

Characterising the Immune Response to Salmonella and Salmonella Surface Antigens during a Systemic Infection

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Abstract

Immunity to *Salmonella enterica* serovar Typhimurium (STm) is complex and requires both cell mediated and humoral immunity at different stages of infection. In infants in sub-Saharan Africa infection with non-typhoidal *Salmonella* (NTS), such as STm, can commonly cause fatal invasive disease. Evidence indicates that disease may be preventable by antibody, which makes vaccine development against these devastating infections a promising option.

This work has explored the cell-mediated and humoral response to STm and its component antigens, their intrinsic properties, and capacity to act as protective immunogens in a mouse model. In particular, responses to surface exposed structures such as the outer membrane proteins (Omps) and the flagellar protein FliC, which are potent, immunodominant antigens and frequent targets of antibody, that may offer potential as vaccine candidates have been examined. Immunisation with soluble flagellin (sFliC) induces a potent Th2 response. Despite this, immunisation with sFliC results in accelerated clearance of STm after the first week of infection in an antibody independent, but T-bet-regulated manner. This suggests that the Th2 responses to flagellin are flexible since they can promote Th1 mediated clearance of STm. This moderate protection conferred by sFliC contrasts with the potent benefit conferred by porins. These proteins induce, and can mediate protection through a T-independent B1b cell population. In particular, antibody to OmpD is key for this protection. These results suggest that vaccines that induce protective antibody to STm may be more effective than vaccines that induce T cell-mediated protection, since they reduce bacterial numbers at the earliest stages of infection. Lastly, experiments using *N. brasiliensis* show that infectious history can impact on the host's ability to control primary STm infection and the efficacy of antibody-mediated protection against infection. These projects further our understanding of the relationship between host and pathogen and the mechanisms used to control infection, but also identify the need to consider the impact of infectious history on the host's capacity to implement protective immunity.

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Author Note

Work discussed in this thesis has led to two publications:

Chapter 3 “Flagellin induces a primary Th2 response yet promotes T-bet regulated Th1 clearance of STm upon challenge”

This is currently in Press in the European Journal of Immunology and the data was compiled by Saeeda Bobat and Dr. Adriana-Flores Langarica who are named as equal contributors.

Chapter 4: “The porin OmpD is a key target for a protective B1b cell antibody response against NTS”

This work was published in 2009 in Proceedings of the National Academy of Sciences and the data was compiled by Dr. Cristina Gil-Cruz, Saeeda Bobat and Dr. Jennifer L Marshall who are named as equal contributors.

Abbreviations

AAM	Alternative Activated Macrophages
B Cell	Bursa of Fabricius Cell
BCR	B Cell Receptor
DC	Dendritic Cell
CAM	Classically Activated Macrophages
CD	Cluster of Differentiation
ELISA	Enzyme-Linked Immunosorbent Assay
EF	Extra follicular
Fig.	Figure
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein Isothiocyanate
GATA-3	GATA-binding protein 3
GC	Germinal Centre
HK-STm	Heat killed <i>Salmonella</i>
IFN γ	Interferon-gamma
IL-X	Interleukin
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
<i>N. brasiliensis</i> and Nb	<i>Nippostrongylus brasiliensis</i>
NES	<i>Nippostrongylus brasiliensis</i> Antigen
NI	Non-Immunised
NF κ B	Nuclear factor of kappa light polypeptide gene enhancer in B cells
NLR	Nod-Like Receptor
NTS	Non-Typhoidal <i>Salmonella</i>
Omps	Outer Membrane Proteins
PAMP	Pathogen associated molecular patterns
PRR	Pattern recognition receptors
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP Cy 5.5	Peridinin Chlorophyll Protein (Cyanin Dye)
Rag	Recombination activating gene

ST	<i>Salmonella enterica</i> serovar Typhi
STm	<i>Salmonella enterica</i> serovar Typhimurium
sFliC	Soluble recombinant Flagellin
STAT	Signal transducer and activator of transcription
T-bet	T-box expressed in T cells
TCR	T Cell Receptor
T Cell	Thymus Cell
TD	Thymus-Dependent
Th	T-Helper Cell
TI	Thymus-Independent
TI-1	Thymus-Independent Type 1 antigen
TI-2	Thymus-Independent Type 2 antigen
TLR	Toll Like Receptor
WT	Wild Type

1.0 Introduction

1.1 The Immune System

The immune system is divided into two categories: innate and adaptive immunity, which are complementary and work in a co-ordinated manner. It can respond to a broad range of antigens throughout the body with responses which are both general and tailored to the antigen encountered. This requires interplay between these two components of the immune system to orchestrate an effective response through cellular contacts and the secretion of soluble mediators, known as cytokines produced by various cell types.

1.2 The Innate Immune Response

Innate immunity is the first line of defence against invading pathogens, and critical for immediate and rapid control. It is the same on the first encounter with a pathogen and upon subsequent encounter. It is efficient at either preventing an infection or greatly reducing the pathogenic load, and many invading pathogens are effectively eliminated by innate effectors (Janeway and Medzhitov, 2002). Innate immunity can be considered as consisting of a number of cell types that recognise foreign antigens through a series of receptors.

1.2.1 Cellular Components of Innate Immunity

1.2.1.1 Neutrophils

Neutrophils are one of the first immune effectors to enter sites of inflammation in response to chemotactic signals, such as IL-8 (Peveri et al., 1988). Although short-lived, they are indispensable for defence against invading pathogens, through secreting chemokines which promote the recruitment and/or activation of other innate immune effectors, including dendritic cells and macrophages (Bennouna et al., 2003; Chertov et al., 1997; Wittamer et al., 2005). Neutrophils kill micro-organisms

upon phagocytosis through exposure to microbicidal agents liberated from granules and secretory vesicles, such as hydrolytic enzymes (cathelicidins, defensins and lysozymes) or antimicrobial peptides (lactoferrin), which destroy bacterial cell wall integrity or antagonise bacterial growth, respectively (Masson et al., 1969). Additionally, they kill many microbes upon internalisation through oxygen consumption in the phagocytic vacuole, in a reaction termed the respiratory burst (Segal, 2005).

1.2.1.2 Dendritic Cells

DC are professional antigen presenting cells which form a bridge between innate and adaptive immunity. As immature cells, they reside in non-lymphoid tissues where they are likely to encounter antigen which they recognise through pattern recognition receptors. Upon pathogen recognition by Toll like receptors, DC undergo maturation and differentiation to become antigen presenting cells, characterised by increased expression of MHC and up-regulation of co-stimulatory molecules such as CD40, CD80 and CD86. Thus, they acquire the capacity to migrate to the T zones of secondary lymphoid tissues, where they present processed antigens to naïve re-circulating T cells in the context of MHC (Banchereau and Steinman, 1998). This enables DC to stimulate primary immune responses by inducing activation and differentiation of naïve T-helper cells (Banchereau and Steinman, 1998; Jankovic et al., 2001; Mizel et al., 2003). Distinct DC populations exist and can be resident in lymphoid or non-lymphoid tissues.

1.2.1.3 Macrophages Populations

Macrophages are tissue-resident mononuclear phagocytes that contribute to the effector phase of the immune response, and have anti-microbial functions as well as other roles. Activation of macrophages during Th1 responses induces development of classically activated macrophages (CAM) (Aderem and Underhill, 1999), which promote phagocytic internalisation and intracellular bacterial killing through induction of cytotoxic reactive nitrogen species, such as iNOS and nitric oxide. In the presence of IL-4 and IL-13, key Th2 cytokines produced during allergy and parasite infections, a distinct population of

non-phagocytic macrophages are induced, known as alternatively activated macrophages (AAM) (Goerdts and Orfanos, 1999; Stein et al., 1992). AAMs play an important role in moderating inflammation and promoting tissue repair (Goerdts and Orfanos, 1999; Loke et al., 2007; Loke et al., 2000). Furthermore, AAMs can enhance the host defence against parasites. Due to these diverse activities proposed for AAMs their functional significance in vivo remains controversial.

1.2.2 Pattern Recognition Receptors

Innate cells recognise conserved signature molecules on the invading pathogens, called **Pathogen Associated Molecular Patterns (PAMP)** through germline encoded **Pattern Recognition Receptors (PRR)** (Janeway and Medzhitov, 2002). PRR enable the host to distinguish between different types of pathogens and initiate a robust and rapid defence, and include **Nucleotide Binding and Oligomerisation Domain (NOD)-Like Receptors (NLR)** and **Toll-Like Receptors (TLR)** amongst others. Induction of innate immunity may involve the activation of multiple PRR and their collaboration (Underhill, 2007).

1.2.2.1 Toll-Like Receptors

Many bacterial PAMP signal through **Toll-Like Receptors (TLR)**, which are a family of at least twelve conserved germline-encoded transmembrane glycoprotein receptors expressed on antigen presenting cells. TLR recognise a broad range of microbe derived ligands in the extracellular milieu or in the lumen of endocytic vesicles (Table 1.1). Each member of the TLR family detects a unique or limited set of microbial PAMP which collectively allows the host to efficiently sense most microbes. TLR are thought to be largely responsible for recognition and activation of innate responses (Mizel et al., 2003; Underhill, 2007).

1.2.2.2 Nod-Like Receptors

Nucleotide Binding and Oligomerisation Domain (NOD)-Like Receptors (NLR) are a family of innate pattern recognition cytoplasmic receptors. They contain a central nucleotide binding and oligomerisation

domain, an N-terminal signal domain and a C-terminal leucine-rich repeat domain involved in recognition. The stimuli activating NLR receptors are not clear, and unlike TLR, NLR recognise pathogen derived molecules that gain access to intracellular compartments, or detect products that are released into the cytoplasm by processes such as phagocytosis (Strober et al., 2006; Underhill, 2007). The NLR-family comprises more than 20 members and includes: ICE protease-activating factor (Ipad) (also known as CARD12, CLAN or NLRC4), which can recognise flagellin when intracellularly localised after phagocytosis (Franchi et al., 2006; Miao et al., 2006).

Table 1.1: The TLR Family of Receptors and their Ligands:

Toll-Like Receptor	Ligand
TLR1	Triacyl lipoproteins (Takeuchi et al., 2002)
TLR2	Lipoproteins and Lipopeptides from various pathogens (Aliprantis et al., 1999) Peptidoglycan and lipoteichoic acid from Gram negative bacteria (Schwandner et al., 1999)
TLR3	Double-stranded RNA (Viruses) (Alexopoulou et al., 2001)
TLR4	Lipopolysaccharide (LPS) (Poltorak et al., 1998)
TLR5	Flagellin (Hayashi et al., 2001)
TLR6	Diacyl lipoproteins (Takeuchi et al., 2001)
TLR7	Small synthetic compounds and single stranded RNA (Heil et al., 2004; Hemmi et al., 2002)
TLR8	Small synthetic compounds and single stranded RNA (Heil et al., 2004; Jurk et al., 2002)
TLR9	Un-methylated bacterial DNA - CpG DNA (Hemmi et al., 2000)
TLR10	Ligand unknown
TLR11	Profilin expressed by protozoan pathogens (Yarovinsky et al., 2005) Uropathogenic bacteria (Zhang et al., 2004)
TLR12	
TLR13	Located on neurons but ligand not determined

1.3 The Adaptive Immune Response

For infections not controlled by innate responses, effective resolution requires the induction of adaptive immunity. Adaptive responses are characterised by their specificity and immunological memory necessary for rapid induction upon antigen re-encounter. Adaptive immunity is central to the concept of vaccination. There are two main cell types which are the effectors of the adaptive response: lymphocytes derived from the thymus (T cells) and bone marrow (B cells). The key difference between innate and adaptive effectors is that each T or B cell is mono-specific. The adaptive response may encompass T cell-induced cell-mediated immunity, B cell-driven humoral-immunity, or a combination of both of these for successful eradication.

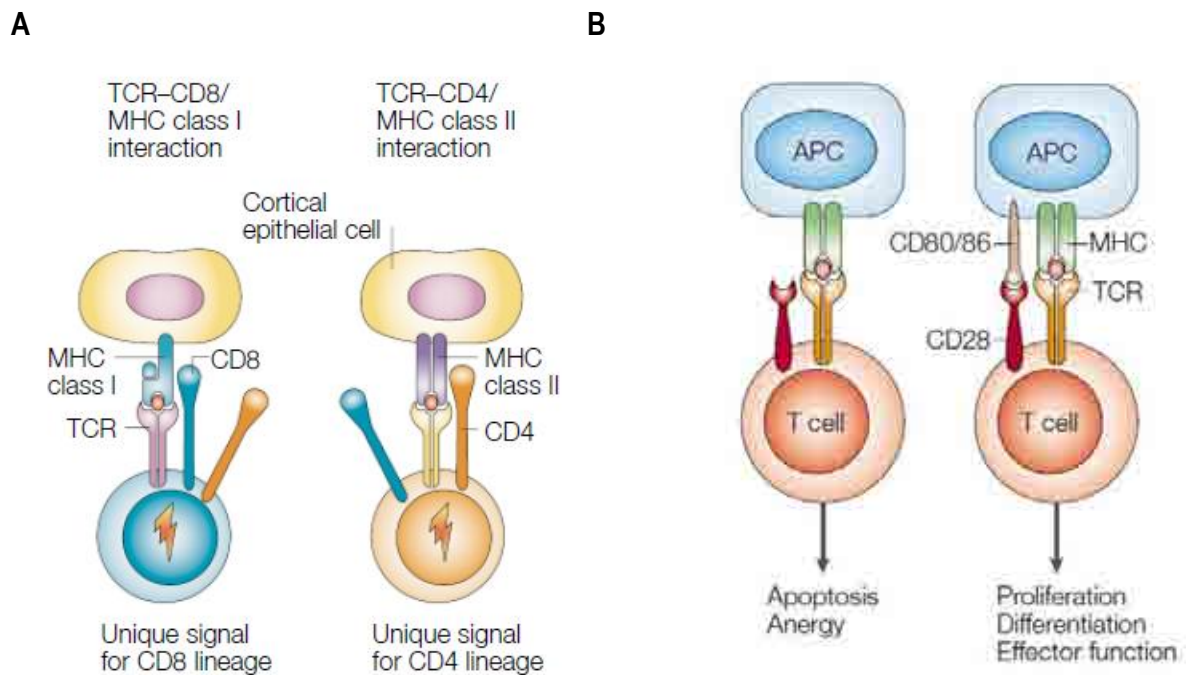
1.3.1 Principles of Cell-Mediated Immunity

Cell-mediated immunity involves T cells, recruited after antigen recognition of peptides presented through MHC (Major Histocompatibility Complex) on the surface of antigen presenting cells by their T cell receptor (TCR) (Fig. 1.1). MHC class I molecules are expressed on most nucleated cells, whilst MHC class II expression is restricted to professional antigen presenting cells (DC, macrophages and B cells). There are many classes of T cells, but the better characterised effector cells include CD8 cytotoxic T-lymphocytes (CTL) and CD4 T-helper lymphocytes which are MHC-class I and MHC-class II restricted, respectively (Germain, 1994). As the role of CD8 T cells is not important for infections with *Salmonella*, they will not be discussed in detail in this thesis.

1.3.1.1 Priming of Naïve T Lymphocytes by APC

Activation of naïve antigen-specific T cells requires at least two signals (Fig. 1.1). The first signal is delivered through the TCR after its interaction with the peptide-MHC complex on the antigen presenting cell. The second signal is generated through engagement of co-stimulatory molecules expressed by antigen presenting cells with receptors expressed by T cells, such as CD28 on T cells with B7-1 (CD80)

or B7-2 (CD86) on antigen presenting cells for CD4 T cells (Shahinian et al., 1993), and 4-1BBL (CD137L) on DC by CD137 on T cells for CD8 T cells (Shuford et al., 1997). After priming, CD4 T cells start to proliferate and acquire effector characteristics. Primed CD4 T cells differentiate down one or more functionally distinct subsets, and acquire the capacity to interact with B cells at this time (Toellner et al., 1998). Amongst other outcomes, primed CD4 T cells can migrate to the tissues to execute their function, enter follicles where they are involved in the Germinal Centre (GC) reaction (Fuller et al., 1993), or develop into central and effector memory cells which reside in secondary lymphoid organs and tissues, respectively (Sallusto et al., 1999).



(Images from Nature Reviews Immunology)

Figure 1.1: T cell activation requires two signals: (A) CD4 and CD8 T cells recognise antigens through their T cell-receptor (TCR) as peptide fragments displayed on antigen-presenting cells by MHC Class II or MHC Class I molecules, respectively (Germain, 2002). (B) Simultaneous recognition between the TCR and peptide-MHC, and engagement of co-receptors on naïve CD4 T cells (CD28) with their ligands on antigen presenting cells (CD80 or CD86) stimulates priming of naïve T CD4 cells, resulting in proliferation and differentiation. In the absence of CD28 co-stimulation, T cells undergo apoptosis or become anergic (Alegre et al., 2001).

