female BALB/c mice were infected with 500xL3 Nb s.c., infection was cleared with Ivermectin at 2 weeks p.i. (controls were also treated) and females were mated **(Fig 5.1A)**. Their offspring were killed at 3wks and 6wks of age and blood was harvested by cardiac puncture. Mothers were tail bled at 3 weeks post partum to determine the circulating antibodies which may be transferred to offspring via breastmilk. These pups and mothers are now called the serum donors. Sera from the serum donors were used to opsonize $2x10^6$ CFU STm which was used to infect new naive adult BALB/c mice, killed 5 days p.i. to determine splenic bacterial burden **(Fig 5.1B)**.

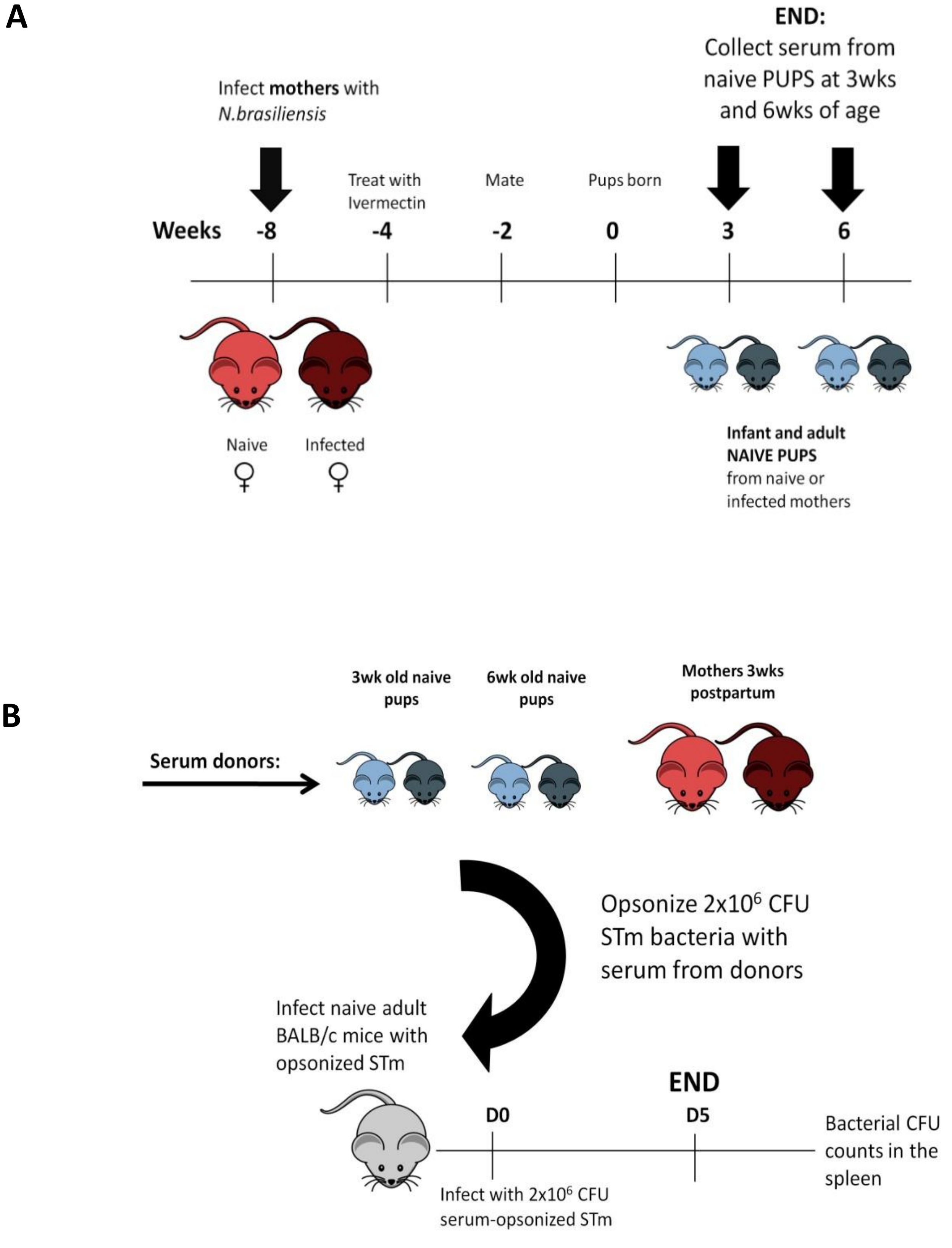


Figure 5.1 Opsonisation experiment plan. (A) 6 week old female BALB/c mice were infected with 500xL3 *N.brasiliensis* (Nb) larvae s.c. or left naive, both groups treated with Ivermectin two weeks post infection, mated and their pups were sacrificed and blood taken three and six weeks after birth. Mothers were also tail bled at three weeks postpartum. **(B)** Serum from three and six week old pups born to naive and infected mothers, as well as from the mothers themselves at 3 weeks postpartum, was used to opsonized $2x10^6$ CFU STm after heat inactivation of complement,. Naive BALB/c mice were then infected with serum-opsonized STm and sacrificed 5 days p.i. for splenic bacterial counts.

5.2.2 Protective ability against STm of naive infant pup serum from 3 week old pups born to naive or infected mothers

Spleen mass and splenic bacterial burden was determined from mice infected with STm opsonized with serum from naive infant pups 3wks of age, born to naive or infected mothers, as well as serum from the mothers themselves at 3 weeks postpartum. This was compared to control serum which was taken from adult mice infected with STm and killed at day 35 p.i. when humoral immunity to STm has developed **(Fig 5.2)**.

An increased spleen mass was seen from an average of 0.44g to an average of 0.53g when STm was opsonized with serum from pups born to infected mothers, but this was not statistically significant **(Fig 5.2A)**. Spleen mass was largely equivalent in the maternal group (an average or 0.44g in naive mothers and an average of 0.42g in infected mothers), where donor serum was from maternal tail bleeds at 3 weeks postpartum, and this was also similar to the pup group. In the control group where serum was expected to be highly protective, the spleen mass was reduced to an average of 0.21g, suggesting a lesser degree of lymphocyte expansion and a higher degree of infection control by ADCC.

Splenic bacterial burden showed that, as expected, the control serum from *Salmonella* infected mice was protective against STm infection with a CFU count of 2.25×10^4 CFU per spleen **(Fig 5.2B)**. Humoral protection against STm is achieved by increasing the efficiency of macrophage phagocytosis and clearance of antibody coated bacteria.[256] There was no statistically significant difference in bacterial clearance between opsonization with serum from infant pups born to naive or infected mothers. No difference in bacterial burden was found in recipients of bacteria opsonized with maternal serum derived from naïve or Nb infected mothers.

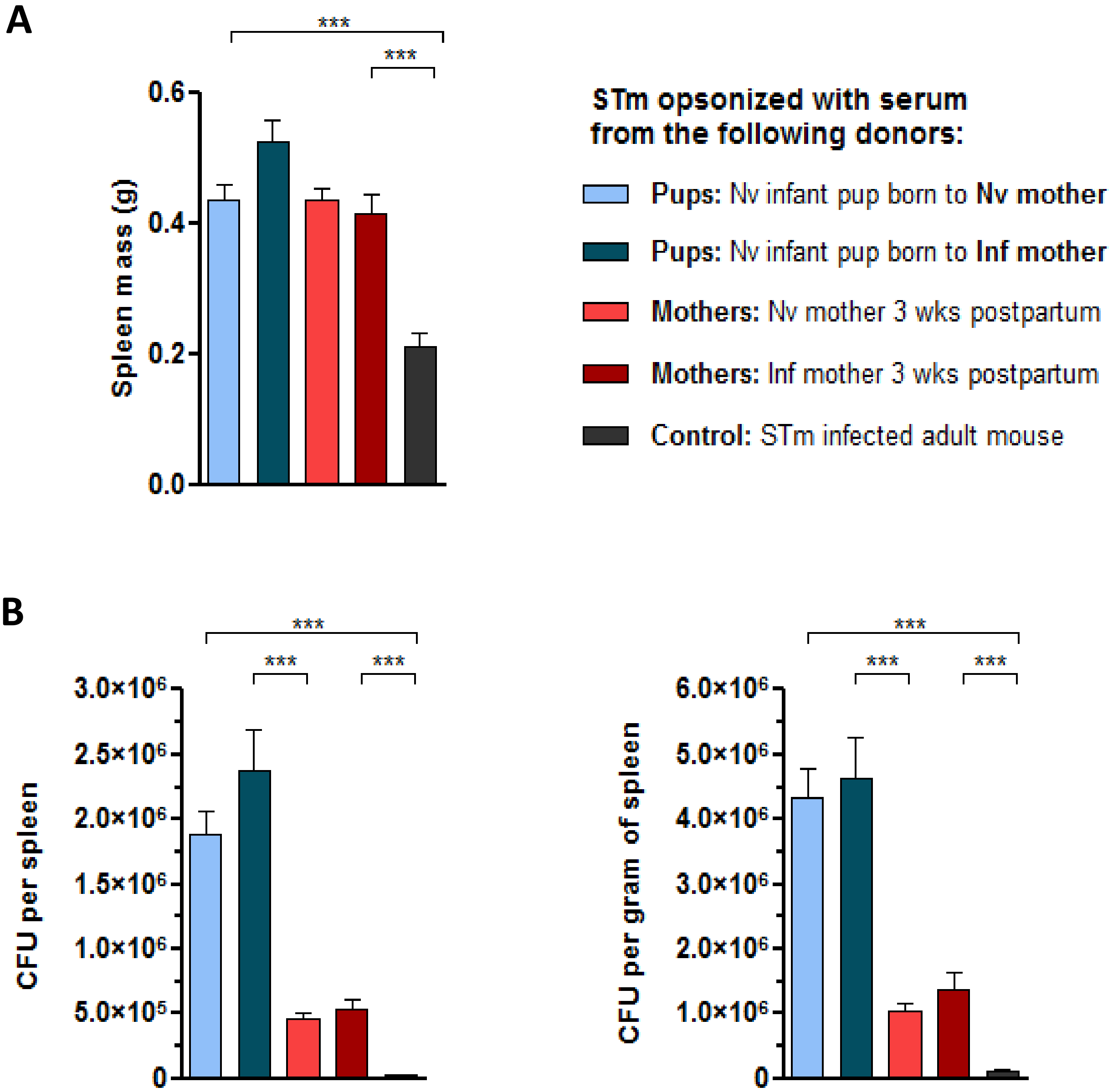


Figure 5.2 Protective ability against STm of naive infant pup serum from 3wk old pups born to naive or infected mothers. (A) Spleen mass of naive adult BALB/c mice infected with STm opsonized with serum from naive 3wk old pups born to naive or infected mothers, or from the mothers themselves at 3 weeks postpartum, or from adult mice infected with STm and sacrificed 35 days p.i. **(B)** STm CFU count per spleen and per gram of spleen from mice in **(A)**. N=8. Experiment was carried out once. ***p<0.001.

When comparing bacterial burden in 3wk old infant pup serum to maternal serum, infant pup serum had an impaired ability to clear bacteria. Where pup serum was used, the average CFU count per spleen for the whole pup group was 2.35×10^6, whereas the average CFU count per spleen for the whole maternal group was 5×10^5. This was also seen in CFU per gram of spleen. This finding may be a reflection of the Th2 biased immune system and the poorly developed humoral immune system of the neonate. Where adult mothers will have a normal ability to mount a Th1 response and may have more natural polyreactive and antigen-specific IgM from B1a B cells and MZ B cells,[58] and antigen specific IgA from B1a B cell priming by commensal bacetria,[64,65] neonates still posses the *in utero* derived Th2 biased response and have yet to fully develop adult humoral immunity as well as their normal commensal bacteria.[257]

5.2.3 Protective ability against STm of naive adult pup serum from 3 week old pups born to naive or infected mothers

Spleen mass and splenic bacterial burden was determined from mice infected with STm opsonized with serum from naive adult pups 6wks of age, born to naive or infected mothers, as well as serum from the mothers themselves at 3 weeks postpartum **(Fig 5.3)**.

The NES- and STm-specific antibody repertoires of the donor adult pup and maternal sera were determined **(Fig 5.3A)**, showing the presence of NES-specific IgG1 levels in adult pups born to Nb infected mothers and its absence in adult pups born to naive mothers. STm-specific IgG1 was low and equivalent between the groups. NES-specific IgG1 was also present in the maternal serum when mothers were infected with Nb, but absent in naive mothers. STm-specific IgG1 and IgG2a levels were low and equivalent between the groups.

When these donor sera were used to opsonize STm and infect new naive adult BALB/c mice, no differences were seen in spleen mass **(Fig 5.3B)**. Interestingly, splenic bacterial burdens revealed that when serum from naive adult pups born to naive mothers was used, the CFU count per spleen was 1.0×10^6 where CFU count per spleen was 2.2×10^6 when serum from naive adult pups born to infected mothers was used **(Fig 5.3 C)**. This impaired protective ability is also seen in CFU counts per gram of spleen.

Maternal serum from infected mothers cleared the infection better than serum from naive mothers. While this result was not seen in the previous opsonization experiment where maternal serum was also used from 3 weeks postpartum, this difference was not great (2.9x10 CFU per spleen with naive maternal serum compared to 2.3x10 CFU per spleen with infected maternal serum) and may be accounted for by freeze-thawing of the serum.

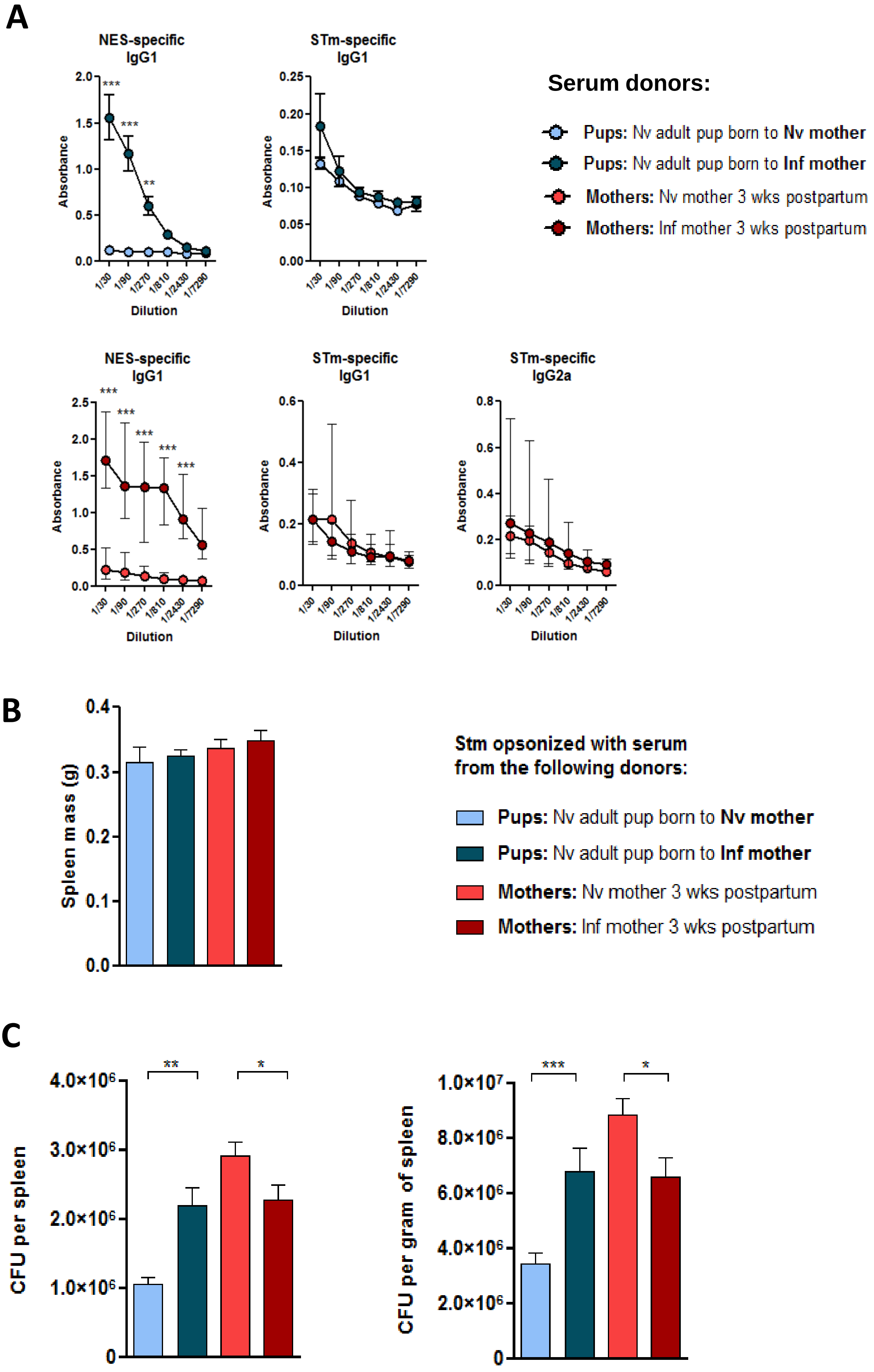


__Figure 5.3__ **Protective ability against STm of adult pup serum from 6wk old pups born to naive and infected mothers. (A)** Relative antibody levels from donor serum used to opsonize 2x10^6 CFU STm: NES-specific IgG1 and STm-specific IgG1 from naive adult 6wk old pups born to naive or infected mothers, NES-specific IgG1, STm-specific IgG1 and STm-specific IgG2a from naive or infected mothers themselves tail bled at 3 weeks postpartum. **(B)** Spleen mass from adult BALB/c mice infected with serum-opsonized STm from pups and mothers in **(A)**. **(C)** STm CFU counts per spleen and per gram of spleen from mice in **(B)**. N=8. Experiment was carried out once. *p<0.05. **p<0.01. ***p<0.001.

When maternal serum was used to opsonize the STm, splenic CFU counts were largely similar to the adult pup serum donor group (an average of $2.6\text{x}10^6$ CFU per spleen with maternal serum, and $1.6\text{x}10^6$ CFU per spleen with adult pup serum). Thus adult pups do not have the impaired clearance ability compared to adult maternal serum seen when infant pup serum was used in section 5.2.2. As Expected, this indicates that the 6wk old pups have overcome the neonatal Th2 bias and have developed their humoral immune system and natural antibody repertoire to match that of adult maternal serum, and demonstrates the high susceptibility of young infants to bacterial infection.

5.3 Discussion

Our opsonization model looks solely at the effects of maternal Nb infection on the ability of the serum antibody of naive offspring to opsonize STm and enhance clearance by phagocytes of a naive adult mouse. Table 5.1 summarizes the serum groups used to opsonize STm and infect naive adult mice. Serum was heat inactivated so changes in pups' complement system are not relevant here (though complement-mediated bactericidal activity of mouse serum is ineffective in any case).

Table 5.1 Summary of serum used to opsonize STm for infection of naive adult mice.

Serum donor	Infection status	Age	Infection status of mother			
Pups	Naive	3 wks or 6 wks	Naive	**Opsonize STm**	→	**Infect naive adult mouse**
Pups	Naive	3 wks or 6 wks	Infected			
Mothers of pups	Naive	3 wks postpartum	Naive			
Mothers of pups	Infected	3 wks postpartum	Naive			

In summary of this experiment, our findings were: 1) The serum of 3 week old naive pups born to Nb infected mothers had the same opsonizing ability of STm as the serum of pups born to naive mothers; 2) The serum of 6 week old naive pups born to Nb infected mothers had impaired opsonizing ability of STm compared to the serum from pups born to naive mothers; 3) Maternal serum was much better at opsonizing STm than 3 week old pup serum; and 4) This difference between maternal and pup serum was negated when 6 week old pup serum was used.

MatAb are known to interfere with neonatal and infant humoral responses to infection and vaccination by epitope masking of immunodominant vaccine epitopes and also by the uptake of MatAb:antigen immune complexes by neonatal APCs.[78] However, in this case the antibodies are antigen-specific and the mother and offspring have the same or overlapping antigen experience. OmpD-specific IgG2a protects against STm while NES-specific IgG1 is elicited in Nb infections and (we assume) transferred from mother to offspring. The presence of MatAb in offspring wanes with age and is stronger in younger pups which have just been weaned (i.e. 3wk old pups) than in adult pups (6wk old pups). In this experiment, we see an impaired opsonizing ability of pup serum due to maternal Nb infection only once the pup has reached adulthood and not when high titres of MatAb are present. Therefore the impaired opsonizing ability of pup serum is not dependent on the titre of MatAb. Our results suggest that the maternal Nb infection has a long term effect on the offspring antibody repertoire and only affects control of STm later, once the offspring's adaptive response is fully mature. This may be mediated by the influence of MatAb on offspring B cell repertoire development and subsequent production of normal antibody against commensal bacteria, including STm, or polyreactive antibody by B1a and MZ B cells, which may then affect opsonization of STm.