1.3.1.2 Lineage Decisions of CD4 T-helper Cells

Primed CD4 T cells were originally shown to differentiate into two functionally distinct subsets, designated T helper 1 (Th1) and (Th2) (Mosmann et al., 1986; Mosmann and Coffman, 1989) (Fig 1.2). Phenotypically, Th1 and Th2 cells can be distinguished by their specific function, through the selective cytokines they secrete, and in mice, the switched antibody isotype they induce in B cells. Furthermore, although less prominent, several other CD4 T cell subsets exist alongside these conventional CD4 T cells, such as Th17 (Harrington et al., 2005; Park et al., 2005) and regulatory T cells (Tregs) (Chen et al., 2003) each of which have distinct functions and cytokine profiles.

1.3.1.2.1 CD4 T-helper 1 Responses

Th1 responses are induced after co-culture of naïve CD4 T cells with Interferon-gamma (IFN γ) (Hsieh et al., 1992) and interleukin-12 (IL-12) (Hsieh et al., 1993; Manetti et al., 1993). IFN γ is the hallmark cytokine associated with Th1 immunity as is the secretion of Tumour Necrosis Factor (TNF α) and Lymphotoxin- α (LT α) (Zhu et al., 2010). IFN γ signals through STAT-1, and together with TCR signalling drives Th1 differentiation by inducing T-bet (T-box expressed in T cells) in naïve T cells (Lighvani et al., 2001). T-bet is a member of the T-box family of transcription factors and is a key Th1 transcription factor (Szabo et al., 2000). Its expression enables remodelling of the gene encoding IFN γ to facilitate its secretion (Kobayashi et al., 1989), and expression of T-bet correlates with expression of IFN γ . In addition to initiating Th1 lineage commitment, T-bet simultaneously represses opposing Th2 programs in early developing, and fully committed Th2 cells (Szabo et al., 2000).

In mice, during cognate interaction between Th1 cells and antigen-specific naïve B-cells, IFN γ secretion promotes T-dependent immunoglobulin class-switching to IgG2a (DeKruyff et al., 1989; Mosmann et al., 1986). Th1 lymphocytes are important for cell-mediated and inflammatory immunity, and delayed type hypersensitivity reactions. Typically, Th1 responses are associated with the resolution of viral, intracellular bacterial and protozoan infections. Indeed, IFN γ is a potent activator of the intrinsic

antimicrobial functions of CAMs and other professional phagocytes, having the inherent ability to induce bactericidal activity of *Salmonella*-infected macrophages through increased phagosome-lysosome fusion, and enhanced secretion of reactive oxygen intermediates (ROI) (Kagaya et al., 1989). Certainly, defects in the IFN γ pathway are characterised by the increased susceptibility to severe infections with poorly pathogenic mycobacteria and non-Typhi *Salmonella* (Lammas et al., 2002).

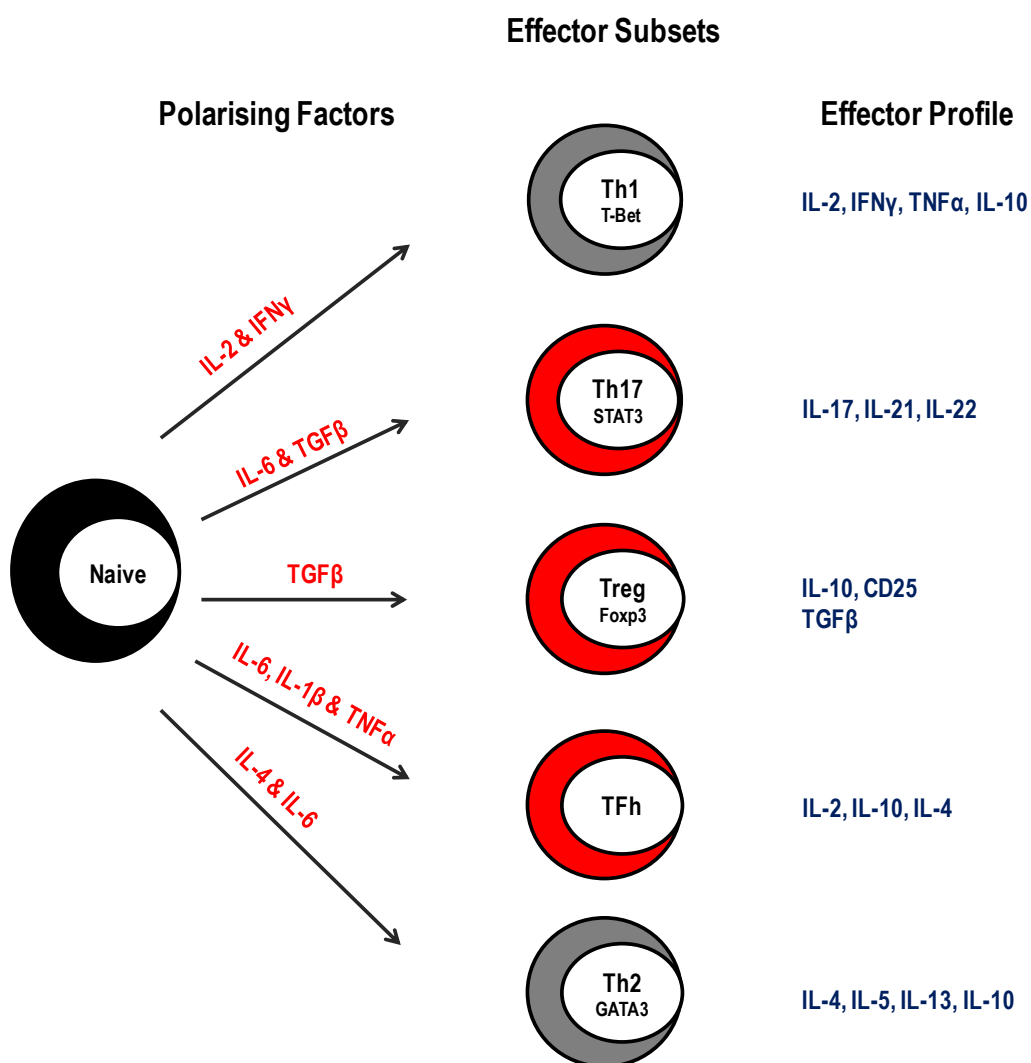


Figure 1.2: CD4 T cell subsets and the factors reported to induce them and their effector profile: During CD4 T cell activation in response to a particular cytokine environment, naive CD4 T cells may differentiate into one of several functionally distinct lineages, including the classically-recognised CD4 T cells, Th1 and Th2. Other effector populations of CD4 T cells, including: Th17 cells, regulatory T cells and follicular (T_{fh}) T cells represent additional CD4 T cell fates. They are defined by their function, expression of specific transcription factors, and pattern of cytokine secretion (Image adapted from (Jelley-Gibbs et al., 2008)).

1.3.1.2.2 CD4 T-helper 2 Responses

Th2 responses are classically associated with humoral immunity, necessary for resolution of extracellular pathogens such as helminths (discussed in detail elsewhere) (Arthur and Mason, 1986; Urban et al., 1991), the induction of allergic reactions (Holgate, 1999), and antibody responses to alum-precipitated proteins. The antibody isotypes associated with Th2 responses are IgE, and in mice IgG1 as well (Mosmann et al., 1986; Vitetta et al., 1985). Th2 responses are also associated with promotion of AAM development, activation of mast-cells, as well as tissue infiltration by both basophils and eosinophils, and goblet cell hyperplasia (Madden et al., 1991). Together, these processes mediate physiological changes such as airway hyper-responsiveness, airway mucus hypersecretion and inflammation (Kuperman et al., 1998), important in allergic type responses such as asthma.

The signature cytokines of Th2 cells are IL-4 and IL-13 (Hsieh et al., 1992). IL-4 is largely produced by Th2 lymphocytes but basophils, mast cells, NK T cells (Yoshimoto and Paul, 1994), eosinophils (Moqbel et al., 1995) and $\gamma\delta$ T cells (Abbas et al., 1996), have also been demonstrated to produce IL-4. IL-13 can be produced by the same cells which secrete IL-4 (Hershey, 2003) and their biological activities have been found to overlap. The Th2 defining transcription factors are: signal transducer and activator of transcription 6 (STAT-6) (Andrews et al., 2002) and GATA-binding protein 3 (GATA-3), which promote secretion of the Th2 selective cytokines: IL-4, IL-5, IL-9 and IL-13 (Banchereau and Steinman, 1998; Lanzavecchia and Sallusto, 2000; Le Gros et al., 1990; Paul and Seder, 1994). Other cytokines have also been associated with Th2 polarisation, particularly IL-6 (Diehl and Rincon, 2002) and IL-10 (Mosmann, 1994). GATA-3 is important for the development and maintenance of Th2 responses (Pai et al., 2004; Zhu et al., 2004) through inducing Th2 activity and terminating the opposing Th1 genetic programs (Ouyang et al., 2000).

Although IL-4 and IL-13 are important for directing the development of Th2 characteristics in vitro (Kuhn et al., 1991; Mohrs et al., 2000), their importance is dispensable in vivo, as Th2 development can occur

independently of both these cytokines (Brewer et al., 1996; Kopf et al., 1993). Indeed, the induction of IL-4 and IgG1 switching is maintained to wild-type levels in the absence of IL-4 signalling, or upon the dual loss of IL-4 and IL-13 signalling through the IL-4 receptor alpha pathway (Cunningham et al., 2002; Kuhn et al., 1991) (Fig 1.3). These studies suggest *in vivo* CD4 T cell IL-4R α independent pathway(s) for IL-4 and IL-13 production exist (Cunningham et al., 2002; Mohrs et al., 2000; Noben-Trauth et al., 1997). Despite Th2 priming being IL-4 independent *in vivo*, IL-4 production is necessary for confirming the induced Th2 state, and supporting its subsequent development through selectively expanding Th2 clones (Cunningham et al., 2002).

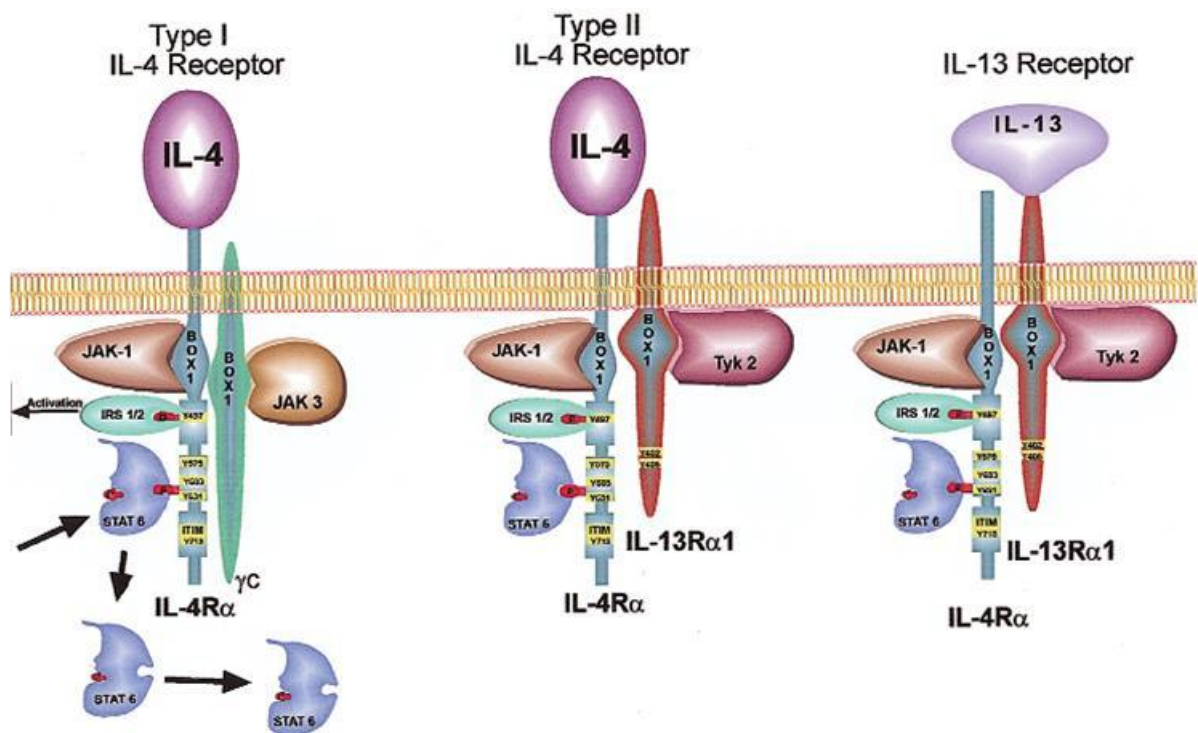


Figure 1.3: IL-4 and IL-13 receptor signalling complexes: Th2 immune effector responses are characterised by IL-4 and IL-13 dependent signalling through heterodimeric transmembrane receptors containing an IL-4 receptor alpha (IL-4R α) subunit (Mohrs et al., 1999). IL-4 signals through both the type 1 and type 2 receptor complexes, whilst IL-13 signals via the type 2 complex. Both type 1 and 2 receptors share a common IL-4R α chain which dimerises with either the γ C chain to form a type 1 receptor or the IL-13R α 1 to form a type 2 receptor. Ligand binding of these complexes results in initiation of the signal cascade and activation of STAT-6 transcription factor which upregulates transcription of Th2 associated genes. IL-13R α 2 (not shown) does not dimerise with IL-4R α , is only bound by IL-13 and does not initiate activation of STAT-6 (Image taken from ((Hershey, 2003))).

1.3.1.2.3 Other CD4 T cell subsets

A major function of CD4 T cells is to help B cells produce antibody in response to T-dependent antigens. CD4 T cells that specifically enter GC to mediate their helper function for antibody production are designated T follicular helper (Tfh) cells. They reside in GC (Reinhardt et al., 2009) and produce IL-4 and IFN γ , but no other signature cytokines (Zaretsky et al., 2009). Although not fully characterised, they can express Programmed-Death Receptor-1 (PD1) and B cell lymphoma 6 protein (BCL-6) (Yu et al., 2009).

CD4 regulatory T cells (Tregs) are both promoted by and differentiated into producers of Transforming growth factor- β (TGF- β 1), utilising the transcriptional regulator Forkhead box p3 (Foxp3) for differentiation (Chen et al., 2003). They play an important role in regulating and preventing over-activity of the immune system, and their deficiency results in a lethal multi-organ autoimmune syndrome, and hyper-reactivity to commensal microbes (Groux and Powrie, 1999; Powrie and Mason, 1990). T cells can also differentiate to become Th17 cells after IL-6 and TGF- β 1, and this requires the transcriptional factor STAT-3. Their signature cytokines are IL-17, IL-22 and IL-23 (Harrington et al., 2005; Park et al., 2005). They are found in the gut, in particular, the lamina propria where specific commensal bacteria can promote their differentiation (Ivanov et al., 2008; Ivanov et al., 2006). Th17 cells are potent effectors of inflammation, thus key mediators of autoimmune diseases. Moreover, they also play critical roles in protecting the host from pathogenic yeast, fungi and extracellular bacteria, particularly at mucosal surfaces (Aujla et al., 2008; Bettelli et al., 2006).

1.3.2 B Cell-Mediated Immunity

B cells can differentiate into plasma cells to secrete antibody or into memory B cells. Antibody is important against extracellular pathogens. Unlike T cells, antigen recognition by B cells does not require antigen processing and presentation through MHC. B cells recognise and interact with soluble antigens

in their native form through their B Cell Receptor (BCR). The principal function of B cells is to synthesise and secrete antibodies which contribute to immunity in multiple ways. Firstly, antibody can neutralise the effect of a pathogen or toxin through binding to its surface and thereby blocking its interaction or effect with its target. Indeed, immunisation with modified bacterial toxins, called toxoids, induce protective neutralising antibody against the potential pathogenicity of Diphtheria and Tetanus toxins (Robbins and Robbins, 1986). Alternatively, antibodies can opsonise pathogens to facilitate their destruction by phagocytic cells or thirdly, antibodies can activate complement to enable lysis of certain pathogens. A further mechanism, Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC), which is probably less pronounced in mice, works by targeting and destroying antibody-coated cells by NK T cells (Takai, 1996). This latter mechanism is important for the action of therapeutic monoclonal antibodies utilised in anti-cancer therapy (Clynes et al., 2000).

1.3.2.1 B Cell Subsets and their Antigenic Targets

The nature of the antigen influences antibody production by B cells. Nearly all protein antigens assessed require cognate interaction between T and B cells in order to initiate antibody responses, and are called Thymus-dependent (TD) antigens. However, protective antibody responses to some bacterial antigens, such as capsular polysaccharides can arise in the absence of T cells and CD40 ligation (Fikrig et al., 1996; McKisic and Barthold, 2000), and are termed Thymus-independent (TI) antigens. 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 (Fagarasan and Honjo, 2000; Noelle and Snow, 1991).