Our opsonization data also revealed that neonatal serum is less able to opsonize STm than adult maternal serum. This may be explained by the Th2 bias of neonatal immunity, imprinted onto the fetus *in utero* by the maternal immune system to avoid rejection of the fetal allograft by an inflammatory Th1 response.[258] With a Th2 biased antibody response (producing IgG1 and IgE rather than IgG2a), as well as a generally weak humoral response and underdeveloped colonization by commensal bacteria, the neonatal immune system may not be able to produce natural IgM and IgG antibodies that adults can and this may be responsible for the impaired opsonization ability of STm by neonatal serum.

6. Concluding Remarks

6.1 Summary of results

In SSA pregnant women are often infected with one or more helminth during pregnancy. This maternal infection is strongly correlated with impaired vaccine efficacy of standard vaccinations like Hib in their children.[111] In SSA a high prevalence of helminth infections overlaps with a high prevalence of other multi drug resistant infections such as NTS,[259] particularly in young children. Children between the ages of 6 and 24 months are highly susceptible to NTS which results in progressive bacteraemia and even death.[201,202,260] Maternal helminth infection appears to alter the immune development of their offspring with deleterious effects on protective responses to other pathogens. This effect may further exacerbate the high susceptibility of young children to infections such as NTS in areas where helminth infections are common. Few studies have addressed how a maternal helminth infection may alter offspring immunity.

Our study showed that in mice, maternal infection with the helminth *N.brasiliensis* impacts on immune development of offspring. There was a pronounced presence of NES-specific IgG1 in offspring born to and/or fostered by Nb infected mothers, suggesting that MatAb is transferred *in utero* and through breastmilk. NES-specific IgG1 is still detectable in 8 week old offspring. Offspring have early GC reactions in the spleen and altered splenic T and B cell populations. Other studies have shown that MatAb can alter the development of the B cell repertoire of offspring,[195,200] promoting an activated phenotype during infection and the selection of B cells into follicles and that this effect is long-lasting.[200] Maternally derived IgG has been shown to have effects on isotype switching in offspring, suppressing the production of IgE in response to allergen.[197] Maternal NES-specific IgG1 may therefore influence the development of offspring B cells, promoting activation of GCs and the production of Type 2 antibodies like IgG1.

We hypothesized that a maternal infection with a highly Th2 polarizing pathogen like Nb may impair control of a highly polarizing Th1 pathogen like *Salmonella enterica* serovar *Typhimurium* in offspring. We found that maternal Nb infection in BALB/c mice does not affect control of STm in offspring or HK-STm vaccine efficacy. It does, however, still results in a pronounced presence of NES-specific IgG1 as well as subtle changes in splenic T and B cell population, breastmilk associated increased cytokine production and the presence of NES-specific IL-13 (the critical Type 2 cytokine involved in clearance of Nb).

BALB/c mice are Th2 biased where C57BL/6 mice are Th1 biased. When we changed our mouse model to C57BL/6, we found that maternal Nb infection in C57BL/6 mice impairs control of STm in young offspring. Since C57BL/6 and BALB/c mice are both susceptible to STm infection and both clear Nb infection at the same rate, further experiments are needed to determine why we see this impaired control in C57BL/6 mice but not in BALB/c mice.

Control of STm infections differs between mice and humans. In the murine model the strain used is attenuated (AroA-/- SL3261) and is not lethal whereas human infections are with invasive virulent strains of STm. Virulent strains are also used in experimental mouse models but these are fatal. Where the CD4+ T cell response is critical in the control of STm in mice[149] and a humoral response is dispensable in a primary infection,[238] antibody and CD4+ T cells are required to control bacteraemia and tissue infection in humans.[153] These are important aspects to consider when assessing effects of maternal helminth infection on STm in offspring as changes in B cell and antibody repertoires may play a larger role in human STm infection.

Our opsonization experiments showed that serum from naive adult offspring born to Nb infected mothers had reduced natural ability to opsonize STm than serum from offspring born to naive mothers. We did not see any effect when we used the serum of young offspring when higher titres of MatAb are present. This suggests that functional effects of maternal Nb infection on offspring antibody repertoire in terms of STm clearance by antibody dependent cell mediated cytotoxicity may be long-term, having an impaired effect on the development of a natural humoral response.

6.2 Future work

This project has shown that a maternal Nb infection has significant effects on the immune development and response in offspring which may extend to an impaired response to other pathogens. Further mechanistic experiments should be carried out in order to identify how maternal immune components prime and potentially bias the immune response of their offspring.

Immunohistochemistry and B cell selection in the spleen

We hypothesize that MatAb affect the development of B cells in offspring, promoting selection into B cell follicles and early GC formation. This can be further investigated by immunohistochemical (IHC) analysis of spleen sections to see the progression and expansion of GC development with age in offspring born to Nb infected mothers and to characterize the antibody specificity of these GCs. IHC should also be used to determine whether NES immune complexes are transferred from the Nb infected mother and retained on FDCs in the follicles of offspring as these, along with MatAb, may activate offspring B cells.

The significance of maternal antibodies on offspring B cell repertoire

To determine the significance of maternal antibodies, breeding strategies with homozygous B cell deficient µMT/µMT or heterozygous µMT /+ females mated with µMT/µMT or µMT/+ males, respectively, could be carried out. We could thus compare normal or B cell deficient pups born from Ig- deprived (Ig-) or phenotypically normal mothers (Ig+). This has already been carried out in previous studies with naive mothers.[195] In our study we could assess the impact of the maternal Nb infection on the influence of maternal antibody on offspring B cell repertoire and antibody production. We could further analyze the functional effects of the serum of STm infected offspring born to Nb infected B cell deficient or normal mothers by carrying out opsonization experiments with STm.

Mechanistic experiments in C57BL/6 mice

Experiments to determine how the maternal Nb infection modulates offspring's immune system in C57BL/6 mice should be carried out. This includes determining the antibody, cytokine and lymphocyte repertoire in C57BL/6 offspring. The macrophage population in STm infected pups should also be characterized to determine whether there is a bias to helminth-induced AAM population rather than the protective CAM population which may then affect ADCC activity through altered macrophage-antibody activity. The efficacy of the porins vaccine in offspring born to Nb infected mothers should also be tested in these mice.

Gene expression in the intrauterine environment

Maternal-offspring experiments are difficult to control and highly variable results are common among individual mouse and human neonates. In mice, factors like lactation, litter size and maternal stress vary and can influence experiments. This has also been reported in other studies.[78] A better, more controlled way to understand the intrauterine effects of a maternal helminth infection on the fetus would be to perform RT-PCR on genes of interest on placental and embryonic tissues. With the advantage of pooled litter samples and controls like housekeeping genes, these results may provide important direction to how the fetal immune development is being affected by maternal imprinting.

7. Appendices

7.1 Appendix A – STm protein preparations

7.1.1 Isolation of purified porins for immunization

Purified porins from STm (strain ATCC 14028) were extracted by Dr. Cristina- Gil Cruz through repeated extraction with SDS. In brief, STm were incubated in minimal salts medium (MAM) containing 0.1% yeast extract, 0.5% glucose and 0.1% $MgSO_4$ at 37°C with agitation (200rpm) until late log phase (OD_{600nm} of 1.0). Cells were then diluted 1:10 in 1.5L MAM and further incubated until late log phase. Cells were harvested at 6000 x g at 4°C for 15min and washed in wash buffer. Cells were then resuspended in wash buffer and disrupted using a French Press at 20,000 psi. Unbroken cells were removed by centrifugation (6,000 x g at 4°C for 20min) and the supernatant was centrifuged again at (30,000 x g at 4°C for 40min). The resulting pellet now contained the envelope fraction and was resuspended in 100ml solubilisation buffer and incubated for 2hrs at 37°C with agitation (120 rpm). The soluble inner membrane fraction was then separated from the insoluble outer membrane (OM) by centrifugation (30,000 x g at 4°C for 45min). This extraction step was performed twice. Pelleted cells (OM envelope) were resuspended in Nikaido buffer and incubated at 37°C for 1 hour with agitation (120rpm) before centrifugation at 30,000 x g at 20°C for 1hr. The resulting supernatant isolated after this third extraction contained the OM fraction.

Final purification was achieved by FPLC gel filtration on a Sephacryl S-200 column with Nikaido's purification buffer. Fractions with an OD at λ280 nm of >0.2 were pooled and extensively dialysed against PBS containing 0.1% (w/v) SDS. Purity was assessed by SDS-PAGE and protein concentration using the BCA assay. The Limulus Amoebocyte Lysate (LAL) assay was used to detect bacterial Endotoxin from purified proteins as per manufacturer's guidelines (Sigma Aldrich, Poole, UK). The LAL assay showed LPS contamination to be 0.06EU/480µg protein. Protein identity was confirmed by trypsin digest and Quadrupole

Time of Flight (QTOF) mass spectrometry at the School of Biosciences Functional Proteomics Unit (University of Birmingham). Protein was stored at -80°C and transported from the UK on dry ice.

7.1.2 Isolation of STm outer membrane proteins for ELISA antigen coating

This process is similar to that described for porins purification. A volume of 5ml LB broth was inoculated with STm bacteria culture (strain *AroA-/-* SL3261) and grown overnight at 37°C with agitation (180rpm). The following day 100ml of sterile LB broth was inoculated with a 1/1000 dilution of the overnight bacterial culture and grown up to late log phase (OD_{600nm} of 1.0) at 37°C with agitation (150rpm). Cells were harvested by centrifugation (10,000 x g at 4°C for 10min) using the 250ml high speed centrifuge pots. The supernatant was discarded and the pellet resuspended in 20ml of 10mM Tris buffer pH7.4 containing 2mM PMSF. Cells were then disrupted using a French Press at 20,000 psi three times. This suspension was then centrifuged (6,000 x g at 4°C for 10mins) to remove unbroken cells. The supernatant was poured off into another 50ml centrifuge tube and the pellet discarded. This supernatant was the centrifuges once more (30,000 x g at 4°C for 90mins) to separate the envelope and cytoplasmic fractions. The supernatant was discarded and the pelleted envelopes were resuspended in 10ml of 10mM Tris Buffer pH 7.4 with 2% (v/v) TritonX-100 and incubated in a water bath at 25°C for 15mins. This allows solubilisation of the inner membrane. The envelopes, now dissolved in the TritonX-100, were harvested by centrifugation (30,000 x g at 4°C for 90 min). The supernatant was discarded and the pellet washed three times and resuspended in 1ml of 10mM Tris Buffer pH 7.4. The pelleted material now contains predominantly outer membrane. This was then aliquoted into 250µl quantities and store at -20°C or -80°C. Samples were run on a gel at neat, 1/2 and 1/5 concentrations at 150V to check purity. The concentration of outer membrane proteins (Omps) was calculated by performing a BCA protein assay.