Mature B cells can be divided into distinct subsets which respond to TD and TI antigens and are designated: Follicular (FO), Marginal Zone (MZ), B1a and B1b B cells (Fig 1.4). Each subset has a distinct function in the immune system and can be further distinguished by their anatomical location, and by the surface markers they express.

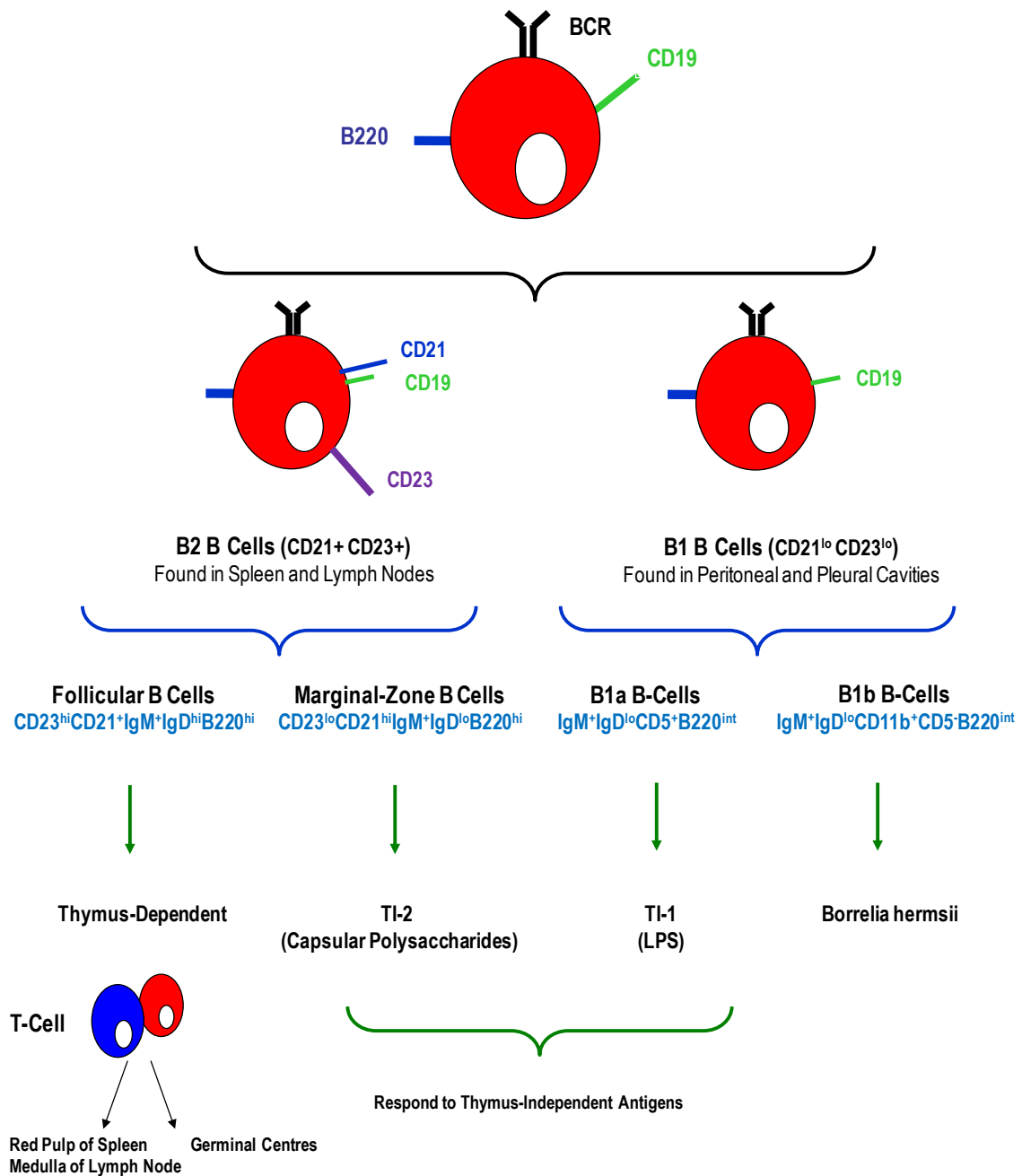


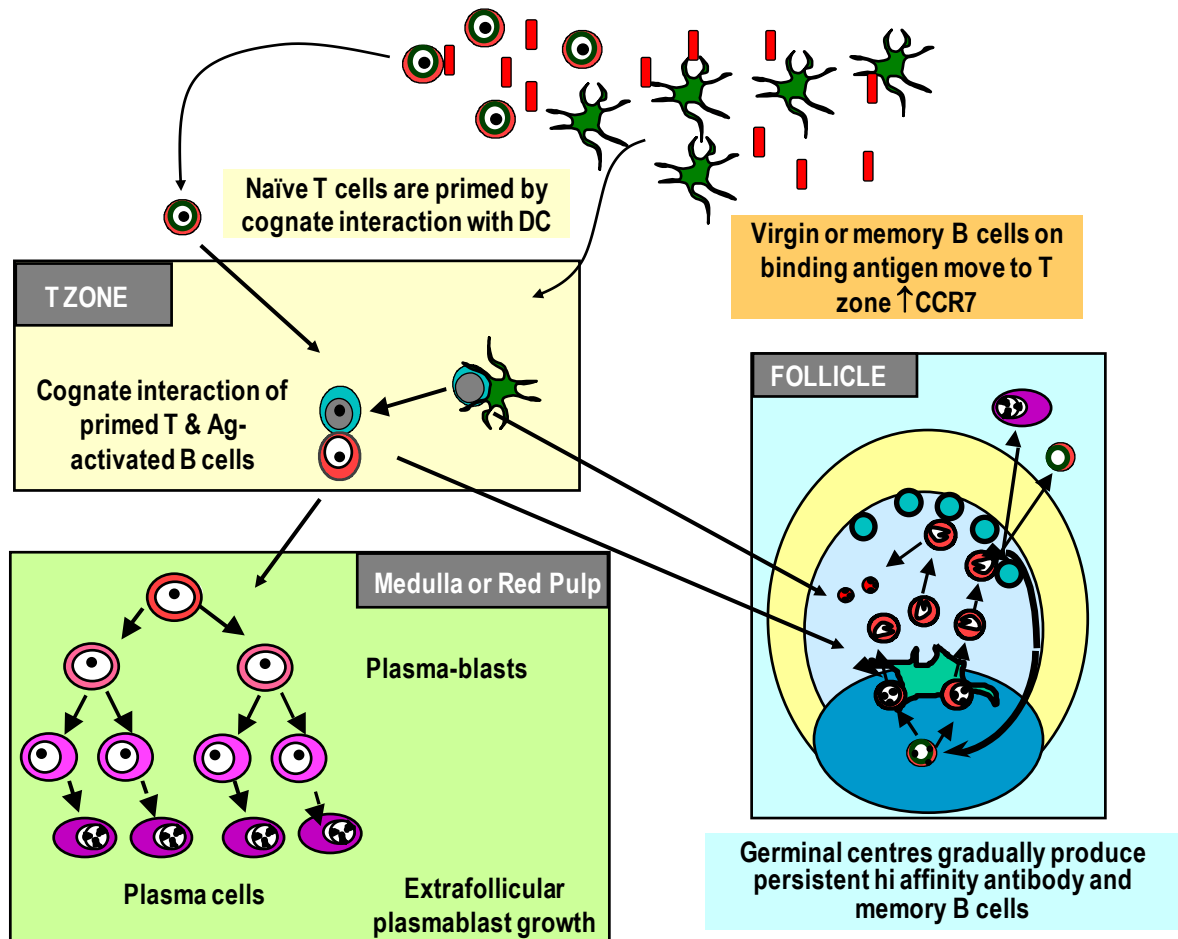
Figure 1.4: Distinct B cell populations respond to TD and TI antigens: B2 cells represent the greatest proportion of the adult B cell population and are subdivided into Follicular (FO) and Marginal Zone (MZ) B cells, which secrete antibodies towards TD and TI-2 antigens, respectively. Although, FO B cells may respond to TI-1 antigens if they express the appropriate accessory receptor. A smaller subset of B cells, B1 B cells are distinguished from B2 cells by their anatomical location (Herzenberg and Tung, 2006; Kantor et al., 1992). They were originally defined by CD5 surface expression and high IgM expression compared to FO B cells (Hayakawa et al., 1983). B1 B cells can be subdivided into B1a and B1b cells which are CD5-positive and CD5-negative, respectively. B1a cells respond to TI-1 antigens, but the role of B1b cells is not clear, although they are important in the TI response to relapsing fever caused by *Borrelia hermsii* (Alugupalli et al., 2004). B cells do not respond in isolation as pathogens present various antigens which has the capacity to invoke a range of responses.

1.3.2.1.1 T-Dependent (TD) Antibody Responses

Naïve FO B2 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 (MacLennan et al., 2003). This process ceases if B cells are recruited into the antibody response. Activation of FO B cells requires two signals, the first signal is provided by antigen recognition through the BCR during which the antigen is internalised, processed and presented to naïve antigen specific CD4 T cells through MHC II, where it can be recognised by T cells (Renshaw et al., 1994). The second co-stimulatory signal is provided through interaction between CD154 (CD40L) on T cells and CD40 on B cells which is central in the control of both humoral and cellular immunity (Grewal et al., 1995). Unlike TD antigens, TI antigens can directly induce B cell proliferation (Cambier et al., 1982) as the second signal can be provided by the antigen itself.

The T cells involved in TD antibody responses have previously been primed by DC in the T-zone (Fig 1.5). As a result of this priming, T cells acquire the capacity to interact with B cells, whereupon they promote proliferation of B cells and their induction of switched germ-line transcripts, influenced by type-specific cytokines produced by T cells. Activated FO B2 cells can either proliferate within follicles where they form GC, or differentiate into proliferating antibody secreting cells called plasmablasts, and then into non-proliferating antibody secreting cells called plasma cells in the extra follicular (EF) response (MacLennan et al., 2003). GC induction is required for memory and high affinity antibody production. This is achieved through affinity maturation by somatic hypermutation (SHM) of the immunoglobulin variable region genes, and changes in immunoglobulin isotype by class switch recombination, giving rise to different subclasses of B cells. The resulting B cells are subjected to careful screening for their reactivity to self-antigens and self-reactive B cells are eliminated by apoptosis. A proportion of positively-selected GC B cells differentiate into plasma cells that can be long-lived and some become memory B cells. Long-lived plasma cells migrate into specialised compartments in the bone marrow or splenic red pulp, where contact with the surrounding tissue may be instrumental for their survival (Manz

et al., 1997; Slifka et al., 1998). Nevertheless, in TD responses the majority of EF plasma cells are short lived.



(Image adapted from Ian MacLennan)

Figure 1.5: Development of TD Antibody Responses: DC primed by antigen in the periphery migrate to the T zones of secondary lymphoid tissues where they prime naïve CD4 T cells. Primed T cells acquire the capacity to interact with B cells. Activated FO B2 cells can either proliferate within follicles where they form GC, or as plasmablasts, before differentiating into plasma cells in the extra follicular (EF) response (MacLennan et al., 2003). EF plasma cells reside in the medullary cords of lymph nodes or the red pulp of the spleen (Jacob et al., 1991). EF plasma cells secrete antibodies that show no evidence of affinity maturation, and are of modest affinity. Despite this, the EF response provides a rapid first line of defence against invading pathogens (Cunningham et al., 2007). Induction of TD EF responses requires T cells and CD154, but once established, continue in the absence of further T cell help (Cunningham et al., 2004b; Toellner et al., 1996). Follicular DC and T cells are critical for GC B cell responses. They provide crucial interactions necessary for screening the resulting immunoglobulin to eliminate low-affinity or self-reactive B cells (Toellner et al., 2002). Without this selection, GC collapse by mass apoptosis (Vinuesa et al., 2000). Upon selection, FO B cells exit GC and differentiate into plasma cells which secrete high-affinity antibodies, or they differentiate into memory B cells.

1.3.2.1.2 T-Independent (TI) Antibody Responses

B1 and MZ B cells respond to antigen in the absence of T cell help (Fig. 1.4). Such responses are characterised by the absence of GC (MacLennan et al., 2003; Toellner et al., 1998) and the production of low-affinity germ-line encoded antibodies. Nevertheless, TI-antibody responses offer the capacity to respond to specific foreign antigens rapidly, thereby providing immediate protection through secretion of antibodies with a broad-specificity against ubiquitous micro-organisms, before the establishment of adaptive immunity. Despite their importance in infection, little is known about how they are recruited for antigenic clearance, and the mechanisms by which they are activated and regulated.

B1 B cells can develop during the foetal and neonatal period (Hayakawa et al., 1983) and are the major population of B cells in infancy. They are notable for their capacity to proliferate and self-renew, a property that FO and MZ B cells do not have. They are found in the peritoneal and pleural cavities (Hayakawa et al., 1983) but can migrate to other tissues, including the mesenteric lymph nodes (MLN), spleen, and gut associated lymphoid tissue where they can differentiate into plasma cells (Bos et al., 1996). B1a B cells constitutively produce natural antibody (Baumgarth et al., 1999) and respond to TI-1 antigens which trigger innate signalling receptors on B cells. The most obvious example is LPS (Fagarasan and Honjo, 2000). The role of B1b cells in immunity remains to be fully elucidated, but they can protect against infection with the relapsing fever bacterium *Borrelia hermsii* (Alugupalli et al., 2004). Furthermore, transfer of purified B1b cells from *B. hermsii* immune mice into Rag-deficient mice can provide long-lasting immunity through the rapid induction of IgM, suggesting they may invoke a memory response (Alugupalli et al., 2004).

MZ B cells are non-recirculating B2 cells that localise to the marginal sinus, at the border of the splenic white and red pulp. There is evidence that they may arise from the differentiation of re-circulating FO B cells (Kumararatne and MacLennan, 1981; Vinuesa et al., 2003). MZ B cells can respond to TI-II antigens, which typically, but not exclusively, are antigens that contain polysaccharide chains

comprising repetitive sequences, such as the capsular polysaccharides of pneumococci. These antigens can extensively crosslink many BCRs on the same B cell to induce a proliferative antibody response (Guinamard et al., 2000). Antibody responses to certain capsular bacteria, such as *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* are not generated in the early years of life, and vaccines against these pathogens are ineffective in infants therefore, MZ B cells are crucial for immunity against this important group of pathogens.

1.4 Salmonella enterica serovar Typhimurium

1.4.1 The species *Salmonella enterica*

Salmonella are non-spore forming, facultative intracellular, Gram-negative (GN) bacilli. Classification of *Salmonella* was initially based on clinical considerations, for example *S. choleraesuis* (swine cholera). However, nomenclature is now based on DNA sequences which has left only two species: *S. enterica* and *S. bongori*. These species are further divided into subspecies and serovars, which are defined by antigenic structure and biotype in accordance with the Kauffman-White classification scheme.

S. enterica is clinically important and contains over 2000 serovars, including *Salmonella enterica* serovar Typhi (ST) and *Salmonella enterica* serovar Typhimurium (STm). Genetically, almost 98% of known DNA sequences between ST and STm are identical at the sequence level, highlighting the homology between the two serovars (Crosa et al., 1973; Zahrt and Maloy, 1997). However, 20% of their genes are different and unique to each species (Lan and Reeves, 1996; McClelland et al., 2001; Parkhill et al., 2001) which may contribute to their respective host specificity and infectivity (discussed in 1.4.2). The most striking structural difference between them is the absence of a capsular polysaccharide in STm, known as Vi antigen in ST and paratyphi serovars.

1.4.2 Disease Burden of *Salmonella* Infection

Salmonella are important human and animal pathogens. In particular, *Salmonella enterica* serovar Typhi (ST) and *Salmonella enterica* serovar Paratyphi A, B and C which are human specific, cause typhoid fever and paratyphoid fever, respectively. Typhoid is a serious systemic infection which can be fatal if left untreated. According to WHO figures, typhoid fever caused 21, 650 974 illnesses and 216, 510 fatalities during 2000 (Crump et al., 2004). Typhoid is spread by the faecal-oral route by ingestion of food or water contaminated by excreta from patients or chronic carriers (Parry, 2004). It is closely associated with poor hygiene, lack of clean drinking water and inadequate sanitation. Typhoid is commonly characterised by abdominal pain, the sudden onset of sustained fever, severe headache and nausea. Furthermore, an asymptomatic carrier state may follow acute infection. Paratyphoid fever is clinically similar to typhoid fever but milder, and with a lower fatality rate.