7.2 Appendix B – Buffers

Flow cytometry:

MACS buffer

2mM EDTA

0.5% BSA

Dissolve the above reagents in a final volume of 1000ml of 1X PBS and store at 4°C.

ELISA:

ELISA blocking buffer

20g fat-free milk powder

0.2g NaN_3

Dissolve reagents in a final volume of 1000ml 1X PBS and store at 4°C.

ELISA wash buffer (20X)

20g KCL

20g $KH_2HPO_4.2H_2O$

800g NaCl

50ml Tween-20

100ml 10% NaN_3

Make up to 5L with ddH_2O and store at room temperature. Dilute in 1:20 in ddH_2O.

ELISA dilution buffer

10g BSA

0.2g NaN_3

Dissolve the above reagents in a final volume of 1000ml of 1X PBS and store at 4°C.

ELISA substrate buffer (for horseradish peroxidase conjugates)

TMB Peroxidase Substrate Solution A (Roche Diagnostics GmbH)

Peroxidase Substrate Solution B (Roche Diagnostics GmbH, Mannheim, Germany)

Just before use, mix equal volumes of TMB Peroxidase Substrate (Solution A) with Peroxidase Substrate Solution B.

Table 7.1 Cytokine ELISA antibodies and solutions (discussed in Section 2.8.2)

Cytokine	Coating 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	1/500	1/500	100ng/ml	
Company	R&D Systems	R&D Systems	BD Biosciences	
Clone	38213.11	Polyclonal	n/a	

Immunohistochemistry:

Tris buffer pH7.6

1L of 200mM Tris base

1.5L of 154mM physiological NaCl

1.0L of 0.1 N HCl

Check pH.

Tris buffer pH9.2

As above but at pH 9.2.

Initially pH 200 ml to give pH9.2 using HCl (dropwise with 1M or greater) and make up to desired volume with NaCl.

Horseradish peroxidase substrate

Dissolve a single 3-3'-diaminobenzidine tetrahydrochloride (DAB) tablet (Sigma Aldrich) in 15ml Tris buffer pH7.6. Filter to 10ml and add 1 drop of hydrogen peroxide (Sigma Aldrich). A single drop of HRP substrate was added to each section until the desired level of positivity was reached, whereupon slides were washed in Tris buffer pH7.6.

Alkaline Phosphate Substrate

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

8. References

1. Murphy, K., Travers, P. & Walport, M. *Janeway's Immunobiology*. (Garland Science: New York, 2010).

2. Abbas, A. K., Jr, C. A. J. & Francisco, S. Immunology : Improving on Nature in the Twenty-First Century of Medicine. **100**, 129–138 (2000).

3. Mak, T. W. & Saunders, M. E. *Primer to the immune response*. (Academic Press, Elsevier: Oxford, 2008).

4. Beutler, B. Innate immunity: an overview. *Molecular Immunology* **40**, 845–859 (2004).

5. Litman, G. W., Anderson, M. K. & Rast, J. P. Evolution of antigen binding receptors. *Annual review of immunology* **17**, 109–47 (1999).

6. Flajnik, M. F. & Kasahara, M. Comparative genomics of the MHC: glimpses into the evolution of the adaptive immune system. *Immunity* **15**, 351–62 (2001).

7. Cooper, M. D. & Alder, M. N. The evolution of adaptive immune systems. *Cell* **124**, 815–22 (2006).

8. Sun, J. C., Lopez-Verges, S., Kim, C. C., DeRisi, J. L. & Lanier, L. L. NK cells and immune "memory". *Journal of immunology (Baltimore, Md. : 1950)* **186**, 1891–7 (2011).

9. Akira, S. & Takeda, K. Toll-like receptor signalling. *Nature reviews. Immunology* **4**, 499–511 (2004).

10. Janeway, C. A. & Medzhitov, R. Innate Immune Recognition. 197–216 (2002).doi:10.1146/annurev.immunol.20.083001.084359

11. McSorley, H. J. & Maizels, R. M. Helminth infections and host immune regulation. *Clinical microbiology reviews* **25**, 585–608 (2012).

12. Gabay, C. & Kushner, I. Acute-phase proteins and other systemic responses to inflammation. *The New England journal of medicine* **340**, 448–54 (1999).

13. Murray, P. J. & Wynn, T. a Protective and pathogenic functions of macrophage subsets. *Nature reviews. Immunology* **11**, 723–737 (2011).

14. Mosser, D. M. & Edwards, J. P. Exploring the full spectrum of macrophage activation. *Nature reviews. Immunology* **8**, 958–69 (2008).

15. Maizels, R. M. & Yazdanbakhsh, M. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nature reviews. Immunology* **3**, 733–44 (2003).

16. Gordon, S. & Martinez, F. O. Alternative activation of macrophages: mechanism and functions. *Immunity* **32**, 593–604 (2010).

17. Neill, D. R. *et al.* Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* **464**, 1367–70 (2010).

18. Banchereau, J. & Steinman, R. M. Dendritic cells and the control of immunity. **392**, 245–252 (1998).

19. Janeway, C. A. J., Travers, P. & Walport, M. *Immunobiology: The immune system in health and disease*. (Garland Science: New York, 2001).

20. Von Boehmer, H. Positive selection of lymphocytes. *Cell* **76**, 219–28 (1994).

21. Kazansky, D. B. MHC restriction and allogeneic immune responses. *Journal of immunotoxicology* **5**, 369–84 (2008).

22. Nossal, G. J. Negative selection of lymphocytes. *Cell* **76**, 229–39 (1994).

23. Giltiay, N. V, Chappell, C. P. & Clark, E. A. B-cell selection and the development of autoantibodies. **14**, 1–13 (2012).

24. Hardy, R. R. & Hayakawa, K. B cell development pathways. *Annual review of immunology* **19**, 595–621 (2001).

25. Pillai, S., Mattoo, H. & Cariappa, A. B cells and autoimmunity. *Current opinion in immunology* **23**, 721–31 (2011).

26. Pillai, S., Cariappa, A. & Moran, S. T. Positive selection and lineage commitment during peripheral B-lymphocyte development. *Immunological reviews* **197**, 206–18 (2004).

27. Germain, R. N. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. *Cell* **76**, 287–99 (1994).

28. DeFranco, A. L., Locksley, R. M. & Robertson, M. *Immunity: the immune response in infectious and inflammatory disease*. (New Science Press: 2007).

29. Toellner, K. M. *et al.* T helper 1 (Th1) and Th2 characteristics start to develop during T cell priming and are associated with an immediate ability to induce immunoglobulin class switching. *The Journal of experimental medicine* **187**, 1193–204 (1998).

30. Prete, G. D. E. L. The Concept of Type-I and Type-2 Helper TCells and Their Cytokines in Humans. **I**, (1998).

31. Mullen, A. C. *et al.* Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science (New York, N.Y.)* **292**, 1907–10 (2001).

32. Mosmann, T. R. & Coffman, R. L. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual review of immunology* **7**, 145–73 (1989).

33. Svetić, A. *et al.* A primary intestinal helminthic infection rapidly induces a gut-associated elevation of Th2-associated cytokines and IL-3. *Journal of immunology (Baltimore, Md. : 1950)* **150**, 3434–41 (1993).

34. Kopf, M. *et al.* Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature* **362**, 245–8 (1993).

35. Holgate, S. T. The epidemic of allergy and asthma. *Nature* **402**, B2–4 (1999).

36. Barlow, J. L. & McKenzie, A. N. J. Nuocytes: expanding the innate cell repertoire in type-2 immunity. *Journal of leukocyte biology* **90**, 867–74 (2011).

37. Ferber, I. A. *et al.* GATA-3 significantly downregulates IFN-gamma production from developing Th1 cells in addition to inducing IL-4 and IL-5 levels. *Clinical immunology (Orlando, Fla.)* **91**, 134–44 (1999).

38. Paul, W. E. & Zhu, J. How are TH2-type immune responses initiated and amplified? **10**, (2010).

39. Vitetta, E. S. *et al.* Serological, biochemical, and functional identity of B cell-stimulatory factor 1 and B cell differentiation factor for IgG1. *The Journal of experimental medicine* **162**, 1726–31 (1985).

40. Chung, F. Anti-inflammatory cytokines in asthma and allergy: interleukin-10, interleukin-12, interferon-gamma. *Mediators of inflammation* **10**, 51–9 (2001).

41. Bettelli, E. *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* **441**, 235–8 (2006).

42. Ivanov, I. I. *et al.* Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell host & microbe* **4**, 337–49 (2008).

43. Zaretsky, A. G. *et al.* T follicular helper cells differentiate from Th2 cells in response to helminth antigens. *The Journal of experimental medicine* **206**, 991–9 (2009).

44. Groux, H. & Powrie, F. Regulatory T cells and inflammatory bowel disease. *Immunology today* **20**, 442–5 (1999).

45. Powrie, F. & Mason, D. OX-22high CD4+ T cells induce wasting disease with multiple organ pathology: prevention by the OX-22low subset. *The Journal of experimental medicine* **172**, 1701–8 (1990).

46. Hostetter, J., Kagan, R. & Steadham, E. Opsonization effects on Mycobacterium avium subsp. paratuberculosis--macrophage interactions. *Clinical and diagnostic laboratory immunology* **12**, 793–6 (2005).

47. Clynes, R. A., Towers, T. L., Presta, L. G. & Ravetch, J. V Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nature medicine* **6**, 443–6 (2000).

48. Giacomin, P. R. *et al.* The role of complement in innate, adaptive and eosinophil-dependent immunity to the nematode Nippostrongylus brasiliensis. *Molecular immunology* **45**, 446–55 (2008).

49. Gao, J. *et al.* Novel functions of murine B1 cells: active phagocytic and microbicidal abilities. *European journal of immunology* **42**, 982–92 (2012).

50. Su, T. T., Guo, B., Wei, B., Braun, J. & Rawlings, D. J. Signaling in transitional type 2 B cells is critical for peripheral B-cell development. *Immunological reviews* **197**, 161–78 (2004).

51. Pillai, S., Cariappa, A. & Moran, S. T. Marginal zone B cells. *Annual review of immunology* **23**, 161–96 (2005).

52. Loder, F. *et al.* B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. *The Journal of experimental medicine* **190**, 75–89 (1999).

53. Harris, N. L. & Ronchese, F. The role of B7 costimulation in T-cell immunity. *Immunology and cell biology* **77**, 304–11 (1999).