Non-Typhoidal *Salmonella* (NTS) serovars include, *Salmonella enterica* serovar Typhimurium (STm) and *Salmonella enterica* serovar Enteritidis. The serovar STm includes numerous pathovars some of which can infect different livestock hosts and humans, whereas others exhibit a restricted host range (Rabsch et al., 2002). In developed countries, in humans, NTS infection caused by STm is normally associated with a self-limiting gastroenteritis with symptoms resolving by a week (Zhang et al., 2003). Systemic disease with STm is rare in most parts of the world, although disease can be more severe in the young, elderly or immunocompromised. However, in sub-Saharan Africa (SSA) there has been a dramatic increase in NTS-associated invasive disease, frequently associated with STm isolates (Gilks et al., 1990; Graham et al., 2000; Vugia et al., 1993). Here, NTS infections show a dramatically more severe presentation and often in the complete absence of gastro-intestinal symptoms. These invasive NTS infections are a common cause of bacteraemia in infants between 6-24 months (MacLennan et al., 2008). Furthermore, NTS infections are a major problem in people of all ages with HIV/AIDs, where it is often fatal, and mortality is largely associated with bacteraemia. This suggests that invasive NTS

infections in SSA may differ to classical NTS infections seen in the West where bacteraemia is a rare complication (Gordon, 2008; Hohmann, 2001).

Interestingly, multilocus sequence analysis of invasive NTS strains in SSA recognised an isolate of STm which is rarely reported outside of Africa. Whole genome sequencing of this isolate identified a virulence associated plasmid encoding multiple drug resistance genes. Furthermore, sequencing found evidence of genome degradation, including the formation of pseudogenes and chromosomal deletions when compared to other STm genome sequences (Kingsley et al., 2009). This suggests that host-restriction and change of niche (gut to systemic disease) is linked to the accumulation of large numbers of pseudogenes and reduction in genome size. Therefore, STm isolates that cause invasive disease are phylogenetically distinct from STm isolates that cause gastroenteritis.

1.4.3 Entry and Colonisation in the host

Infections with ST are well characterised and our understanding of disease progression in *Salmonella* is based on ST infection, where ingestion and transit through the gastrointestinal (GI) tract forms the natural means of infection (Fig 1.6). After ingestion of contaminated food or water, the initial stages of infection involve bacterial survival within the acidic environment of the stomach, surviving bacteria spread rapidly along the GI tract. Ingested *Salmonella* must cross the intestinal epithelial barrier to initiate systemic infection. This is achieved by transit across the mucosal barrier, primarily through Membranous epithelial (M) cells (Jepson and Clark, 2001; Jones et al., 1994) in the specialised follicle-associated epithelium (FAE) overlying organised mucosa-associated lymphoid tissues, such as the Peyer's patches (PP). *Salmonella* can utilise M cell-independent routes of extraintestinal dissemination, including phagocytes and epithelial cells (Vazquez-Torres et al., 1999).

Regardless of the route of entry, following penetration bacteria colonise the lamina propria and PP where *Salmonella* rapidly invade host cells within these sites. If local host defences fail to kill the

bacteria then bacteria can spread systemically. Initially, bacteria are translocated to the MLN through the lymphatics where they multiply, from here they can escape and enter the blood through the thoracic duct, leading to a transient bacteraemia. This primary bacteraemia is cleared by the reticuloendothelial system (RES), and intracellular replication in cells of the RES results in a secondary bacteraemia to the spleen and non-lymphoid organs, such as the liver, where bacteria replicate in macrophages (Richter-Dahlfors et al., 1997; Salcedo et al., 2001). Colonisation of the liver results in spread to the biliary tract promoting colonisation of the gall bladder, and large numbers of bacteria in the small intestine, where necrosis and ulceration may occur. The key feature with typhoid and NTS is that severity requires bacterial dissemination, and colonisation of sites, remote from where infection occurred.

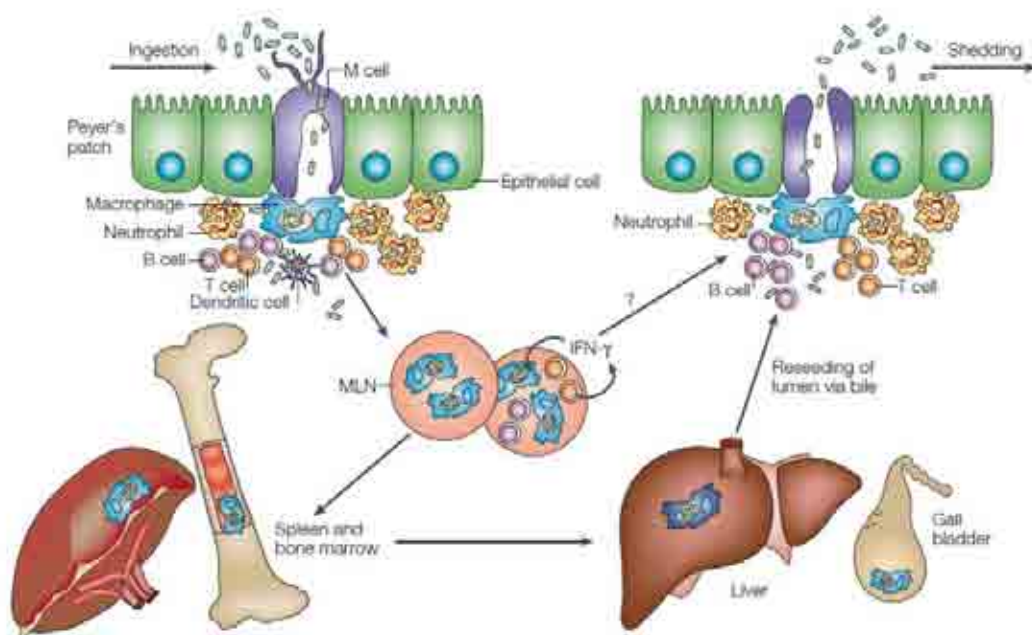


Figure 1.6 Model of how ST infection is established: Ingested bacteria translocate across the mucosal barrier through M cells to induce a systemic infection. The apical membranes of M cells are relatively accessible to luminal bacteria, and their increased pinocytic activity is exploited by *Salmonella* (Jepson and Clark, 2001). Successful translocation enables bacteria to enter Peyer's patches where bacteria can specifically target host cells, such as DCs and macrophages which enables dissemination through the lymphatics and circulation. Bacteria can persist in the MLN, bone marrow and gall bladder for life. (Image taken from (Monack et al., 2004)).

1.4.4 Structural Features STm

The hallmark of GN bacteria is the composition of their cell envelope comprising three morphologically distinct layers (Fig 1.7). It is characterised by a double membrane (the cytoplasmic and the outer membrane (OM)) separated by a highly viscous compartment known as the periplasm. The OM has an array of different components involved in numerous bacterial processes that are also important potential immunological targets.

1.4.4.1 Lipopolysaccharide (LPS)

LPS consists of three components: Lipid A, the core and the O-antigen (Fig 1.7). Lipid A is a glucosamine disaccharide coated with multiple fatty acids which anchor LPS to the OM of the cell wall. The structure of lipid A is highly conserved among GN bacteria and it is largely responsible for much of the toxicity associated with LPS, including endotoxic shock (Raetz and Whitfield, 2002). The core is a surface-exposed negatively-charged oligosaccharide. The O-antigen or O-polysaccharide extends from the core and comprises the outermost domain of LPS. It is composed of heteropolysaccharide side chains of repeating oligosaccharide units. The composition of the sugars in the O-chain varies markedly between species and even different GN bacteria. Indeed, antigenic variation of the O-antigen enables the existence of multiple serotypes of a given bacterium and forms the basis of serotyping by antibody. Furthermore, variation in the repeating sugar units associated with the O-chain determines whether LPS is considered rough or smooth. Loss of the O specific region by mutation results in the strain becoming a "rough" strain (due to the appearance of the colony on agar) and such bacteria are designated LPS rough (Lerouge and Vanderleyden, 2002).

1.4.4.2 Outer Membrane Proteins

The GN cell envelope contains numerous highly organised β -barrel proteins called Outer Membrane Proteins (OMPs). They span the membrane with an even number of amphipathic β -strands with

antiparallel topology, connected by alternating short periplasmic turns and longer extracellular loops that form a closed barrel-like structure (Ruiz et al., 2006) (Fig 1.7).

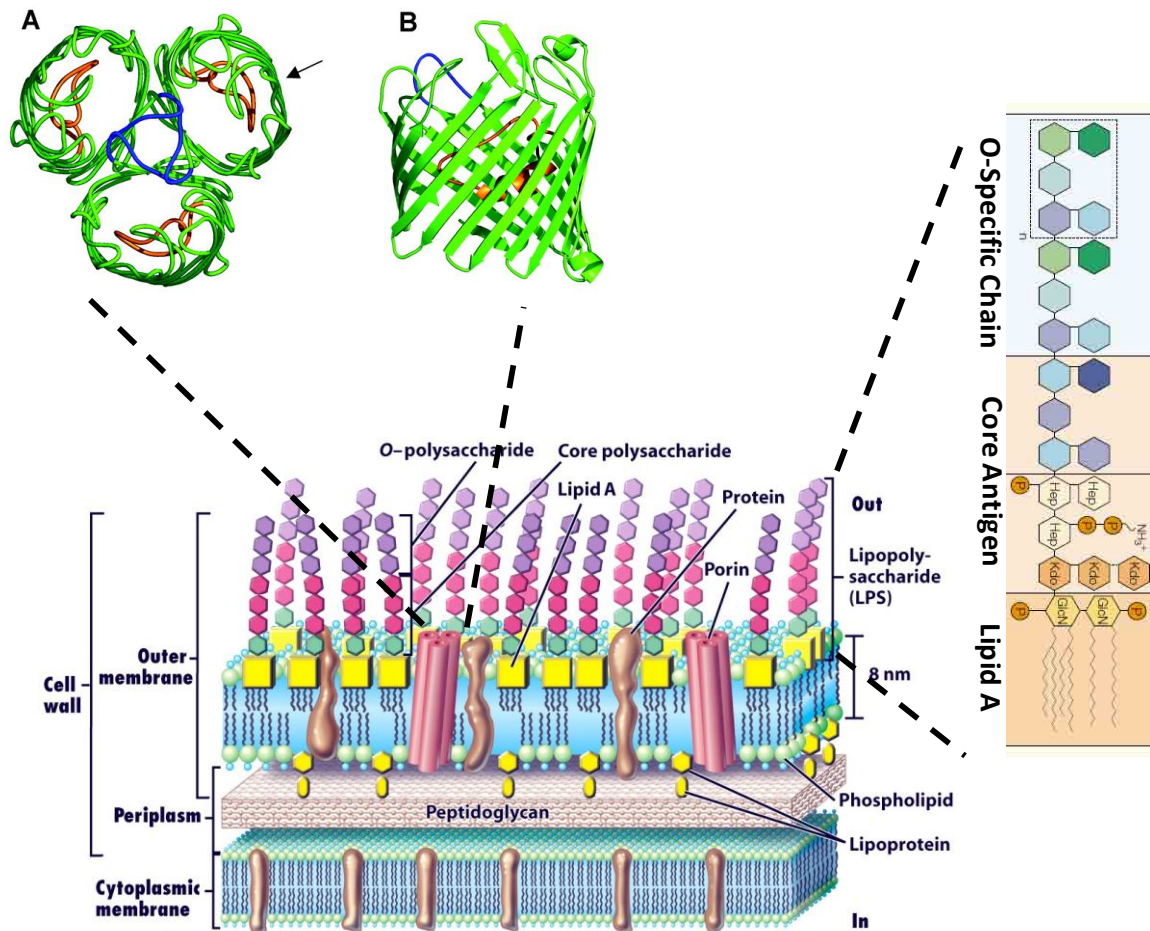


Figure 4-35a Brock Biology of Microorganisms 11/e
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Figure 1.7 Composition of the Gram-negative cell wall: The inner membrane (IM) is a symmetrical phospholipid bilayer. The outer membrane (OM) is a highly asymmetrical bilayer comprising phospholipids and lipopolysaccharides (LPS) in its inner and outer leaflets, respectively. The membranes are separated by an aqueous compartment known as the periplasm, which contains soluble proteins and a thin peptidoglycan layer (Ruiz et al., 2006). The peptidoglycan layer functions as an extracytoplasmic cytoskeleton which maintains cellular architecture (Bos and Tommassen, 2004). The cell wall is a dynamic structure containing several components necessary for bacterial function, including Outer membrane proteins (OMPs) and LPS. OMPs are cylindrical β -barrel proteins which largely function in the exchange of molecules across the cell wall (OMP's image taken from: (Nikaido, 2003)). LPS is composed of three regions: The Lipid A portion extends from the OM and is attached to the core antigen which is attached to the O-antigen. The O-antigen is a repeating unit (n) of specific sugar residues. LPS forms strong lateral interactions between neighbouring molecules which enables LPS to create a compact and almost gel-like coat (LPS image taken from: (Beutler and Rietschel, 2003)).

A subset of these proteins, called porins, span the membrane as protein trimers forming water-filled channels which facilitate the transport of small hydrophilic molecules (Nikaido and Vaara, 1985). Porins have numerous surface-exposed epitopes and monoclonal antibodies raised against them have been used to characterise porins between different species (Singh et al., 1992). Porins are important antigenic determinants, and their importance in immunity to STm is discussed later.

The OmpC, OmpD, and OmpF genes encode the three major porins of STm, which are 36 kDa, 34 kDa, and 35 kDa respectively. Expression of OmpC and OmpF expression is regulated by growth medium osmolarity and other factors. Under conditions of high osmolarity, OmpC expression is elevated and OmpF is repressed. Under conditions of low osmolarity, the reverse occurs (Hall and Silhavy, 1981; Jovanovich et al., 1988; Norioka et al., 1986). OmpR encodes a positive regulator which controls OmpC and OmpF expression, but control of OmpD expression is OmpR independent and less well understood (Head et al., 1998; Mizuno and Mizushima, 1990), but may be regulated by multiple environmental factors. Indeed, OmpD expression decreases in low pH and increases in anaerobic conditions. Changes in response to anaerobic conditions appears to be controlled post-transcriptionally by the global transcriptional regulator, Fnr. However, Fnr is not responsible for changes in OmpD expression in response to pH alterations (Santiviago et al., 2003). All *Salmonella* serovars, except ST express OmpD, and it is highly expressed in the OM (Santiviago et al., 2001). The OmpD primary amino acid sequence is more than 99% conserved between serovars.

1.4.4.3 Flagellin

STm is motile by means of peritrichous flagella which extend from the GN bacterial cell wall (Yoshikawa et al., 1980). Flagella filaments are composed of monomers of flagellin, which are exported through the type III secretion system (T3SS) to the cell wall where they polymerise to form long protofilaments. Each flagellum is composed of 11 flagella protofilaments, each with 30, 000 or more flagellin monomers (Yonekura et al., 2002, 2003) (Fig 1.8A).

STm expresses two antigenically distinct flagellin genes which are translated into FliC (Phase 1) and FliB (Phase 2) proteins. Only one of the two flagellin types is produced at any given time and a bacterium never naturally expresses both gene products simultaneously (Wyant et al., 1999), through a complex system of phase variation achieved by gene inversion (Fig 1.8B). No clear biological function for phase variation has been described, and the literature remains conflicting. Both proteins can signal through TLR5 similarly (Simon and Samuel, 2007), and can adhere and invade mouse epithelial cells to the same capacity (Ikeda et al., 2001). However, in vivo FliC-locked strains replicate faster and after oral infection of mice with WT STm, most invasive bacteria express FliC (Ikeda et al., 2001).

The predicted amino acid sequences of the two flagellins are very similar. The *fliC* and *fliB* genes encode 495 and 506 amino acid sequences respectively which are highly conserved at the N and C-terminus between different *Salmonella* species. Each flagellin monomer is composed of four globular domains: D0, D1, D2 and D3. The D0 and D1 domains of flagellin are formed from these conserved regions which are important for TLR5 recognition (Namba et al., 1989; Smith et al., 2003a) and flank a central more divergent region (D2 and D3) (Fig 1.8C). The D2 and D3 domains are dispensable for flagella function (Eaves-Pyles et al., 2001). They are surface exposed and confer antigenic differences to the flagella which forms part of the basis of STm serotyping (Brown and Hormaeche, 1989). Flagellin is an important target of the immune response to STm. Indeed, it is recognised by the innate receptors: TLR5 and Ipaf (Amer et al., 2006; Franchi et al., 2006; Hayashi et al., 2001). In addition, the adaptive immune system generates antibodies to the variable domains of the monomer (Frankel et al., 1989) and responds to MHC-II presented peptide derived from flagellin during infection (Cookson and Bevan, 1997) (discussed in detail elsewhere).

1.4.4.4 Virulence Genes associated with *Salmonella*

Salmonella possess many virulence genes which largely have defensive functions against host immunity. *Salmonella* has also developed two complex virulence functions to actively interact with the

host and modify cellular functions. These virulence functions are linked to horizontally acquired regions of DNA called pathogenicity islands (PAIs), and are termed *Salmonella* Pathogenicity Islands (SPIs) (Marcus et al., 2000). There are several known SPIs. The better characterised, SPI1 and SPI2 each encode a type III secretion system (T3SS) which translocate bacterial effector proteins directly into host cells.