54. Stein, K. E. Thymus-independent and thymus-dependent responses to polysaccharide antigens. *The Journal of infectious diseases* **165 Suppl** , S49–52 (1992).

55. García de Vinuesa, C., O'Leary, P., Sze, D. M., Toellner, K. M. & MacLennan, I. C. T-independent type 2 antigens induce B cell proliferation in multiple splenic sites, but exponential growth is confined to extrafollicular foci. *European journal of immunology* **29**, 1314–23 (1999).

56. Steiniger, B. Introductory article: Spleen. *Encyclopedia of Life Sciences* (2005).doi:10.1038/npg.els.0003982

57. Cesta, M. F. Normal structure, function, and histology of the spleen. *Toxicologic pathology* **34**, 455–65 (2006).

58. MacLennan, I. C. M. *et al.* Extrafollicular antibody responses. *Immunological reviews* **194**, 8–18 (2003).

59. Allen, C. D. C., Okada, T. & Cyster, J. G. Germinal-center organization and cellular dynamics. *Immunity* **27**, 190–202 (2007).

60. MacLennan, I. C. Germinal centers. *Annual review of immunology* **12**, 117–39 (1994).

61. Millikin, P. D. Anatomy of germinal centers in human lymphoid tissue. *Archives of pathology* **82**, 499–505 (1966).

62. Shapiro-Shelef, M. & Calame, K. Regulation of plasma-cell development. *Nature reviews. Immunology* **5**, 230–42 (2005).

63. Radbruch, A. *et al.* Competence and competition: the challenge of becoming a long-lived plasma cell. *Nature reviews. Immunology* **6**, 741–50 (2006).

64. Fagarasan, S. T-Independent Immune Response: New Aspects of B Cell Biology. *Science* **290**, 89–92 (2000).

65. Berland, R. & Wortis, H. H. Origins and functions of B-1 cells with notes on the role of CD5. *Annual review of immunology* **20**, 253–300 (2002).

66. Alugupalli, K. R. *et al.* B1b lymphocytes confer T cell-independent long-lasting immunity. *Immunity* **21**, 379–90 (2004).

67. Adkins, B., Leclerc, C. & Marshall-Clarke, S. Neonatal adaptive immunity comes of age. *Nature reviews. Immunology* **4**, 553–64 (2004).

68. Wood, N. & Siegrist, C.-A. Neonatal immunization: where do we stand? *Current opinion in infectious diseases* **24**, 190–5 (2011).

69. Neuzil, K. M., Mellen, B. G., Wright, P. F., Mitchel, E. F. & Griffin, M. R. The effect of influenza on hospitalizations, outpatient visits, and courses of antibiotics in children. *The New England journal of medicine* **342**, 225–31 (2000).

70. Morein, B., Blomqvist, G. & Hu, K. Immune responsiveness in the neonatal period. *Journal of comparative pathology* **137 Suppl** , S27–31 (2007).

71. Aschkenazi, S. *et al.* Differential regulation and function of the Fas/Fas ligand system in human trophoblast cells. *Biology of reproduction* **66**, 1853–61 (2002).

72. Hunt, J. S., Petroff, M. G. & Burnett, T. G. Uterine leukocytes: key players in pregnancy. *Seminars in cell & developmental biology* **11**, 127–37 (2000).

73. Gupta, S. K. & International Congress of Reproductive Immunology *Reproductive immunology : [VII International Congress of Reproductive Immunology ... held in New Delhi in October 1998]*. (Kluwer Academic Publ. [u.a.]: Boston, Mass. [u.a.], 1999).

74. Hodgins, D. C. & Shewen, P. E. Vaccination of neonates: problem and issues. *Vaccine* **30**, 1541–59 (2012).

75. Hsieh, C. S. *et al.* Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science (New York, N.Y.)* **260**, 547–9 (1993).

76. Dakic, A. *et al.* Development of the dendritic cell system during mouse ontogeny. *Journal of immunology (Baltimore, Md. : 1950)* **172**, 1018–27 (2004).

77. Lee, S. M. *et al.* Decreased interleukin-12 (IL-12) from activated cord versus adult peripheral blood mononuclear cells and upregulation of interferon-gamma, natural killer, and lymphokine-activated killer activity by IL-12 in cord blood mononuclear cells. *Blood* **88**, 945–54 (1996).

78. Siegrist, C. A. Neonatal and early life vaccinology. *Vaccine* **19**, 3331–46 (2001).

79. Johnston, R. B., Altenburger, K. M., Atkinson, A. W. & Curry, R. H. Complement in the newborn infant. *Pediatrics* **64**, 781–6 (1979).

80. Pihlgren, M. *et al.* Influence of complement C3 amount on IgG responses in early life: immunization with C3b-conjugated antigen increases murine neonatal antibody responses. *Vaccine* **23**, 329–35 (2004).

81. Zinkernagel, R. M. Maternal antibodies, childhood infections, and autoimmune diseases. *The New England journal of medicine* **345**, 1331–5 (2001).

82. Glezen, W. Effect of maternal antibodies on the infant immune response. *Vaccine* **21**, 3389–3392 (2003).

83. Siegrist, C. Mechanisms by which maternal antibodies influence infant vaccine responses: review of hypotheses and definition of main determinants. *Vaccine* **21**, 3406–3412 (2003).

84. Crowe, J. E., Firestone, C. Y. & Murphy, B. R. Passively acquired antibodies suppress humoral but not cell-mediated immunity in mice immunized with live attenuated respiratory syncytial virus vaccines. *Journal of immunology (Baltimore, Md. : 1950)* **167**, 3910–8 (2001).

85. Nohynek, H. *et al.* Effect of transplacentally acquired tetanus antibodies on the antibody responses to Haemophilus influenzae type b-tetanus toxoid conjugate and tetanus toxoid vaccines in Filipino infants. *The Pediatric infectious disease journal* **18**, 25–30 (1999).

86. De Vries, E. *et al.* Longitudinal survey of lymphocyte subpopulations in the first year of life. *Pediatric research* **47**, 528–37 (2000).

87. Marshall-Clarke, S., Tasker, L. & Parkhouse, R. M. Immature B lymphocytes from adult bone marrow exhibit a selective defect in induced hyperexpression of major histocompatibility complex class II and fail to show B7.2 induction. *Immunology* **100**, 141–51 (2000).

88. Gatto, D. *et al.* Complement receptors regulate differentiation of bone marrow plasma cell precursors expressing transcription factors Blimp-1 and XBP-1. *The Journal of experimental medicine* **201**, 993–1005 (2005).

89. Siegrist, C.-A. & Aspinall, R. B-cell responses to vaccination at the extremes of age. *Nature reviews. Immunology* **9**, 185–94 (2009).

90. Timens, W., Rozeboom, T. & Poppema, S. Fetal and neonatal development of human spleen: an immunohistological study. *Immunology* **60**, 603–9 (1987).

91. Pihlgren, M. *et al.* Unresponsiveness to lymphoid-mediated signals at the neonatal follicular dendritic cell precursor level contributes to delayed germinal center induction and limitations of neonatal antibody responses to T-dependent antigens. *Journal of immunology (Baltimore, Md. : 1950)* **170**, 2824–32 (2003).

92. Timens, W., Boes, A., Rozeboom-Uiterwijk, T. & Poppema, S. Immaturity of the human splenic marginal zone in infancy. Possible contribution to the deficient infant immune response. *Journal of immunology (Baltimore, Md. : 1950)* **143**, 3200–6 (1989).

93. Klein Klouwenberg, P. & Bont, L. Neonatal and infantile immune responses to encapsulated bacteria and conjugate vaccines. *Clinical & developmental immunology* **2008**, 628963 (2008).

94. Timens, W., Boes, A., Rozeboom-Uiterwijk, T. & Poppema, S. Immaturity of the human splenic marginal zone in infancy. Possible contribution to the deficient infant immune response. *Journal of immunology (Baltimore, Md. : 1950)* **143**, 3200–6 (1989).

95. Mancuso, G. *et al.* Role of interleukin 12 in experimental neonatal sepsis caused by group B streptococci. *Infection and immunity* **65**, 3731–5 (1997).

96. Urban, J. F. *et al.* IL-12 protects immunocompetent and immunodeficient neonatal mice against infection with Cryptosporidium parvum. *Journal of immunology (Baltimore, Md. : 1950)* **156**, 263–8 (1996).

97. Arulanandam, B. P., Van Cleave, V. H. & Metzger, D. W. IL-12 is a potent neonatal vaccine adjuvant. *European journal of immunology* **29**, 256–64 (1999).

98. Donckier, V. *et al.* IL-12 prevents neonatal induction of transplantation tolerance in mice. *European journal of immunology* **28**, 1426–30 (1998).

99. Pihlgren, M. *et al.* Delayed and deficient establishment of the long-term bone marrow plasma cell pool during early life. *European journal of immunology* **31**, 939–46 (2001).

100. Pihlgren, M. *et al.* Reduced ability of neonatal and early-life bone marrow stromal cells to support plasmablast survival. *Journal of immunology (Baltimore, Md. : 1950)* **176**, 165–72 (2006).

101. Namikawa, R. *et al.* Ontogenic development of T and B cells and non-lymphoid cells in the white pulp of human spleen. (1986).

102. Friedberg, S. H. & Weissman, I. L. Lymphoid tissue architecture. II. Ontogeny of peripheral T and B cells in mice: evidence against Peyer's patches as the site of generation of B cells. *Journal of immunology (Baltimore, Md. : 1950)* **113**, 1477–92 (1974).

103. Barrios, C. *et al.* Neonatal and early life immune responses to various forms of vaccine antigens qualitatively differ from adult responses: predominance of a Th2-biased pattern which persists after adult boosting. *European journal of immunology* **26**, 1489–96 (1996).

104. Brandt, C. *et al.* Protective immunity against respiratory syncytial virus in early life after murine maternal or neonatal vaccination with the recombinant G fusion protein BBG2Na. *The Journal of infectious diseases* **176**, 884–91 (1997).

105. World Health Organization *Prevention and control of schistosomiasis and soil-transmitted helminthiases*. Technical Report Series 912 (Geneva, 2002).

106. Brooker, S. *et al.* The co-distribution of Plasmodium falciparum and hookworm among African schoolchildren. *Malaria journal* **5**, 99 (2006).

107. Else, K. J. & Finkelman, F. D. Intestinal nematode parasites, cytokines and effector mechanisms. *International journal for parasitology* **28**, 1145–58 (1998).

108. Sakti, H. *et al.* Evidence for an association between hookworm infection and cognitive function in Indonesian school children. *Tropical medicine & international health : TM & IH* **4**, 322–34 (1999).