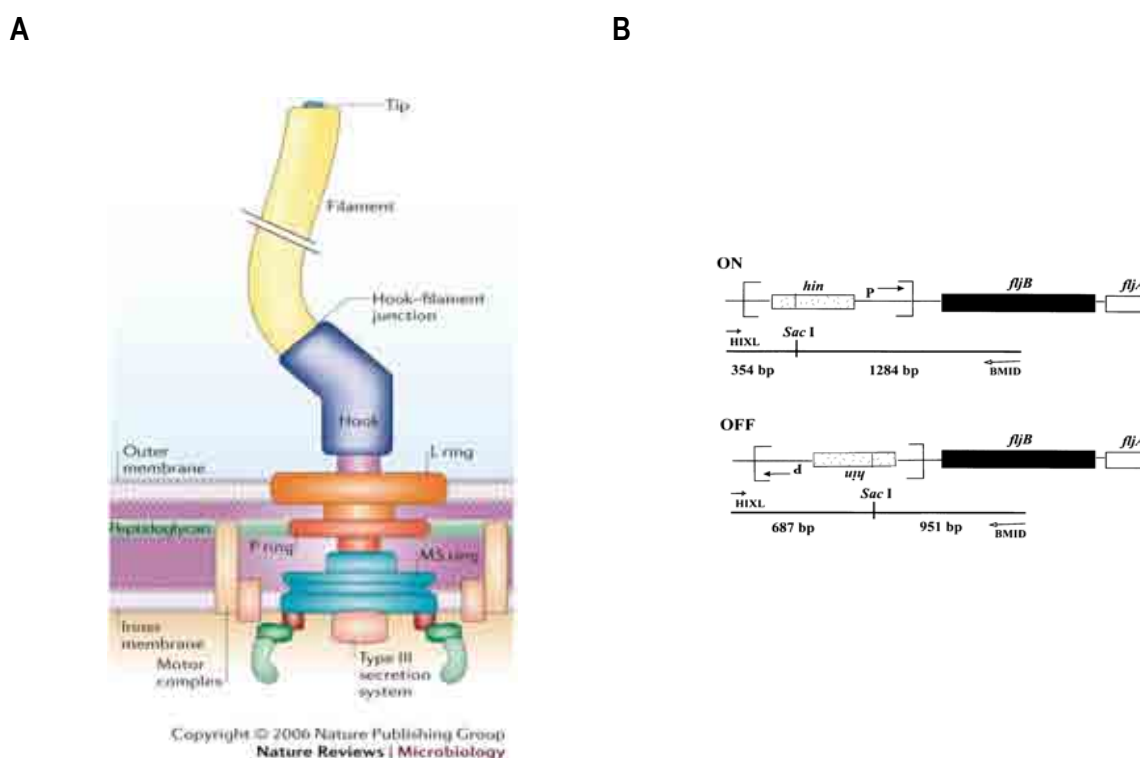


Figure 1.8 Structure and properties of STm flagellin: (A) The bacterial flagellum consists of several components. The motor complex traverses both membranes where it joins a flexible hook structure at the cell surface. The hook is connected to the filament which can grow by self-assembly of as many as 30, 000 flagellin subunits forming long protofilaments. Filament growth occurs at the distal end where a cap protein is stably attached at its tip. Each flagellum is a tubular structure of 11 protofilaments. (Image from (Pallen and Matzke, 2006)) (B) STm phase variation is achieved through alternative inversion of 1 kb of DNA containing the promoter region of fljB and fljA (an inhibitor of fliC) (Bonifield and Hughes, 2003; Zieg et al., 1977). In one orientation, the promoter is situated upstream of the fljBfljA operon (termed ON) enabling transcription of fljB (Phase 2) and fljA, whilst transcription of fliC, is repressed through fljA. In the opposite orientation (termed OFF), fljB and fljA are not transcribed, enabling fliC (Phase 1) expression. This invertible DNA segment is flanked by inverted repeats which encode a recombinase required for switching (hin) (Johnson and Simon, 1985). (Image from (Ikeda et al., 2001)).

T3SS-1 is encoded within SPI1, it is required for the initial stages of infection, the invasion of epithelial cells which is orchestrated through rearrangements of the actin cytoskeleton (Lostroh and Lee, 2001). SPI1 mutants are not attenuated for systemic virulence after intraperitoneal infection of mice (Galan and Curtiss, 1989a). T3SS-2 is encoded within SPI2, which is required for the secretion of effector proteins across the membrane of *Salmonella*-containing vacuoles (SCV). Thus, SPI2 is required for later stages of the infection to enable intracellular accumulation of STm and systemic dissemination (Waterman and Holden, 2003). The role of SPI2 for survival and replication in macrophages is important for this phase of pathogenesis, and SPI2 mutants are severely attenuated in virulence in mouse models of systemic infections (Cirillo et al., 1998; Shea et al., 1999; Shea et al., 1996). Interestingly, a great deal of attenuating mutations in STm are located within SPI1 and SPI2 (Carnell et al., 2007).

1.4.5 Murine Model of STm

STm infection in mice can resemble typhoid fever in many respects and is therefore, widely accepted as a good experimental system for studying human typhoid (Santos et al., 2001). Mice show varied levels of susceptibility to intracellular pathogens such as STm which is associated with a macrophage divalent cation transporter called Slc11a1 (formerly Nramp1) (Goswami et al., 2001). In mice a single mutation in Slc11a1 determines resistance (Gly169) or susceptibility (Asp169) to numerous intracellular pathogens (Vidal et al., 1995). Innately resistant mice can control low doses of virulent organisms which reach a plateau without T cell intervention (Hormaeche et al., 1990). In contrast, innately susceptible mice cannot control infection with virulent strains of STm. They succumb to overwhelming sepsis following parenteral injection of a very few microorganisms (the LD₅₀ is less than 10 organisms) (Plant and Glynn, 1976; Santos et al., 2001). STm infection in these animals results in rapid death (usually less than 1 week) whereas death in human typhoid is rare, and if apparent, late in infection. Most laboratory mouse strains carry the susceptible Asp169 allele and to overcome this enhanced susceptibility live attenuated STm can be used, allowing infection of genetically modified strains, generally only available on the

highly susceptible C57BL6/J or BALB/c background. One advantage of using this system is that adaptive immunity is required for protection which enables immune responses to be followed from induction through to resolution of infection.

Many routes of infection exist for STm in the mouse model. Intraperitoneal or intravenous infection of susceptible strains of mice with attenuated STm provides a model to study synchronised disseminated infection that resembles many features of disseminated infection in resistant mice, such as induction of a low grade bacteraemia. However, intravenous infection is not reduced by the presence of immune sera thus, intraperitoneal immunisation has the added benefit of enabling the role of antibody in protection to be assessed. This may be because complement is not effective at killing STm in cell-free systems in the mouse (Siggins et al., 2011). Ideally, oral infection would be a suitable route as it resembles the natural route of infection and would enable mucosal responses to be assessed. However, attenuated strains do not necessarily colonise effectively when orally administered and other factors, such as resistance to innate barriers, including gastric acid or bile can affect interpretation of results.

1.4.6 Innate Immune Regulation of *Salmonella* Infection

During the early phase of infection (the first week) of mice with STm numerous phagocytic cells, including neutrophils, macrophages, and natural killer (NK) cells rapidly increase in infected sites. These innate cells act to restrict bacterial growth and dissemination before establishment of adaptive immunity (Jepson and Clark, 2001; Johansson et al., 2006; Mastroeni and Sheppard, 2004). This is achieved through mechanisms such as intracellular killing and the actions of numerous soluble mediators, including cytokines and complement components.

1.4.6.1 Role of Cytokines

Early inflammation is orchestrated by these soluble innate effectors such as tumour-necrosis factor alpha (TNF-alpha), which is required for the formation and maintenance of macrophage-rich focal

lesions in the tissue called granulomas, which help contain infection (Mastroeni et al., 1999). The pro-inflammatory cytokines, IL-12 and IL-18, produced by DC and activated inflammatory cells in response to pathogens (Janeway and Medzhitov, 2002) are crucial for immunity against virulent STm (Mastroeni et al., 1999; Mastroeni et al., 1996). The importance of IL-12 and IL-18 in immunity against STm has been attributed to their ability to promote release of IFN γ (Janeway and Medzhitov, 2002; Mastroeni et al., 1999; Mastroeni et al., 1996), which is instrumental in co-ordinating an effective immune response to STm through the activation of CAMs. Disruption of the IFN γ pathway impairs the ability to eliminate STm infection in mice (Hess et al., 1996; Mastroeni et al., 1996). The innate response initiated against STm is complex and requires both soluble mediators and innate effectors to act in a co-ordinated manner. This generates a potent response against invasive pathogens which breach basic immune mechanisms. The key function of innate immunity is to contain infection as innate immunity alone cannot resolve infection. Indeed, mice lacking functional T cells can contain STm infection for almost three weeks, after which uncontrollable bacterial growth occurs (Hess et al., 1996; Ravindran et al., 2005).

1.4.6.2 Pattern Recognition Receptors in Innate Immunity to *Salmonella*

1.4.6.2.1 Innate Recognition of Flagellin

Flagellin is an important target of the innate and adaptive immune response to STm. Cytosolic flagellin is recognised by Ipaf and extracellular or endosomal flagellin, through TLR5. TLR5 recognises flagellin from both GN and Gram-positive bacteria (Hayashi et al., 2001; Means et al., 2003; Smith et al., 2003a). Amino acids residues 386-407 of TLR5 are necessary for recognition of flagellin, whilst the extracellular domain encompassing amino acids 1-386 appear important for signalling (Mizel et al., 2003). TLR5 is not expressed on conventional mouse DC or bone marrow derived macrophages (Means et al., 2003), but is expressed on the basolateral surfaces of murine CD11b+CD11c+ intestinal lamina propria DC (Jepson and Clark, 2001; Uematsu et al., 2006). TLR5 expression is not restricted to cells of the immune system as it is abundantly expressed on most types of epithelial cells as outlined in Table 1.2.

Mutating flagellin reveals that TLR5 recognises an evolutionary conserved site in flagellin which has been mapped to amino acids 89-96 of the N-terminal D1 domain (Andersen-Nissen et al., 2005; Smith et al., 2003a). This region is only exposed in monomeric flagellin, and during filament assembly contacts between adjacent flagellin monomers buries this site in the centre of the filament. Therefore, only monomeric flagellin induces TLR5 activity (Smith et al., 2003a). This TLR5 recognition site is also important for flagella motility, as mutations in this region compromise bacterial motility, as well as filament assembly (Andersen-Nissen et al., 2005). Interestingly, the α - and ϵ -Proteobacteria, including *Campylobacter jejuni* and *Helicobacter pylori*, which require flagella-mediated motility for colonisation and persistence in their hosts, synthesise flagellin molecules which circumvent recognition by TLR5, yet retain motility. This has been shown to be due to differences in this site that permit TLR5 evasion, but retain motility (Andersen-Nissen et al., 2005).

Table 1.2: Cell types known to express TLR5

Cell Type	Host	Reference
Gastric Epithelial Cells	Human Cell Lines	(Smith et al., 2003b)
Intestinal Epithelial Cells	Mouse Human Cell Lines	(Gewirtz et al., 2001) (Rhee et al., 2004)
Lung Epithelial Cells	Human Cell Lines	(Blohmke et al., 2008)
Uterine Epithelial Cells	Human Cell Lines	(Schaefer et al., 2004)
Keratinocytes	Human Cell Lines	(Le et al., 2010)
Melanocytes	Human cell Lines	(Jin and Kang, 2010)
Intestinal Lamina Propria Dendritic Cells	Mouse	(Uematsu and Akira, 2009)
Neutrophils	Mouse	(O'Mahony et al., 2008)
Monocytes	Mouse	(O'Mahony et al., 2008)

Upon flagellin binding, TLR5 dimerises enabling signalling through the adaptor molecule myeloid differentiation factor 88 (MyD88) and the serine kinase IRAK. This leads to the release of numerous pro-inflammatory mediators including TNF- α , IL-1, IL-6 and iNOS, which depends on activation and translocation of the nuclear factor transcriptional factor NF- κ B into the nucleus (Eaves-Pyles et al., 2001; Janeway and Medzhitov, 2002; Underhill, 2007).

In addition to detection by TLR5, intracellular flagellin within the macrophage inflammasome is recognised through the cytoplasmic NLR Ipaf (Franchi et al., 2006; Miao et al., 2006). Ipaf is activated in response to flagellin delivered through the SPI1 T3SS. Therefore, Ipaf may respond to bacteria expressing both flagellin and virulence factors. Additionally, NAIP5 (NLR apoptosis-inhibitory protein 5) which forms hetero-oligomers with Ipaf (Damiano et al., 2004), has been proposed to be involved in sensing cytosolic flagellin (Molofsky et al., 2006) through the conserved carboxy-terminal domain of flagellin (Lightfield et al., 2008). Therefore, TLR5 and Ipaf both recognise different, but conserved domains of flagellin. Ipaf activation leads to processing of pro IL-1 β by caspase 1 and release of pro-inflammatory cytokines upon activation of the inflammasome (Franchi et al., 2006; Kanneganti et al., 2007). Ultimately, the impact of flagellin on the innate immune system is profound. TLRs and NLRs work to activate pro-inflammatory transcription factors, trigger phagocytosis and promote bacterial killing (Vijay-Kumar et al., 2007).

1.4.6.2.2 Innate Recognition of LPS

LPS induces signalling through TLR4 (Poltorak et al., 1998). Recognition of LPS by TLR4 is complex and requires additional accessory molecules, including a soluble acute phase protein, LPS-binding protein (LBP) which recognises LPS and transfers it to the LPS co-receptor CD14 (Tobias et al., 1986). CD14 is a high affinity LPS receptor that can either be secreted into the serum, or expressed as a glycopospho-inositol linked protein on the surface of macrophages (Wright et al., 1990). Signalling also requires MD-2, which is physically associated with the extracellular region of TLR4. Activation of TLR4

can lead to MyD88-dependent or independent signalling to stimulate production of various pro-inflammatory mediators (Kawai et al., 1999).

TLR2 recognises a broad range of microbial products (Table 1.1) by forming heterodimers with either TLR1 or TLR6 (Janeway and Medzhitov, 2002). TLR2 has also been reported to recognise LPS preparations from non-enterobacteria (Smith et al., 2003b; Werts et al., 2001). These LPS structures differ from the typical LPS recognised by TLR4, in the number of acyl chains in Lipid A (Netea et al., 2002). However, the recognition of LPS by TLR2 remains inconclusive (Hashimoto et al., 2004). Indeed, LPS preparations contain trace amounts of endotoxin protein contaminants, and TLR2 is extremely sensitive to these proteins which are difficult to separate when purifying LPS (Hirschfeld et al., 2000).

1.4.7 Cell-Mediated Immune Regulation of *Salmonella* Infection

In terms of enteric infections caused by STm, such infections in humans are entirely controlled by innate immune effectors. However, in relation to the mouse model of NTS infections caused by STm, the innate immune system restricts the initial growth of STm but is incapable of eliminating STm from the host. During infection STm transitions from an extracellular to an intracellular phase, predominantly residing in specialised *Salmonella*-containing vacuoles (SCVs). This enables evasion of host antimicrobial defences (Vazquez-Torres et al., 1999) by preventing phago-lysosomal fusion, and delaying vacuole acidification (Salcedo et al., 2001). The intracellular location of STm requires cell-mediated immunity for control whilst the extracellular dissemination requires humoral control in terms of invasive STm infection.

Clearance of STm in the mouse model requires Th1 CD4 T cells (Pie et al., 1997), but optimal immunity requires both cell mediated immunity and antibody (Mastroeni et al., 2000). T cell deficient mice or mice where total CD4 T cell responses are defective exhibit a persistent burden of infection, and become chronically ill as uncontrollable bacterial growth occurs and mice fail to clear the infection leading to a

progressive, usually fatal increase in bacterial numbers (Mastroeni et al., 1992; McSorley and Jenkins, 2000). In contrast, mice deficient in MHC class-I restricted T cells or $\gamma\delta$ T-cells resolve infection (Hess et al., 1996). This requirement for CD4 T cells has been attributed to the ability of CD4 Th1 cells to induce IFN γ , which activates CAMs to kill STm (Mittrucker et al., 2000).

1.4.7.1 Target antigens of the CD4 T cell response

Infection with STm generates substantial numbers of STm-specific CD4 T cells which expand rapidly following encounter, and these cells persist at high frequency for several weeks until bacteria are eliminated (McSorley et al., 2002a). Few T cell epitopes from STm have been characterised, and of these, flagellin is the one most consistently recognised. Flagellin is a major target of the CD4 T cell response to STm in both humans and mice infected with STm (Bergman et al., 2005; Cookson and Bevan, 1997; McSorley et al., 2000; Szein et al., 1994). Indeed, some T cells isolated from STm infected mice can be detected in the memory population months after infection which remain responsive, and the predominant recall antigen for CD4 T cells from STm infected mice is flagellin (Cookson and Bevan, 1997; Srinivasan et al., 2004).

CD4 T cells responding to flagellin produce IFN γ but not IL-4, reflective of a Th1 response, and this antigen-specific recall response to flagellin is sufficient to protect naïve mice against lethal challenge with a virulent strain of STm (McSorley et al., 2000). Interestingly, the CD4 T cell response to flagellin (or STm) is not identical in every site. It is highly dependent upon the initial site of infection where activation is first seen in the draining secondary lymphoid tissues. This was demonstrated using TCR transgenic FliC-specific CD4 T cells (SM1 T cells), which enables direct visualisation of CD4 T cell responses to STm flagellin in vivo (McSorley et al., 2002a). Following oral STm infection, adoptively transferred SM1 T cells proliferate rapidly in mucosal gut-associated lymphoid tissues (PP and MLN) (within 3 hours), whereas in the spleen and liver SM1 T cells are not activated, even by day 3, despite the presence of bacteria in both these sites (McSorley et al., 2002a). In contrast, after intravenous

infection SM1 T cells were activated in the spleen, but not in the MLN (Srinivasan et al., 2004), demonstrating a more complicated response in vivo.

A number of epitopes have been recognised in flagellin (residues 339-350) as the minimal region capable of stimulating proliferation of CD4 T cells from STm-infected mice (Cookson and Bevan, 1997). Interestingly, these CD4 T cell epitopes within flagellin are localised within the conserved N and C-terminal regions (D0 and D1 domain), the same domains recognised by TLR5 (Bergman et al., 2005). These epitopes are conserved between FliC and FljB and other flagellated gamma-proteobacteria (Alaniz et al., 2006; Bergman et al., 2005; McSorley et al., 2002a; McSorley et al., 2000). Therefore, STm infection generates innate and adaptive immune responses to multiple regions of flagellin, but predominantly targets the highly conserved domains (Bergman et al., 2005; McSorley et al., 2000).

1.4.7.2 Properties of Flagellin

1.4.7.2.1 Effects on the Immune System

Not only is flagellin a target of the innate and adaptive immune system, but it is a powerful and effective adjuvant, as it is capable of enhancing antigen-specific CD4 T cell and B cell responses to itself and co-administered antigen (Bergman et al., 2005; Cuadros et al., 2004; Cunningham et al., 2004a; Salazar-Gonzalez et al., 2007; Uematsu et al., 2006). For instance, immunisation with flagellin-OVA fusion peptides can augment the total number of proliferating OVA specific CD4 T cells relative to immunisation with OVA alone (Bates et al., 2009; McSorley et al., 2002b). The ability of flagellin to modulate CD4 T cell responses is related to its capacity to induce maturation of DC (Huleatt et al., 2007; McSorley et al., 2002b; Sanders et al., 2006), in a TLR5 dependent manner (Bates et al., 2009). Indeed, incubation of splenic and bone marrow-derived murine DC with flagellin can induce up-regulation of MHC II and co-stimulatory markers (CD80 and CD86) (Didierlaurent et al., 2004) which can encourage development of adaptive immune responses.

1.4.7.2.2 Characteristics of the Immune Response to Flagellin

The direction of B cell switching to flagellin when flagellin is presented in its native context, bound to live STm is predominantly to IgG2a, indicative of a Th1 response (Cunningham et al., 2004a). In contrast, when flagellin is presented as a recombinant soluble protein, sFliC, or a polymeric form purified from STm, it is to IgG1 (Cunningham et al., 2004a), which mirrors the response seen against haptenated-protein antigens. Thus, the context in which flagellin is encountered by APC appears to influence the outcome of the immune response. This Th2-bias of responses to flagellin is supported by in vivo studies which demonstrate that activation of TLR5 by flagellin can induce MyD88-dependent OVA-specific CD4 Th2 responses (Didierlaurent et al., 2004).

1.4.7.2.3 Evasion of Cell Mediated Immunity

Several reports have focussed on differential flagellin expression during infection. Although STm expresses flagellin when replicating in vitro (Cummings et al., 2006), once it establishes an intracellular state during infection it is not clear whether flagellin is still actively expressed. Indeed, during transition from an extracellular to an intracellular phase STm triggers changes in antigen expression (Cummings et al., 2006; Lucas and Lee, 2000). This was elegantly demonstrated in a study where FliC-specific CD4 T cell proliferation was assessed ex-vivo, using antigens derived from bacteria grown in conditions which mimicked the extracellular or intracellular environments. This showed that T cell responses to extracellular phase bacterial antigens were greater compared to intracellular phase antigens, which were unable to support expansion of FliC-specific T cell populations in vitro (Cummings et al., 2005). This suggested that the antigen specificity of these T cells was directed against antigens which were only expressed by bacteria during their extracellular growth phase.

During growth inside macrophages at systemic sites such as the spleen and liver (Cummings et al., 2005), intracellular STm repress FliC transcription and its expression at the protein level below the threshold required to stimulate DCs in vitro (Bergman et al., 2005), thereby diminishing innate

recognition. Artificially introducing flagellin during infection enables STm-infected antigen-presenting cells to stimulate FliC-specific T cells (Cummings et al., 2005). This repression of flagellin by intracellular STm may be dependent on the PhoP-PhoQ two-component system (Cummings et al., 2006; Galan and Curtiss, 1989b; Groisman, 2001). Additionally, during intracellular growth STm can reduce the ability of antigen-presenting cells to present FliC to CD4 T cells by confining its expression to SCVs, which are not accessible to antigen-presenting cells, thereby reducing its bioavailability (Alaniz et al., 2006). Thus, intracellular localisation of STm naturally compartmentalises FliC which directly affects DC mediated activation of FliC-specific T cells, which may limit activation of host immune responses.

1.4.8 B Cell Mediated Control of STm Infection

The role of antibody in immunity to STm is incompletely defined. There is an essential role for CD4 T cells (Hess et al., 1996) and a dispensable role for antibody for the effective resolution of primary infection with attenuated STm. Indeed, B cells are not necessary at all to control primary infection with attenuated STm, as mice lacking B cells do not succumb to infection (Mastroeni et al., 2000; McSorley and Jenkins, 2000; Mittrucker et al., 2000).

Although antibody cannot prevent the progress of an established primary infection with attenuated STm in innately susceptible mice, or clear bacteria from the tissues, antibody can increase the efficiency of its clearance during secondary responses (McSorley and Jenkins, 2000). Furthermore, antibody induced during infection can help restrict bacteraemia during primary infection (Cunningham et al., 2007), whilst vaccination of adults against typhoid with purified polysaccharide Vi antigen from ST, a wholly TI antigen is sufficient to protect against invasive disease (Acharya et al., 1987; Guzman et al., 2006; Klugman et al., 1996). Additionally, passive transfer of serum protects genetically resistant mice to challenge with STm but not susceptible mice, supporting a role for antibody in immunity to STm (Eisenstein et al., 1984).

In SSA NTS infections can cause a progressive bacteraemia, killing around 50% of infected individuals. It has been hypothesised, that this bacteraemia may arise as cell-mediated effectors fail to control the intracellular infection, enabling rapid extracellular growth (MacLennan et al., 2008). Cell mediated immunity does not protect against bacteria in the blood, and antibody and complement become critical for controlling this extracellular growth. Indeed, the peak risk of disseminated infection where bacteraemia is pronounced occurs between the ages of 6 and 24 months, correlating with loss of maternal antibody and before acquisition of humoral immunity (MacLennan et al., 2008). Furthermore, although individuals who lack components of IL-12 or IL-23 signalling display more severe STm infections, and have greater disseminated infections in tissues compared to patients with a defect in IFN γ alone (MacLennan et al., 2004), these patients generate serum anti-STm IgG and do not die from NTS infections, suggesting a requirement for humoral immunity against NTS infections.

Nevertheless, optimal immunity is seen when both cell-mediated and antibody are present (McSorley and Jenkins, 2000). Indeed, the simultaneous adoptive transfer of immune serum and cellular immune effectors is necessary to protect naïve innately susceptible mice from challenge with virulent STm. The transfer of immune cells or serum alone cannot confer protection (Mastroeni et al., 1993). Thus, unlike many other intracellular pathogens, both antibody and T cells are involved in resistance to challenge with STm (Mastroeni et al., 2000; Mastroeni et al., 1993; Mittrucker et al., 2000).

1.4.8.1 Mechanism of action of antibody to STm

In humans, both bactericidal and opsonising antibody are important in immunity to STm (Casadevall, 1998; Siggins et al., 2011). Indeed, sera from healthy Malawian children containing anti-STm IgG and IgM antibody displays bactericidal activity against NTS (MacLennan et al., 2008). This study showed a correlation between complement deposition and antibody titre. This suggests that cell-free antibody-dependent complement-mediated killing, through the induction of membrane attack complex, may be important against NTS bacteraemia in humans, but not mice (Siggins et al., 2011). Antibody in mice

probably contributes to protective immunity through opsonising the bacterial cell surface, and thereby facilitating uptake by macrophages (Hsu, 1989).

1.4.8.2 Features of the antibody response to STm

STm induces an atypical antibody response in mice which differs from classical TD antibody responses (Cunningham et al., 2007). It is characterised by a rapid and massive TD EF antibody response, which persists throughout infection and markedly reduces bacteraemia (Cunningham et al., 2007). In contrast, the onset of GC, and therefore high affinity antibodies are not apparent until the infection is almost resolved, which differs significantly from classical Th2 responses in terms of kinetics (Cunningham et al., 2007). This study showed that the key component of antibody protection was the presence of IgG, which augmented protection of IgM, but was independent of the affinity of IgG.

1.4.8.3 Antibody Targets in STm infection

Antibodies against STm target bacterial surface antigens such as LPS, flagellin and lipoproteins (Brown and Hormaeche, 1989) as well as other surface components. Antigenic targets of B cells which confer protection against STm are not clear, and their identification and characterisation is required to aid development of effective vaccines against systemic NTS infections.

Porins

Immunisation with porin preparations isolated from a rough mutant of STm proved to be an effective immunogen, and could induce protection against STm infection (Kuusi et al., 1979). The protection conferred by porins has been related to their ability to induce protective humoral responses. Indeed, serum from typhoid patients largely recognises these proteins (Calderon et al., 1986; Ortiz et al., 1989), whilst Secundino and colleagues elegantly demonstrated that purified porins isolated from ST promoted marked, persistent antibody responses in mice in the absence of exogenous adjuvants. This long-lasting antibody response demonstrated specific bactericidal activity (Secundino et al., 2006). Interestingly,

despite the high degree of homology among porins isolated from different *Salmonella* serovars, serum from mice immunised with ST porins did not cross protect against STm (Secundino et al., 2006), demonstrating the species specificity of this antibody response. This ability of porins to elicit an immune response which can protect the host against infection has led to studies on the potential of porins from ST as vaccine candidates against typhoid (Salazar-Gonzalez and McSorley, 2005; Singh et al., 1999).

Other Targets

Antibodies against flagellin and LPS are used in serotyping *Salmonella* species (Brown and Hormaeche, 1989; Frankel et al., 1989). However, despite the immunogenicity of flagellin, whether antibody to flagella can provide immunity to *Salmonella* remains unresolved. Historically, antibody to LPS has been shown to be strongly induced in response to infection (Brown and Hormaeche, 1989), which can confer high levels of protection against virulent strains of STm. Furthermore, antibodies directed against the O-antigen of STm LPS have been demonstrated to enhance opsonisation of STm (Jorbeck et al., 1981).

1.4.9 Vaccines

Three vaccines have been developed against ST. The heat-killed phenol-preserved vaccine consisting of whole cells is no longer licensed in the UK, but is still in use today in some countries in spite of its high reactogenicity (Engels et al., 1998). Two newer vaccines are currently licensed for use in humans against typhoid and are widely used today, they are well tolerated and effective. This includes the A subunit of the ST polysaccharide antigen (Vi) (Robbins and Robbins, 1986) and the oral attenuated live bacterial vaccine Ty21a (Clemens et al., 1999; Germanier and Fuer, 1975; Guzman et al., 2006; Levine et al., 1989). Both these vaccines fail to induce long-lasting immunity, the Vi vaccine is considered to be protective for at least three years, whilst Ty21a for 5-7 years. Furthermore, neither Vi nor Ty21a are effective in infants less than 3 years of age. Consequently, vaccines comprising Vi-conjugated to TD protein carriers have been developed (Kossaczka et al., 1999; Lin et al., 2001).

There is currently no available vaccine against NTS for use in humans, and current antibiotic treatments are being confounded by the widespread development of drug-resistant strains. Importantly, many NTS STm isolates causing invasive disease are multiply antibiotic resistant (Gordon et al., 2008). Vaccination is an essential requirement for controlling NTS infections in SSA. In the murine model protective immunity can be conferred by immunisation of susceptible mice with live attenuated STm, but it is not known which antigens are responsible for inducing resistance, and further characterisation of isolated antigens based on specific virulence features, and knowledge of the immune response to them may prove beneficial in vaccine design. Interestingly, despite the effectiveness of antibody-mediated protection to ST induced by vaccines it does not appear that there is much cross-reactivity between ST and NTS. Whilst some antigens such as the Vi antigen are not present in STm, it is less clear why attenuated ST vaccines, which are antigenically more complex, should not cross-react.

1.5 Helminth Infections

1.5.1 Incidence and Pathology

Almost half of SSA's population is affected by one or more soil-transmitted helminth (de Silva et al., 2003) and although mortality is a rare outcome from infection, heavy parasitic helminth burdens are associated with serious morbidity. Indeed, hookworm infections are the leading cause of iron deficiency anaemia and malnutrition worldwide, infecting nearly 800 million people (Hotez et al., 2004) with children disproportionately affected (Brooker et al., 2006). Sequela in this group can include profound physical and mental deficits (Hotez et al., 2008). Moreover, it is common in areas with high prevalence of parasitic nematode infections for individuals to carry multiple infections of several nematode species. Therefore, such infections have a staggering economic impact in endemic areas (Hotez et al., 2008).

Helminths can have immunomodulatory effects on the host which may influence the response to other pathogens or antigens, and these may be positive or negative for the host (Cooper et al., 2001; Su et

al., 2005). Indeed, helminth infections are associated with diminished responses to BCG and cholera vaccination (Elias et al., 2008; Harris et al., 2009). Furthermore, soluble immunomodulatory parasite antigens can cross the placenta and significantly affect responses to antigens included in childhood vaccinations years later (Christian et al., 2004). The effects of helminth infections on the host are not always negative. Interestingly, allergic diseases are lower in helminth endemic areas (Lynch et al., 1993; Scrivener et al., 2001) supporting the notion that childhood exposure to chronic helminths can direct the maturing immune system to develop tolerance to environmental antigens (Yazdanbakhsh et al., 2002). Moreover, helminths may reduce inflammatory disease caused by infection (Fox et al., 2000; Nacher et al., 2001) and offer a potential method of treating both immune dysfunction and auto-immune diseases.

1.5.2 Host Immune Responses to Helminth Infections

1.5.2.1 The classical Th2 response

Helminth immunity requires Th2 cell-mediated responses involving multiple cell types including CD4 T cells, eosinophils, basophils, mast cells, epithelial and smooth muscle cells, and IgE production by B cells whilst Th1 responses can inhibit immunity (Finkelman et al., 1997; Urban et al., 1991). In humans the production of IL-4 and IL-13 correlates with a protective immune response against helminths (Turner et al., 2003) and this is also supported by experimental murine models, which show key roles for these cytokines in effective worm expulsion (Barner et al., 1998; Dehlawi et al., 2006; Horsnell et al., 2007).

1.5.2.2 Th2-induced changes in gut physiology

Clearance of helminths from the intestine requires integrated physiological and immunological responses. In addition to immune cell recruitment, the response is characterised by changes in gut physiology to establish a hostile environment for the parasite (Bancroft et al., 1998; Finkelman et al., 2004; Urban et al., 1992). This includes: enhanced gut motility through hyper-contractility of intestinal

smooth muscle cells (Zhao et al., 2003), increased luminal fluid secretions, enhanced epithelial cell permeability and accelerated cell turn-over, all of which are mediated by Th2 associated factors such as STAT-6 signalling (McKenzie et al., 1998; Zhao et al., 2003). Worm expulsion is facilitated by increased mucus production from goblet cells which affect worm viability by inhibiting parasite motility (Lee and Biggs, 1990) and their ability to feed (Rothwell, 1989). Goblet cell hyperplasia is dependent on IL-4 and IL-13 induced STAT-6 signalling (Hershey, 2003; Khan et al., 2001; McKenzie et al., 1998).