109. Dreyfuss, M. L. *et al.* Hookworms, malaria and vitamin A deficiency contribute to anemia and iron deficiency among pregnant women in the plains of Nepal. *The Journal of nutrition* **130**, 2527–36 (2000).

110. Navitsky, R. C. *et al.* Ancylostoma duodenale is responsible for hookworm infections among pregnant women in the rural plains of Nepal. *The Journal of parasitology* **84**, 647–51 (1998).

111. Labeaud, a D., Malhotra, I., King, M. J., King, C. L. & King, C. H. Do antenatal parasite infections devalue childhood vaccination? *PLoS neglected tropical diseases* **3**, e442 (2009).

112. Hotez, P. Hookworm and poverty. *Annals of the New York Academy of Sciences* **1136**, 38–44 (2008).

113. Loukas, A., Bethony, J., Brooker, S. & Hotez, P. Hookworm vaccines: past, present, and future. *The Lancet infectious diseases* **6**, 733–41 (2006).

114. Loukas, A. *et al.* Vaccination with recombinant aspartic hemoglobinase reduces parasite load and blood loss after hookworm infection in dogs. *PLoS medicine* **2**, e295 (2005).

115. Camberis, M., Le Gros, G. & Urban, J. Animal model of Nippostrongylus brasiliensis and Heligmosomoides polygyrus. *Current protocols in immunology / edited by John E. Coligan ... [et al.]* **Chapter 19**, Unit 19.12 (2003).

116. Reece, J. J. *et al.* Hookworm-induced persistent changes to the immunological environment of the lung. *Infection and immunity* **76**, 3511–24 (2008).

117. DeFranco, A. L., Locksley, R. M. & Robertson, M. *Immunity: the immune response in infectious and inflammatory disease*. (New Science Press: 2007).

118. Finkelman, F. D. *et al.* Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites. *Immunological reviews* **201**, 139–55 (2004).

119. Zhao, A. *et al.* Immune regulation of protease-activated receptor-1 expression in murine small intestine during Nippostrongylus brasiliensis infection. *Journal of immunology (Baltimore, Md. : 1950)* **175**, 2563–9 (2005).

120. Oh, C. K., Geba, G. P. & Molfino, N. Investigational therapeutics targeting the IL-4/IL-13/STAT-6 pathway for the treatment of asthma. *European respiratory review : an official journal of the European Respiratory Society* **19**, 46–54 (2010).

121. Brombacher, F. The role of interleukin-13 in infectious diseases and allergy. *BioEssays : news and reviews in molecular, cellular and developmental biology* **22**, 646–56 (2000).

122. Hoshino, T., Winkler-Pickett, R. T., Mason, A. T., Ortaldo, J. R. & Young, H. A. IL-13 production by NK cells: IL-13-producing NK and T cells are present in vivo in the absence of IFN-gamma. *Journal of immunology (Baltimore, Md. : 1950)* **162**, 51–9 (1999).

123. Nelms, K., Keegan, A. D., Zamorano, J., Ryan, J. J. & Paul, W. E. The IL-4 receptor: signaling mechanisms and biologic functions. *Annual review of immunology* **17**, 701–38 (1999).

124. Horsnell, W. G. C. *et al.* IL-4Rα-responsive smooth muscle cells contribute to initiation of TH2 immunity and pulmonary pathology in Nippostrongylus brasiliensis infections. *Mucosal immunology* **4**, 83–92 (2011).

125. Horsnell, W. G. C. *et al.* Delayed goblet cell hyperplasia, acetylcholine receptor expression, and worm expulsion in SMC-specific IL-4Ralpha-deficient mice. *PLoS pathogens* **3**, e1 (2007).

126. Marillier, R. G. *et al.* IL-4/IL-13 independent goblet cell hyperplasia in experimental helminth infections. *BMC immunology* **9**, 11 (2008).

127. Mearns, H. *et al.* Interleukin-4-promoted T helper 2 responses enhance Nippostrongylus brasiliensis-induced pulmonary pathology. *Infection and immunity* **76**, 5535–42 (2008).

128. Urban, J. F. *et al.* IL-13, IL-4Ralpha, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite Nippostrongylus brasiliensis. *Immunity* **8**, 255–64 (1998).

129. Wakelin, D. Acquired immunity to Trichuris muris in the albino laboratory mouse. *Parasitology* **57**, 515–24 (1967).

130. Anthony, R. M. *et al.* Memory T(H)2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. *Nature medicine* **12**, 955–60 (2006).

131. Harvie, M. *et al.* The lung is an important site for priming CD4 T-cell-mediated protective immunity against gastrointestinal helminth parasites. *Infection and immunity* **78**, 3753–62 (2010).

132. Herbert, D. R. *et al.* Intestinal epithelial cell secretion of RELM-beta protects against gastrointestinal worm infection. *The Journal of experimental medicine* **206**, 2947–57 (2009).

133. Knott, M. L. *et al.* Impaired resistance in early secondary Nippostrongylus brasiliensis infections in mice with defective eosinophilopoeisis. *International journal for parasitology* **37**, 1367–78 (2007).

134. Popoff, M. Y., Bockemühl, J. & Brenner, F. W. Supplement 1998 (no. 42) to the Kauffmann-White scheme. *Research in microbiology* **151**, 63–5

135. Fookes, M. *et al.* Salmonella bongori provides insights into the evolution of the Salmonellae. *PLoS pathogens* **7**, e1002191 (2011).

136. Crosa, J. H., Brenner, D. J., Ewing, W. H. & Falkow, S. Molecular relationships among the Salmonelleae. *Journal of bacteriology* **115**, 307–15 (1973).

137. Zahrt, T. C. & Maloy, S. Barriers to recombination between closely related bacteria: MutS and RecBCD inhibit recombination between Salmonella typhimurium and Salmonella typhi. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 9786–91 (1997).

138. Hohmann, E. L. Nontyphoidal salmonellosis. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **32**, 263–9 (2001).

139. Graham, S. M. *et al.* Nontyphoidal Salmonella infections of children in tropical Africa. *The Pediatric infectious disease journal* **19**, 1189–96 (2000).

140. Vugia, D. J. *et al.* Pathogens and predictors of fatal septicemia associated with human immunodeficiency virus infection in Ivory Coast, west Africa. *The Journal of infectious diseases* **168**, 564–70 (1993).

141. Gilks, C. F. *et al.* Life-threatening bacteraemia in HIV-1 seropositive adults admitted to hospital in Nairobi, Kenya. *Lancet* **336**, 545–9 (1990).

142. Jepson, M. A. & Clark, M. A. The role of M cells in Salmonella infection. *Microbes and infection / Institut Pasteur* **3**, 1183–90

143. Jones, B. D., Ghori, N. & Falkow, S. Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. *The Journal of experimental medicine* **180**, 15–23 (1994).

144. Vazquez-Torres, A. *et al.* Extraintestinal dissemination of Salmonella by CD18-expressing phagocytes. *Nature* **401**, 804–8 (1999).

145. Mastroeni, P. & Sheppard, M. Salmonella infections in the mouse model: host resistance factors and in vivo dynamics of bacterial spread and distribution in the tissues. *Microbes and infection / Institut Pasteur* **6**, 398–405 (2004).

146. Richter-Dahlfors, A., Buchan, A. M. & Finlay, B. B. Murine salmonellosis studied by confocal microscopy: Salmonella typhimurium resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo. *The Journal of experimental medicine* **186**, 569–80 (1997).

147. Monack, D. M., Mueller, A. & Falkow, S. Persistent bacterial infections: the interface of the pathogen and the host immune system. *Nature reviews. Microbiology* **2**, 747–65 (2004).

148. Santos, R. L. *et al.* Animal models of Salmonella infections: enteritis versus typhoid fever. *Microbes and infection / Institut Pasteur* **3**, 1335–44

149. Pie, S. *et al.* Th1 response in Salmonella typhimurium-infected mice with a high or low rate of bacterial clearance . Th1 Response in Salmonella typhimurium-Infected Mice with a High or Low Rate of Bacterial Clearance. **65**, (1997).

150. Cunningham, A. F. *et al.* Salmonella induces a switched antibody response without germinal centers that impedes the extracellular spread of infection. *Journal of immunology (Baltimore, Md. : 1950)* **178**, 6200–7 (2007).

151. Hess, J., Ladel, C., Miko, D. & Kaufmann, S. H. Salmonella typhimurium aroA- infection in gene-targeted immunodeficient mice: major role of CD4+ TCR-alpha beta cells and IFN-gamma in bacterial clearance independent of intracellular location. *Journal of immunology (Baltimore, Md. : 1950)* **156**, 3321–6 (1996).

152. Johansson, C., Ingman, M. & Jo Wick, M. Elevated neutrophil, macrophage and dendritic cell numbers characterize immune cell populations in mice chronically infected with Salmonella. *Microbial pathogenesis* **41**, 49–58

153. Mastroeni, P., Simmons, C., Fowler, R., Hormaeche, C. E. & Dougan, G. Igh-6(-/-) (B-cell-deficient) mice fail to mount solid acquired resistance to oral challenge with

virulent Salmonella enterica serovar typhimurium and show impaired Th1 T-cell responses to Salmonella antigens. *Infection and immunity* **68**, 46–53 (2000).

154. Salcedo, S. P., Noursadeghi, M., Cohen, J. & Holden, D. W. Intracellular replication of Salmonella typhimurium strains in specific subsets of splenic macrophages in vivo. *Cellular microbiology* **3**, 587–97 (2001).

155. Miles, A. A., Misra, S. S. & Irwin, J. O. The estimation of the bactericidal power of the blood. *The Journal of hygiene* **38**, 732–49 (1938).

156. Slade, H. B. & Schwartz, S. A. Mucosal immunity: the immunology of breast milk. *The Journal of allergy and clinical immunology* **80**, 348–58 (1987).

157. SCHAEDLER, R. W., DUBOS, R. & COSTELLO, R. The development of the bacterial flora in the gastrointestianl tract of mice. *The Journal of experimental medicine* **122**, 59–66 (1965).

158. Hooijkaas, H., Benner, R., Pleasants, J. R. & Wostmann, B. S. Isotypes and specificities of immunoglobulins produced by germ-free mice fed chemically defined ultrafiltered “antigen-free” diet. *European journal of immunology* **14**, 1127–30 (1984).

159. Verhasselt, V. *et al.* Breast milk-mediated transfer of an antigen induces tolerance and protection from allergic asthma. *Nature medicine* **14**, 170–5 (2008).