1.5.2.3 Role of Antibody

In humans, parasitic nematode infections fail to induce immunity to re-infection. Antibodies may protect against re-infection, as shown using maternal-derived antibodies in murine models (Harris et al., 2006). It has been proposed that antibodies may promote recruitment of effector cells to neutralise secretory products produced by helminths. Nevertheless, the importance of antibody in primary and secondary infections, and the potential mechanisms by which they may execute their function is largely unknown.

1.5.3 *Nippostrongylus brasiliensis*

The nematode, *Nippostrongylus brasiliensis* belongs to the phylum Nematoda which also includes whipworms, and filarial parasites (Camberis et al., 2003). *N. brasiliensis* is a natural parasite of rats and has been adapted for use in mice. It provides an acute model of gastrointestinal nematode parasite infection, and is used as a model of hookworm disease, as its lifecycle is analogous to the human hookworms *N. americanus* and *A. duodenale*, important parasitic pathogens that cause the most common neglected tropical disease in SSA (Bethony et al., 2006).

The typical hookworm lifecycle consists of cutaneous invasion of the host by newly hatched infective third-stage (L3) larvae residing in the soil, or less commonly by oral ingestion (Fig 1.9). After infiltration, larvae migrate to the lungs through the vasculature where molting occurs to enable emergence of L4 larvae. These migrate out from capillaries, through the alveoli and into the bronchi where they are

coughed and swallowed into the small intestine. Here worms complete their development into sexually mature adults (L5), which mate and produce eggs which are passed in the faeces (Camberis et al., 2003). The lifecycle of *N. brasiliensis* is analogous to the human hookworm, as larvae reach the lung within hours of infection and establish a definitive adult stage in the proximal half of the small intestine by day 5-8 (Camberis et al., 2003). Pathology occurs in the intestine with considerable damage to the host intestinal architecture (Horsnell et al., 2007).

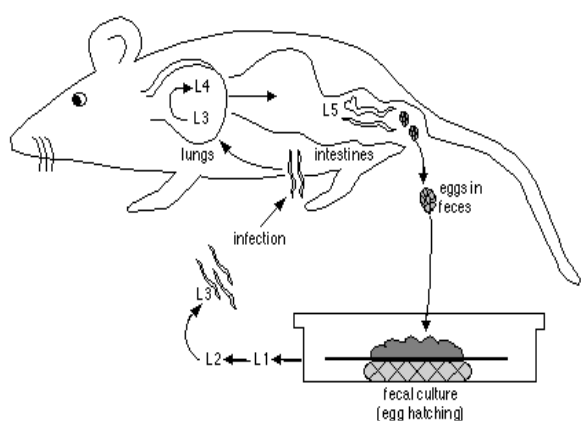


Figure 1.9 Expulsion of *N. brasiliensis* in BALB/c mice after s.c. infection: Eggs are passed in the faeces and 500 infective third-stage (L3) Nb larvae are administered s.c. Larvae infiltrate through connective tissue and migrate through the vasculature to the lungs within hours of infection. Larvae migrate through the trachea and emerge in the proximal half of the small intestine by day 5-8 where worms complete their development and mate to produce eggs which are passed in the faeces. Larvae are absent from the lungs from day 4 of the infection despite a persistent Th2 response. In an immune-competent mouse Nb is normally expelled by day 9-11 (Camberis et al., 2003).

1.5.3.1 *N. brasiliensis* induced Immune Response

N. brasiliensis infection results in potent systemic and mucosal Th2 responses required for parasite expulsion (Finkelman et al., 1997; Horsnell et al., 2007; Mearns et al., 2008). This Th2-response is host protective and can lead to rapid worm expulsion following primary immunisation. IL-4 is dispensable for *N. brasiliensis* expulsion, but clearance does require signalling of IL-13 through IL-4R α and activation of the STAT6 pathway (Barner et al., 1998; Urban et al., 1998). The loss of IL-13 or impairment in IL-13 signalling is characterised by the failure to expel *N. brasiliensis*, and infection is associated with higher fecundity (Barner et al., 1998; McKenzie et al., 1999).

1.6 Summary and Aims

NTS infections are huge, yet poorly-recognised problems in SSA where such infections can be typified by invasive disease associated with high case-fatality rates. Antibiotic resistance is an increasing problem and the development of a vaccine is urgently required. Immunity to STm is complex, and during primary responses protective immunity is provided by CD4 Th1 cells through their ability to invoke macrophage activation. However, during secondary responses optimal protection also requires B cells. This highlights that effective resolution of STm infection encompasses aspects of innate, cell-mediated, and humoral immunity. Therefore, an integrated approach is required to understand how immunity is executed. In addition, helminth infections are endemic in SSA and the likelihood of co-infection with STm is reasonable. This can further complicate immunity to infection given the documented immunomodulatory influences of helminths on host immunity, demonstrating a highly complicated relationship between the host and pathogen.

This study aims to examine the adaptive immune response to STm, and in particular, the individual contribution of component surface antigens in immunity, and to identify their influence on protective immunity during primary and secondary STm infections. Specifically, this study will focus on the cell mediated and humoral immune response to flagellin and OMPs. Furthermore, this thesis aims at identifying whether intestinal parasitic co-infection with STm, confounds the development of the appropriate cellular mechanisms required for effective immunity against both STm and *N. brasiliensis*.

2.0 Materials and Methods

2.1 Animals Used

For animal studies mice were age and sex-matched and between the ages of 6-12 weeks. Wild type (WT) C57BL6/J or BALB/c mice were obtained from HO Harlan OLAC Ltd. (Bicester, U.K) or from colonies maintained in the University of Birmingham Biomedical Services Unit (BMSU) for experiments performed in the UK. For experiments conducted in Cape Town WT mice were sourced from in-house colonies from the animal facility at The University of Cape Town (UCT). All genetically modified mice used were sourced from in-house colonies in the BMSU and the original source is provided in Table 2.1. All mice were bred and maintained under specific-pathogen free conditions.

At indicated time points, mice were sacrificed by a schedule 1 method in accordance with Home Office guidelines (UK) or sacrificed by CO₂ asphyxiation in accordance with UCT guidelines (Cape Town). Animal studies were performed with ethical approval from both the UK and UCT Research Animal Ethics Committees.

2.2 Antigen Preparation for Immunisation

2.2.1 Bacterial Antigens

The attenuated *Salmonella enterica* serovar Typhimurium SL3261 (STm) strain was used throughout. SL3261 is a well-studied vaccine strain which confers protection to re-challenge with virulent STm (Hormaeche, 1991). All STm strains used and their sources have been identified in Table 2.2. Most of the strains used were provided by Dr. RA Kingsley (Wellcome Trust Sanger Institute, Cambridge) and the methods used to create them have been described in Appendix B.

Table 2.1: Genetically modified mouse strains used and their sources

Table provides the source and phenotype of mice used throughout the study for experiments performed in the UK.

Mouse Strain	Description	Source and Reference
SM1 (Rag 2-deficient background)	$\alpha\beta$ TCR transgenic specific for STm flagellin peptide residues 427-441 bred to Rag-2 deficient mice	Source: Professor Paul Garside University of Strathclyde. (McSorley et al., 2002a)
Rag 1-deficient (C57BL6/J background)	Disruption of Rag 1 gene causing early arrest of B and T cell development	Source: Professor Peter Lane University of Birmingham (Mombaerts et al., 1992b)
OTII Boy J (Cogenic CD45.1)	$\alpha\beta$ TCR transgenic specific for chicken ovalbumin peptide residues 323-339 in the context of H-2 I-A ^b which has been crossed to CD45.1 ⁺ C57BL6/J cogenic mice	Source: Charles River (Clarke et al., 2000)
IL-4R α -deficient (BALB/c background)	Impaired IL4 and IL-13 signalling via deficiency of the IL-4 receptor alpha chain following Cre-mediated recombination of the IL4-receptor alpha allele through the cre/loxP system.	Source: Professor James Alexander University of Strathclyde (Mohrs et al., 1999)
IL-10 deficient (C57BL6/J background)	Homologous recombination of IL-10 with a neomycin cassette Mice develop chronic enterocolitis	Source: Charles River (Kuhn et al., 1993)
T-bet deficient (Cogenic CD45.2+)	Disruption of T-bet gene by homologous recombination	Source: Jackson Laboratory (Szabo et al., 2002)
TCR $\beta\delta$ -deficient (C57BL6/J background)	Absent $\gamma\delta$ and $\alpha\beta$ T cells through targeted deletion of beta and delta TCR genes	Source: Jackson Laboratory (Mombaerts et al., 1992a)
IgH deficient	B-cell deficient. Generated from breeding out the QM IgH transgene from QM mice, which have the other IgH locus inactivated.	Source: Dr Kai Toellner University of Birmingham (Cascalho et al., 1996)
TLR5-deficient (C57BL/J x 129/Sv background)	Targeted deletion of TLR5 Reduction of serum cytokines in response to flagellin	Source: Professor Bernhard Ryffel; Toulouse France (Vijay-Kumar et al., 2007)

Table 2.2: Bacterial strains used

Table lists all the STm strains used throughout the study. The methods used to generate specific strains are outlined in Appendix B.

Strain	Reference and/or Source	Characteristics
SL3261	(Hoiseh and Stocker, 1981)	Attenuated <i>Salmonella</i> Typhimurium aroA ⁻ deficient strain Attenuated through insertional mutagenesis of aroA using aroA554::Tn10 Deletion of aroA confers a defect in the aromatic biosynthetic pathway
SL3261 Sal OVA	Adam Cunningham (Serre et al., 2008)	Attenuated <i>Salmonella</i> Typhimurium aroA ⁻ deficient strain Constitutively expresses OVA under Tac promoter SL3261 aroA pettacOVA Amp ^R
SL3261 Sal GFP	Adam Cunningham	Attenuated <i>Salmonella</i> Typhimurium aroA ⁻ deficient strain Constitutively expresses GFP under Tac promoter SL3261 aroA pettacGFPhis Amp ^R
SL1344	(Hoiseh and Stocker, 1981)	Virulent strain of <i>Salmonella</i> Typhimurium – an aroA ⁺ strain
RAK 121	Robert A. Kingsley	Attenuated <i>Salmonella</i> Typhimurium aroA ⁻ deficient strain Phase-locked to express FliB only (termed locked-ON) SL3261 aroA ON
RAK 123	Robert A. Kingsley	Attenuated <i>Salmonella</i> Typhimurium aroA ⁻ deficient strain Phase-locked to express FliC only (termed locked-OFF) SL3261 aroA OFF
SW 564	Robert A. Kingsley	Attenuated <i>Salmonella</i> Typhimurium aroA ⁻ deficient strain Does not produce FliC or FliB so does not make flagella– (termed Aflagellated) SL3261 aroA ON ΔfliB::aph ΔfliC::cat
RAK 126	Robert A. Kingsley	OmpD-deficient attenuated <i>Salmonella</i> Typhimurium aroA ⁻ deficient strain Inactivation of OmpD through Tn10 Insertion SL3261 aroA OmpD::Tn10 (tet ^R)
RAK 083	Robert A. Kingsley	OmpR-deficient attenuated <i>Salmonella</i> Typhimurium aroA ⁻ deficient strain Deletion of OmpR through red recombinase technology SL3261 aroA OmpR::Tn10 (tet ^R)

2.2.1.1 Bacterial Growth Conditions

STm for infection studies was incubated overnight in Luria-Bertani (LB) broth (Invitrogen, Paisley, UK) at 37°C with aeration (180 rpm) and STm for bacterial culture was grown on plates at 37°C. LB broth was supplemented with 2% w/v granulated agar (Melford Laboratories, Ipswich, UK) for plates. Media for growth was prepared by dissolving powders in deionised water (dH₂O) and autoclaving at 121°C for 15 minutes. Media was left to cool at room temperature prior to use. Where appropriate, culture medium was supplemented with antibiotics following filtration through a 0.22 µm filter (Millipore, MA, USA). Ampicillin was used at 50 µg/ml whilst Kanamycin and Tetracycline at 25 µg/ml.

2.2.1.2 Infection with STm

For studies addressing immune responses to flagellin (Chapter 3.0), following incubation STm was harvested as bacteria entered late log/early stationary phase (OD_λ600 nm of 1.2-1.4) when flagellin expression is optimal. Cultures that had OD_λ600 nm of >1.4 were disregarded due to the potential of having increased levels of dead cells. For studies addressing antibody responses to porins (Chapter 4.0) STm was harvested at mid-exponential phase (OD_λ600 nm of 0.5-0.8) for optimal OmpD expression, whilst STm was harvested at OD_λ600 nm of 1.0 for the dual infection study (Chapter 5.0).

For immunisation into the peritoneum 1 ml of bacteria were harvested by centrifugation at 6,000 x g for 5 minutes at 4°C. Pelleted cells were washed twice in sterile Phosphate Buffered Saline (PBS) (pH 7.4) (Sigma Aldrich, Dorset, UK) at 6,000 x g for 5 minutes at 4°C prior to re-suspension in 1 ml PBS. Bacteria were diluted to give the appropriate infectious dose in a final volume of 200 µl PBS. Mice were infected at doses of 5x10⁵ or 5x10⁶ per mouse with attenuated bacteria (SL3261 strains) or infected with virulent bacteria (SL1344 strains) at a dose of 3x10³ per animal. For oral infection studies with attenuated STm, STm was grown overnight until it reached OD_λ600 nm of 1.4. Bacteria were then harvested at 3,000 x g for 15 minutes at 4°C and re-suspended in PBS to a density of 1x10¹⁰ per ml. Bacteria were then diluted 1:1 with 3% NaHCO₃ to obtain a dose of 1x10⁹ per mouse (5x10⁹ per ml).

Mice were immediately infected by oral gavage in a final volume of 200 µl. In each experiment the administered dose was confirmed using the Miles and Misra CFU counting technique by plating serial dilutions onto agar plates and incubating overnight at 37°C (Miles et al., 1938).

2.2.1.2.1 Infection with Heat-Killed STm

For infection with heat-killed STm bacteria were harvested and washed as described. Once bacteria were diluted to 2.5×10^6 organisms per ml, cells were heat inactivated at 72°C for 1 hour and mice were infected thereafter. Bacteria were plated to confirm the absence of live organisms.

2.2.1.2.2 Infection with Serum-Opsonised STm

For STm opsonisation studies the serum to be tested was heated at 56°C for 30 minutes to inactivate complement prior to use. In each experiment two mice were tested with each serum, and at least three different sera were tested per group. After heat-inactivation, complement-inactivated sera were mixed with bacteria which had previously been harvested, washed and the bacterial concentration adjusted to 2.5×10^6 organisms per ml. Sera was added at dilutions where antibody was at sub-saturation as determined by ELISA. Bacteria and sera were mixed under gentle agitation for 30 minutes at room temperature. Mice were infected with 5×10^5 bacteria and bacteria were cultured to ensure no loss of viability and to assess lack of agglutination.

2.2.2 Parasite Antigens

2.2.2.1 Maintenance of *N. brasiliensis* Life Cycle

N. brasiliensis worms (originally provided by Klaus Erb, Wurzburg, Germany) were maintained by routine passage through Wister Rats every two weeks. Live third stage (L3) larvae were prepared in 0.65% NaCl at 10,000 larvae per ml. Six to eight rats were injected subcutaneously into the nape of the neck with 5000 larvae (500 µl) using 20G needles. At day 5 post-infection rats were transferred to gridded cages for faecal collection. The cages were lined with damp paper to keep the faecal pellets

moist. Faecal pellets from infected mice were collected daily from day 6 to 8 post-infection and soaked each day in MilliQ water containing fungizone (Gibco® Life Technologies). Pellets were emulsified and mixed into a paste-like consistency with an equal volume of activated charcoal and spread onto culture dishes. A culture dish consisted of a large petri dish containing a moist filter paper and square gauze, moistened in fungizone. The dishes were incubated in humidified containers at 25°C for at least 5 days to allow L3 larvae to migrate to the edge of the filter paper. The outer layers of the filter paper containing the worms were removed and transferred to fresh culture dishes for further incubation where infective live L3 larvae were used for infectious studies.

2.2.2.2 Preparation of *N. brasiliensis* for Infection

For infection studies pieces of filter paper containing fresh L3 larvae were washed briefly in 0.65% NaCl to release the worms. Worms were counted using a dissecting microscope and diluted to 2500 worms per ml in NaCl. Mice were injected subcutaneously with 500 L3 larva in a final volume of 200 µl NaCl in an Animal Biosafety Level 2 Laboratory (Animal Unit, UCT).

2.2.3 Protein Antigens

1. Endograde® Ovalbumin was commercially obtained from Hyglos, Regensburg, Germany
2. Soluble recombinant flagellin (sFliC) and sFliC bearing a modified TLR5 ligating motif (Δ TLR5) were generated as described in 2.2.3.1 and 2.2.3.2 respectively.
3. Surface-isolated polymeric flagella was generated as described in 2.2.3.3
4. Purified porins from ST (strain ATCC 9993) or STm (strain ATCC 14028) were generated by Dr. Cristina-Gil Cruz through repeated extraction with sodium dodecyl sulphate (SDS). The detailed methods used to generate them and the techniques used to functionally characterise them are outlined in Appendix C.