160. Spear, P. G., Wang, A. L., Rutishauser, U. & Edelman, G. M. Characterization of splenic lymphoid cells in fetal and newborn mice. *The Journal of experimental medicine* **138**, 557–73 (1973).

161. Siegrist, C., Barrios, C., Martinez, X., Brandt, C. & Marco, C. Influence of maternal antibodies on vaccine responses : inhibition of antibody but not T cell responses allows successful early prime-boost strategies in mice. 4138–4148 (1998).

162. OSBORN, J. J., DANCIS, J. & JULIA, J. F. Studies of the immunology of the newborn infant. II. Interference with active immunization by passive transplacental circulating antibody. *Pediatrics* **10**, 328–34 (1952).

163. Aase, J. M., Noren, G. R., Reddy, D. V & Geme, J. W. Mumps-virus infection in pregnant women and the immunologic response of their offspring. *The New England journal of medicine* **286**, 1379–82 (1972).

164. Hara, T. *et al.* Human V delta 2+ gamma delta T-cell tolerance to foreign antigens of Toxoplasma gondii. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 5136–40 (1996).

165. Gill, T. J. *et al.* Transplacental immunization of the human fetus to tetanus by immunization of the mother. *The Journal of clinical investigation* **72**, 987–96 (1983).

166. Baker, C. J. *et al.* Immunization of pregnant women with a polysaccharide vaccine of group B streptococcus. *The New England journal of medicine* **319**, 1180–5 (1988).

167. McCormick, J. B. *et al.* Antibody response to serogroup A and C meningococcal polysaccharide vaccines in infants born of mothers vaccinated during pregnancy. *The Journal of clinical investigation* **65**, 1141–4 (1980).

168. Eloi-Santos, S. M. *et al.* Idiotypic sensitization in utero of children born to mothers with schistosomiasis or Chagas' disease. *The Journal of clinical investigation* **84**, 1028–31 (1989).

169. Agarwal, M., Prasad, G. B., Harinath, B. C. & Bhatia, B. D. Transplacental transfer of filarial infection. *Indian pediatrics* **23**, 169–74 (1986).

170. Weil, G. J. *et al.* Prenatal allergic sensitization to helminth antigens in offspring of parasite-infected mothers. *The Journal of clinical investigation* **71**, 1124–9 (1983).

171. King, C. L. *et al.* B cell sensitization to helminthic infection develops in utero in humans. *Journal of immunology (Baltimore, Md. : 1950)* **160**, 3578–84 (1998).

172. Guadalupe, I. *et al.* Evidence for in utero sensitization to Ascaris lumbricoides in newborns of mothers with ascariasis. *The Journal of infectious diseases* **199**, 1846–50 (2009).

173. Malhotra, I. *et al.* Helminth- and Bacillus Calmette-Guérin-induced immunity in children sensitized in utero to filariasis and schistosomiasis. *Journal of immunology (Baltimore, Md. : 1950)* **162**, 6843–8 (1999).

174. Figueiredo, C. A. *et al.* Chronic intestinal helminth infections are associated with immune hyporesponsiveness and induction of a regulatory network. *Infection and immunity* **78**, 3160–7 (2010).

175. Van Riet, E., Hartgers, F. C. & Yazdanbakhsh, M. Chronic helminth infections induce immunomodulation: consequences and mechanisms. *Immunobiology* **212**, 475–90 (2007).

176. Elias, D., Britton, S., Aseffa, A., Engers, H. & Akuffo, H. Poor immunogenicity of BCG in helminth infected population is associated with increased in vitro TGF-beta production. *Vaccine* **26**, 3897–902 (2008).

177. Van Riet, E., Hartgers, F. C. & Yazdanbakhsh, M. Chronic helminth infections induce immunomodulation: consequences and mechanisms. *Immunobiology* **212**, 475–90 (2007).

178. Stobo, J. D. & Paul, W. E. Functional heterogeneity of murine lymphoid cells. II. Acquisition of mitogen responsiveness and of theta antigen during the ontogeny of thymocytes and "T" lymphocytes. *Cellular immunology* **4**, 367–80 (1972).

179. Gelfand, M. C., Elfenbein, G. J., Frank, M. M. & Paul, W. E. Ontogeny of B lymphocytes. II. Relative rates of appearance of lymphocytes bearing surface immunoglobulin and complement receptors. *The Journal of experimental medicine* **139**, 1125–41 (1974).

180. Nossal, G. J. & Pike, B. L. Differentiation of B lymphocytes from stem cell precursors. *Advances in experimental medicine and biology* **29**, 11–8 (1973).

181. Scher, I., Berning, A. K., Kessler, S. & Finkelman, F. D. Development of B lymphocytes in the mouse; studies of the frequency and distribution of surface IgM and IgD in normal and immune-defective CBA/N F1 mice. *Journal of immunology (Baltimore, Md. : 1950)* **125**, 1686–93 (1980).

182. Bos, N. A. *et al.* Serum immunoglobulin levels and naturally occurring antibodies against carbohydrate antigens in germ-free BALB/c mice fed chemically defined ultrafiltered diet. *European journal of immunology* **19**, 2335–9 (1989).

183. Simister, N. Placental transport of immunoglobulin G. *Vaccine* **21**, 3365–3369 (2003).

184. Dhohyung Kim, B. Mechanism of Maternal Antibody Inhibition and Vaccination Strategies in the presence of Maternal Antibodies. *PhD Dissertation* (2012).

185. Baumgarth, N., Tung, J. W. & Herzenberg, L. a Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. *Springer seminars in immunopathology* **26**, 347–62 (2005).

186. Pit, D. S. S., Polderman, A. M. & Soboslay, P. T. Prenatal immune priming with helminth infections : parasite-speci ® c cellular reactivity and Th1 and Th2 cytokine responses in neonates. 732–739 (2000).

187. Cooper, P. J. *et al.* Impact of early life exposures to geohelminth infections on the development of vaccine immunity, allergic sensitization, and allergic inflammatory diseases in children living in tropical Ecuador: the ECUAVIDA birth cohort study. *BMC infectious diseases* **11**, 184 (2011).

188. Dauby, N., Goetghebuer, T., Kollmann, T. R., Levy, J. & Marchant, A. Uninfected but not unaffected: chronic maternal infections during pregnancy, fetal immunity, and susceptibility to postnatal infections. *The Lancet infectious diseases* **12**, 330–340 (2012).

189. Medicine, P., Health, P. & Branch, T. M. The Cells of Human Colostrum . I . In Vitro Studies of Morphology and Functions. **109**, 103–109 (1968).

190. Bertotto, A., Castellucci, G., Fabietti, G., Scalise, F. & Vaccaro, R. Lymphocytes bearing the T cell receptor gamma delta in human breast milk. *Archives of disease in childhood* **65**, 1274–5 (1990).

191. Bertotto, A. *et al.* Human breast milk T lymphocytes display the phenotype and functional characteristics of memory T cells. *European journal of immunology* **20**, 1877–80 (1990).

192. Skansén-Saphir, U., Lindfors, A. & Andersson, U. Cytokine production in mononuclear cells of human milk studied at the single-cell level. *Pediatric research* **34**, 213–6 (1993).

193. Jain, L. *et al.* In vivo distribution of human milk leucocytes after ingestion by newborn baboons. *Archives of disease in childhood* **64**, 930–3 (1989).

194. Holt, P. G. & Jones, C. a The development of the immune system during pregnancy and early life. *Allergy* **55**, 688–97 (2000).

195. Malanchère, E., Huetz, F. & Coutinho, A. Maternal IgG stimulates B lineage cell development in the progeny. *European journal of immunology* **27**, 788–93 (1997).

196. Siegrist, C. *et al.* Determinants of infant responses to vaccines in presence of maternal antibodies. **16**, 1409–1414 (1998).

197. Seeger, M. *et al.* Antigen-independent suppression of the IgE immune response to bee venom phospholipase A2 by maternally derived monoclonal IgG antibodies. *European journal of immunology* **28**, 2124–30 (1998).

198. Kolbe, L., Heusser, C. H. & Kölsch, E. Isotype-associated recognition of allergen epitopes and its modulation by antigen dose. *Immunology* **84**, 285–9 (1995).

199. Freitas, a a, Viale, a C., Sundblad, a, Heusser, C. & Coutinho, a Normal serum immunoglobulins participate in the selection of peripheral B-cell repertoires. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 5640–4 (1991).

200. Fink, K. *et al.* Long-term maternal imprinting of the specific B cell repertoire by maternal antibodies. *European journal of immunology* **38**, 90–101 (2008).

201. Graham, S. M. *et al.* Nontyphoidal Salmonella infections of children in tropical Africa. *The Pediatric infectious disease journal* **19**, 1189–96 (2000).

202. Graham, S. M. Salmonellosis in children in developing and developed countries and populations. *Current opinion in infectious diseases* **15**, 507–12 (2002).

203. Brent, A. J. *et al.* Salmonella bacteremia in Kenyan children. *The Pediatric infectious disease journal* **25**, 230–6 (2006).

204. Gordon, M. A. *et al.* Non-typhoidal salmonella bacteraemia among HIV-infected Malawian adults: high mortality and frequent recrudescence. *AIDS (London, England)* **16**, 1633–41 (2002).

205. Maclennan, C. A. *et al.* The neglected role of antibody in protection against bacteremia caused by nontyphoidal strains of Salmonella in African children. **118**, 1553–1562 (2008).

206. Nazli, A. *et al.* Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation. *PLoS pathogens* **6**, e1000852 (2010).

207. MacLennan, C. a *et al.* Dysregulated humoral immunity to nontyphoidal Salmonella in HIV-infected African adults. *Science (New York, N.Y.)* **328**, 508–12 (2010).

208. Gil-Cruz, C. *et al.* The porin OmpD from nontyphoidal Salmonella is a key target for a protective B1b cell antibody response. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 9803–8 (2009).

209. Hotez, P. J. *et al.* Review series Helminth infections : the great neglected tropical diseases. **118**, 1311–1321 (2008).

210. Rafi, W., Ribeiro-Rodrigues, R., Ellner, J. J. & Salgame, P. “Coinfection-helminthes and tuberculosis”. *Current opinion in HIV and AIDS* 1–6 (2012).doi:10.1097/COH.0b013e3283524dc5

211. Du Plessis, N. *et al.* Acute helminth infection enhances early macrophage mediated control of mycobacterial infection. *Mucosal immunology* (2012).doi:10.1038/mi.2012.131

212. Nacher, M. *et al.* Intestinal helminth infections are associated with increased incidence of Plasmodium falciparum malaria in Thailand. *The Journal of parasitology* **88**, 55–8 (2002).

213. Spiegel, A., Tall, A., Raphenon, G., Trape, J. F. & Druilhe, P. Increased frequency of malaria attacks in subjects co-infected by intestinal worms and Plasmodium falciparum malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **97**, 198–9 (2003).

214. Le Hesran, J.-Y. *et al.* Severe malaria attack is associated with high prevalence of Ascaris lumbricoides infection among children in rural Senegal. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **98**, 397–9 (2004).

215. Degarege, A., Legesse, M., Medhin, G., Animut, A. & Erko, B. Malaria and related outcomes in patients with intestinal helminths: a cross-sectional study. *BMC infectious diseases* **12**, 291 (2012).

216. Tetsutani, K. *et al.* Concurrent infection with Heligmosomoides polygyrus suppresses anti-Plasmodium yoelii protection partially by induction of CD4(+)CD25(+)Foxp3(+) Treg in mice. *European journal of immunology* **39**, 2822–30 (2009).

217. Legesse, M., Erko, B. & Balcha, F. Increased parasitaemia and delayed parasite clearance in Schistosoma mansoni and Plasmodium berghei co-infected mice. *Acta tropica* **91**, 161–6 (2004).

218. Helmby, H., Kullberg, M. & Troye-Blomberg, M. Altered immune responses in mice with concomitant Schistosoma mansoni and Plasmodium chabaudi infections. *Infection and immunity* **66**, 5167–74 (1998).

219. Potian, J. A. *et al.* Preexisting helminth infection induces inhibition of innate pulmonary anti-tuberculosis defense by engaging the IL-4 receptor pathway. *The Journal of experimental medicine* **208**, 1863–74 (2011).

220. Lyke, K. E. *et al.* Association of Schistosoma haematobium infection with protection against acute Plasmodium falciparum malaria in Malian children. *The American journal of tropical medicine and hygiene* **73**, 1124–30 (2005).

221. Nacher, M. *et al.* Ascaris lumbricoides infection is associated with protection from cerebral malaria. *Parasite immunology* **22**, 107–13 (2000).

222. Hartgers, F. C. & Yazdanbakhsh, M. Co-infection of helminths and malaria: modulation of the immune responses to malaria. *Parasite immunology* **28**, 497–506 (2006).

223. John, T. J. & Jayabal, P. Oral polio vaccination of children in the tropics. I. The poor seroconversion rates and the absence of viral interference. *American journal of epidemiology* **96**, 263–9 (1972).

224. Patriarca, P. A., Wright, P. F. & John, T. J. Factors affecting the immunogenicity of oral poliovirus vaccine in developing countries: review. *Reviews of infectious diseases* **13**, 926–39

225. Nookala, S., Srinivasan, S., Kaliraj, P., Narayanan, R. B. & Nutman, T. B. Impairment of Tetanus-Specific Cellular and Humoral Responses following Tetanus Vaccination in Human Lymphatic Filariasis Impairment of Tetanus-Specific Cellular and Humoral Responses following Tetanus Vaccination in Human Lymphatic Filariasis. (2004).doi:10.1128/IAI.72.5.2598

226. Sabin, E. a, Araujo, M. I., Carvalho, E. M. & Pearce, E. J. Impairment of tetanus toxoid-specific Th1-like immune responses in humans infected with Schistosoma mansoni. *The Journal of infectious diseases* **173**, 269–72 (1996).

227. Cooper, P. J. *et al.* Albendazole treatment of children with ascariasis enhances the vibriocidal antibody response to the live attenuated oral cholera vaccine CVD 103-HgR. *The Journal of infectious diseases* **182**, 1199–206 (2000).

228. Cooper, P. J. *et al.* Human Infection with Ascaris lumbricoides Is Associated with Suppression of the Interleukin-2 Response to Recombinant Cholera Toxin B Subunit

following Vaccination with the Live Oral Cholera Vaccine CVD 103-HgR Human Infection with Ascaris lumbricoides Is. (2001).doi:10.1128/IAI.69.3.1574

229. Esen, M. *et al.* Reduced antibody responses against Plasmodium falciparum vaccine candidate antigens in the presence of Trichuris trichiura. *Vaccine* 1–4 (2012).doi:10.1016/j.vaccine.2012.10.026

230. Van Riet, E. *et al.* Cellular and humoral responses to influenza in gabonese children living in rural and semi-urban areas. *The Journal of infectious diseases* **196**, 1671–8 (2007).

231. Bobat, S. Characterising the Immune Response to Salmonella and Salmonella Surface Antigens during a Systemic Infection. *PhD Dissertation* (2011).

232. Mastroeni, P. *et al.* Interleukin 18 contributes to host resistance and gamma interferon production in mice infected with virulent Salmonella typhimurium. *Infection and immunity* **67**, 478–83 (1999).

233. Ogilvie, B. M. & Jones, V. E. Parasitological review. Nippostrongylus brasiliensis: a review of immunity and host-parasite relationship in the rat. *Experimental parasitology* **29**, 138–77 (1971).

234. Mcsorley, S. J. & Jenkins, M. K. Antibody Is Required for Protection against Virulent but Not Attenuated Salmonella enterica Serovar Typhimurium Antibody Is Required for Protection against Virulent but Not Attenuated Salmonella enterica Serovar Typhimurium. **68**, (2000).

235. Mastroeni, P., Villarreal-Ramos, B. & Hormaeche, C. E. Adoptive transfer of immunity to oral challenge with virulent salmonellae in innately susceptible BALB/c mice requires both immune serum and T cells. *Infection and immunity* **61**, 3981–4 (1993).

236. Griffin, a J. & McSorley, S. J. Development of protective immunity to Salmonella, a mucosal pathogen with a systemic agenda. *Mucosal immunology* **4**, 371–82 (2011).

237. Mittrücker, H. W., Raupach, B., Köhler, a & Kaufmann, S. H. Cutting edge: role of B lymphocytes in protective immunity against Salmonella typhimurium infection. *Journal of immunology (Baltimore, Md. : 1950)* **164**, 1648–52 (2000).

238. McSorley, S. J. & Jenkins, M. K. Antibody is required for protection against virulent but not attenuated Salmonella enterica serovar typhimurium. *Infection and immunity* **68**, 3344–8 (2000).

239. Smith, K. G., Light, a, Nossal, G. J. & Tarlinton, D. M. The extent of affinity maturation differs between the memory and antibody-forming cell compartments in the primary immune response. *The EMBO journal* **16**, 2996–3006 (1997).

240. Barner, M., Mohrs, M., Brombacher, F. & Kopf, M. Differences between IL-4R alpha-deficient and IL-4-deficient mice reveal a role for IL-13 in the regulation of Th2 responses. *Current biology : CB* **8**, 669–72 (1998).

241. McKenzie, G. J. *et al.* Impaired development of Th2 cells in IL-13-deficient mice. *Immunity* **9**, 423–32 (1998).

242. Eglinton, B. A., Roberton, D. M. & Cummins, A. G. Phenotype of T cells, their soluble receptor levels, and cytokine profile of human breast milk. *Immunology and cell biology* **72**, 306–13 (1994).

243. Verhasselt, V. *et al.* Breast milk-mediated transfer of an antigen induces tolerance and protection from allergic asthma. *Nature medicine* **14**, 170–5 (2008).

244. Hughes, J. M. & Cohen, M. L. *Typhoid Immunization Recommendations of the Advisory Committee on Immunization Practices (ACIP)*. **43**, (Atlanta, 1994).

245. Cunningham, A. F. *et al.* Salmonella induces a switched antibody response without germinal centers that impedes the extracellular spread of infection. *Journal of immunology (Baltimore, Md. : 1950)* **178**, 6200–7 (2007).

246. Infante-Duarte, C. & Kamradt, T. Th1/Th2 balance in infection. *Springer Seminars in Immunopathology* **21**, 317–338 (1999).

247. Watanabe, H., Numata, K., Ito, T., Takagi, K. & Matsukawa, A. Innate Immune Response in Th1- and Th2-Dominant Mouse Strains. *Shock* **22**, 460–466 (2004).

248. Goswami, T. *et al.* Natural-resistance-associated macrophage protein 1 is an H+/bivalent cation antiporter. *The Biochemical journal* **354**, 511–9 (2001).

249. Vidal, S. *et al.* The Ity/Lsh/Bcg locus: natural resistance to infection with intracellular parasites is abrogated by disruption of the Nramp1 gene. *The Journal of experimental medicine* **182**, 655–66 (1995).

250. Simon, R., Tennant, S. M., Galen, J. E. & Levine, M. M. Mouse models to assess the efficacy of non-typhoidal Salmonella vaccines: Revisiting the role of host innate susceptibility and routes of challenge. *Vaccine* **29**, 5094–106 (2011).

251. Xu, H. R., Tan, Y. Y., Hsu, H. S., Moncure, C. W. & Wang, X. M. Comparative antibody response to Salmonella antigens in genetically resistant and susceptible mice. *Clinical and experimental immunology* **91**, 73–7 (1993).

252. Kuroda, E., Kito, T. & Yamashita, U. Reduced expression of STAT4 and IFN-gamma in macrophages from BALB/c mice. *Journal of immunology (Baltimore, Md. : 1950)* **168**, 5477–82 (2002).

253. Gondwe, E. N. *et al.* Importance of antibody and complement for oxidative burst and killing of invasive nontyphoidal Salmonella by blood cells in Africans. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 3070–5 (2010).

254. Roantree, R. J. & Rantz, L. A. A study of the relationship of the normal bactericidal activity of human serum to bacterial infection. *The Journal of clinical investigation* **39**, 72–81 (1960).

255. Siggins, M. K. *et al.* Absent bactericidal activity of mouse serum against invasive African nontyphoidal Salmonella results from impaired complement function but not a lack of antibody. *Journal of immunology (Baltimore, Md. : 1950)* **186**, 2365–71 (2011).

256. Hsu, H. S. Pathogenesis and immunity in murine salmonellosis. *Microbiological reviews* **53**, 390–409 (1989).

257. Butler, J. E., Zhao, Y., Sinkora, M., Wertz, N. & Kacskovics, I. Immunoglobulins, antibody repertoire and B cell development. *Developmental and comparative immunology* **33**, 321–33 (2009).

258. Piccinni, M.-P. T cell tolerance towards the fetal allograft. *Journal of reproductive immunology* **85**, 71–5 (2010).

259. Hotez, P. J. One World Health : Neglected Tropical Diseases in a Flat World. **3**, 3–5 (2009).

260. Gordon, M. a *et al.* Epidemics of invasive Salmonella enterica serovar enteritidis and S. enterica Serovar typhimurium infection associated with multidrug resistance among adults and children in Malawi. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **46**, 963–9 (2008).