Protein antigens were diluted to 100 µg per ml in sterile PBS and mice were immunised intraperitoneally for 35 days with 20 µg unless stated otherwise. For studies with alum precipitated ovalbumin mice were immunised intraperitoneally with 50 µg ovalbumin precipitated in alum in a final volume of 200 µl. Briefly, ovalbumin was mixed with an equal volume of 9% alum (aluminium potassium sulphate (Sigma Aldrich, Dorset, UK)) and the pH was then adjusted to 7.0 using 10 M sodium hydroxide. This was then left to precipitate in the dark for 30 minutes at room temperature. Following precipitation, ovalbumin was washed three times in excess PBS and made up to volume for immunisation. On some occasions mice were co-immunised and infected with sFliC or alum precipitated ovalbumin and SL3261 respectively. Here, sFliC or alum precipitated ovalbumin was prepared at 100 µg per ml or 250 µg per ml, respectively and SL3261 was then added at 5×10^5 .

2.2.3.1 Soluble Recombinant Flagellin (sFliC)

Soluble recombinant flagellin (sFliC) was isolated from STm as described (Cunningham et al., 2004a). In brief, fliC was amplified from SL3261 and ligated into pET22b+ (Merck Chemicals, Nottingham, UK) to create pET22b+ FliC Xho1 which contained a C-terminal His-Tag, a beta-lactamase gene which conferred ampicillin resistance, and expressed FliC under the control of Isopropyl β-D-Thiogalactopyranoside (IPTG) induction of the Lac operon. This was transformed into chemically competent BL21 DE3 and sFliC was isolated following over-expression in LB. Here bacteria were incubated overnight at 37°C with aeration in LB supplemented with ampicillin. After incubation the culture was diluted 1:100 in fresh media and incubated at 37°C until mid-log phase (OD₆₀₀ 0.6-0.8) where IPTG (Promega, Southampton, UK) was added at a final concentration of 1 mM to induce sFliC expression. Culturing was continued post-induction for a further 2.5 hours after which the cell paste was harvested at 6000 x g for 20 minutes at 4°C.

Protein purification was achieved through a two-step process. Initially, cells were re-suspended in Bugbuster (Merck Chemicals, Nottingham, UK) and agitated at room temperature for 20 minutes. The

cell lysate was spun at 16,000 x g for 20 minutes at 4°C to remove cellular debris. The resulting lysate was filtered through a 0.22 µm filter (Millipore, MA, USA) and incubated with Ni-NTA Sepharose (Qiagen, Hilden, Germany) for 45 minutes at room temperature. Flagellin was then purified by Nickel-affinity chromatography using a disposable Polypropylene Column (Qiagen). After the lysate had passed through, the column was washed with five bed volumes of PBS and the His-tagged protein (sFliC) was eluted in 5 ml PBS containing 100 mM Imidazole (Melford Laboratories). sFliC was then extensively dialysed against PBS under gentle agitation at 4°C.

After dialysis, sFliC was filtered and purified to homogeneity by affinity chromatography using an anti-FliC monoclonal antibody in conjunction with Dr Margaret Goodall. Purified sFliC was then further dialysed and protein identity and purity was assessed by SDS-PAGE which revealed sFliC was present at the expected molecular weight (56 kDa), and that no contaminating proteins were detectable post affinity chromatography (Fig 2.1A). Protein concentration was determined using the BCA assay (Thermo Fisher Scientific, MA, USA). LPS contamination was assessed using the Sigma E-TOXATE Kit as per manufacturer's guidelines and shown to be ≤ 1 Endotoxin Unit EU/300 µg. Sterile protein was stored at -20°C. The detailed methods for SDS-PAGE and protein quantification are described in Appendix C.

2.2.3.2 Soluble Recombinant sFliC (Δ TLR5)

Additionally, pET22b+ FliC Xho1 was further modified using the GeneEditor in vitro mutagenesis system (Promega) to delete the putative TLR5 binding site as described (Andersen-Nissen et al., 2005). The methods used to create the construct are outlined in Appendix B. The plasmid clone encoding the modified protein (Δ TLR5 sFliC) conferred resistance to ampicillin and expressed Δ TLR5 sFliC with a C-terminal His-tag. It was over-expressed in BL21 and purified to homogeneity as described for sFliC. Protein identity and purity was determined by SDS-PAGE (Fig 2.1B). This revealed Δ TLR5 sFliC was present at the expected molecular weight and that no contaminating proteins were detectable post affinity chromatography.

2.2.3.3 Surface Isolated Polymeric Flagella

Flagella were isolated from the surface of SL3261 by acid hydrolysis as described (Ibrahim et al., 1985). Briefly, bacteria were grown overnight and were diluted 1:100 in fresh LB the following morning where growth was continued until late-log phase ($OD_{\lambda 600} = 1.4$). Bacteria were harvested at $6000 \times g$ for 20 minutes at $4^{\circ}C$ and then re-suspended for an hour in acid at pH 2.0 under gentle agitation. This suspension was then spun at $100,000 \times g$ for 90 minutes at $4^{\circ}C$. The pH of the supernatant was restored to pH 7.4 before protein fractions were separated by size-exclusion chromatography in conjunction with Dr. Denisse Leyton. LPS content was assessed using the E-TOXATE Kit and shown to be ≤ 1 EU/ $300 \mu g$. Sterile protein was stored at $-20^{\circ}C$.

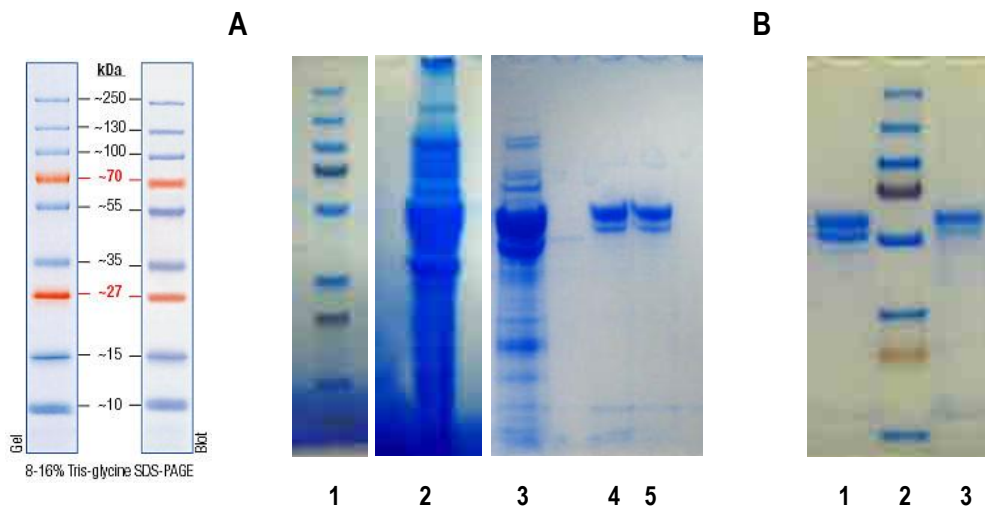


Figure 2.1: Assessment of sFliC by SDS-PAGE: Purification fractions and purified proteins were analysed by SDS-PAGE and visualised using Gel-Code Blue: (A) Soluble Recombinant Flagellin (sFliC) was purified by His-Tag purification and affinity chromatography. Lane 1: Fermentas PageRuler Plus. Lanes 2-3 His-Tag Purification fractions: Flow Through (2) and Elute (3). Lanes 4-5: Purified sFliC post-Affinity Chromatography. (B) Purified Δ TLR5 sFliC and sFliC were purified to homogeneity by His-Tag and affinity chromatography: Lane 1: Purified sFliC, Lane 2: Fermentas Page Ruler Plus and Lane 3: Purified Δ TLR5 sFliC. Proteins were separated using a 4-20% gradient gel (Thermo Fisher Scientific).

2.2.4 Lipopolysaccharide (LPS)

TLR Grade LPS, which is isolated and purified from STm S-form, was commercially obtained (Axxora, Cambridge, UK (ALX-581-011-L002)) and has been highly purified to eliminate any cross-reactivity with TLR2. STm C5 LPS was purchased from List Biologicals, CA, USA (Product 225) and Single-Step Purified LPS (SSP) was prepared from this using the Phenol-Water extraction method originally described by Westphal, and Jann (1965). Additionally, SSP LPS was further purified as a phenol re-extracted preparation through extensive washing in phenol and triethylamine, as described (Hirschfeld et al., 2000). LPS preparations were diluted to 100 µg per ml in PBS (pH 7.4) and mice were immunised intraperitoneally with 20 µg of the immunogen for 35 days unless stated otherwise.

2.3 Experiment End-Point

At defined time-points mice were anaesthetised and peripheral blood obtained by cardiac puncture. Cells were isolated from the peritoneal cavity for FACS (Fluorescence Activated Cell Sorting). The spleen was isolated and weighed for analysis by FACS, cytokine assessment by ELISA, immunohistochemistry and bacterial culture. The liver was also isolated for bacterial culture. Worm burdens were assessed in the small intestine and a section of the jejunum was retained for histology. All organs were isolated under aseptic conditions.

2.4 Bacterial Culture

The bacterial burden in the spleen and liver was determined by direct culturing. Tissues were disrupted in PBS (Sigma Aldrich, Dorset, UK) through a 70 µm nylon-cell strainer (BD Biosciences) and serially diluted in PBS. The diluted suspension was plated on LB agar supplemented with appropriate antibiotics and incubated overnight at 37°C. Bacterial colonies were counted and the total CFU per organ was determined based upon the entire organ mass and dilution.

2.5 Assessment of Worm Burdens

Adult worm burdens were determined from *N. brasiliensis* infected mice. The entire small intestines were removed from sacrificed mice and incised longitudinally before being placed in 10 ml of 0.65% saline. The intestines were incubated at 37°C for 3 hours to enable the worms to migrate into the salt solution. The salt solution was poured into a petri dish and the intestinal fragments were discarded before adult worm burdens were counted under a dissecting microscope.

2.6 Extraction of Cells Ex-Vivo

2.6.1 Preparation of Splenocytes

Single cell suspensions of splenocytes were obtained by disruption of the spleen in RPMI-1640 media supplemented with 10% heat-inactivated foetal calf serum (FCS) and 1% penicillin/streptomycin (P/S), followed by filtration through a 70 µm nylon cell strainer (BD Biosciences). Cells were then harvested at 375 x g for 4 minutes at 4°C and erythrocytes lysed using ammonium chloride buffer for 4 minutes at room temperature (BD Biosciences). Cells were then washed in 10% FCS RPMI as before to remove residual lysis buffer and live cell density was determined using Trypan Blue staining to exclude dead cells.

2.6.2 Preparation of Peritoneal Exudate Cells (PEC)

Cells were isolated from the peritoneal cavity in 5 ml PBS (pH 7.4) to purify peritoneal cell populations. Isolated cells were washed in PBS at 375 x g for 4 minutes at 4°C. If cells were contaminated with blood they were subjected to ammonium chloride lysis, as per splenocytes. Cell density was then determined as described.

2.7 Extracellular FACS Staining by Flow Cytometry

Immunostaining of single cell splenocytes or PEC suspensions was carried out for 20 minutes at 4°C in FACS buffer (PBS containing 1% FCS, 2 mM EDTA and 1 mM HEPES). The antibodies used and their optimised concentrations are listed in Table 2.3. Before staining, cells were diluted in FACS buffer to obtain an equal cell density for each sample. Cells were then pelleted at 375 x g for 4 minutes at 4°C in V-bottom 96-well plates (Corning Life Sciences, Netherlands). Five million cells were used for assessing cell populations and one million cells were used as single colour controls for gating. Cells were incubated with anti-CD16:CD32 for 20 minutes to block CD32/Fcγ1 and CD16/Fcγ2 receptors, prior to staining. Cells were then pelleted and labelled for FACS analysis of B or T cell populations. Cells stained with biotinylated-antibodies were incubated as described and subsequently washed twice in FACS buffer prior to incubation with secondary, fluorochrome-conjugated streptavidin. After incubation, cells were washed prior to re-suspension in FACS buffer for acquisition of data. Samples were acquired on a FACS Calibur Cytometer (Becton Dickinson) and analysed using FlowJo 7.6.1 software (TreeStar).

2.8 Splenocyte Stimulation and Cytokine Detection

Intracellular cytokines were detected following re-stimulation of total splenocytes in-vitro with either anti-CD3 or antigen-specific preparations, and cytokines measured by one of the following methods:

2.8.1 Intracellular FACS Staining (ICS)

Cytokines were detected by ICS using flow cytometry. For anti-CD3 stimulation a 24 well plate was pre-coated overnight at 4°C with 10 µg/ml purified anti-mouse CD3 in PBS. Following incubation anti-CD3 was aspirated and the wells were washed in PBS before cells were plated. For antigen-specific re-stimulation, splenocytes were plated into a 48-well plate and cells were re-stimulated in the absence of anti-CD3, but in the presence of either sFlt1 or somatic NES at 5 µg/ml (the preparation of NES is described in section 2.10.1.3). Cells were plated at a density of 5 million splenocytes per well and all

stimulations were carried out in the presence of anti-CD28 at 1 µg per ml. Furthermore, in each re-stimulation assay control wells containing only anti-CD28 were included. Cells were incubated at 37°C for 2.5 hours in the absence of the protein transport inhibitor monensin (GolgiStop) and then 2.5 hours in the presence of monensin as per manufacturer's guidelines (BD Biosciences).

After stimulation cells were surface stained as described. WT cells were stained for CD3, CD4 and CD62L. Antigen-specific SM1 cells were stained with Vβ₂-biotin and visualised with SA-PerCP Cy 5.5, or CD45.2-phycoerythrin (PE) whilst OTII Boy J cells were stained with CD45.1-PE. Cells were then fixed and permeabilised using the Cytotfix/Cytoperm Plus Kit as per manufacturer's instructions (BD Biosciences). Intracellular staining was achieved by staining cells as described, but in permeabilisation buffer with APC- or PE-conjugated cytokine antibodies, or the isotype control antibodies as outlined in Table 2.3. Cells were then washed prior to re-suspending in FACS buffer for acquisition of data.

Table 2.3: Antibodies used for Immunostaining

Table shows antibodies used for extracellular and intracellular FACS analysis and their sources.

Specificity	Clone	Dilution	Source	Application
Antibodies used for Extracellular FACS				
Purified CD16 CD32	93	1:150	e-Bioscience	Blocking FcγIII and FcγII
CD3 FITC	145-2C11	1:100	e-Bioscience	T Cells
CD4 PerCP Cy 5.5	RM4-5	1:300	BD Biosciences	
CD62L PE	MEL-14	1:500	e-Bioscience	
CD19 APC-Cy 7	ID3	1:100	BD Biosciences	Purify B1b Cells from PEC
IgM PE-Cy 7	11/41	1:500	e-Bioscience	
B220 Pacific Blue	RA3-6B2	1:200	e-Bioscience	
CD5 PE Cy5	Ly-1	1:200	e-Bioscience	
CD23 PE	B3B4	1:200	e-Bioscience	
CD21 FITC	7G6	1:100	e-Bioscience	
CD11b APC	MI-70	1:1000	e-Bioscience	
Antibodies used for ICS				
Purified CD3e (No Azide)	17A2	1:100	e-Bioscience	Ex-Vivo Re-Stimulation
Purified CD28 (No Azide)	JJ319	1:1000	BD Biosciences	
Biotinylated Vβ ₂	B20.6	1:100	BD Biosciences	Detect SM1 T Cells
Streptavidin-PerCP Cy 5.5		1:100	e-Bioscience	To detect vβ ₂
CD45.1 PE	A20	1:200	e-Bioscience	Detect OTII Boy J Cells
IFNγ APC	XMG1.2	1:100	BD Biosciences	Ex-Vivo Re-Stimulation
IL- 5 APC	TRFK5	1:100	BD Biosciences	
IL-13 PE	eBIO13A	1:100	BD Biosciences	
Mouse IgG ₁ Isotype	MOPC-21	1:100	BD Biosciences	

2.8.2 ELISPOT

ELISPOT was performed using anti-mouse IFN γ antibody (XMG 1.2 – a kind gift from Prof. Peter Lane, Birmingham, UK) as capture antibody and a biotin anti-mouse IFN γ for detection. IL-4 ELISPOT was carried out with a mouse IL-4 ELISPOT kit (eBioscience, Hatfield, USA). In brief, plates (MultiScreen, Millipore, MA, USA) were coated overnight at 4°C with capture antibody at 10 μ g/ml in PBS. Following incubation, plates were washed in PBS and blocked in PBS containing 1% BSA for 1 hour at 37°C. Splenocytes were added in triplicate at 4×10^5 cells per well and were stimulated with 5 μ g/ml sFliC in the presence of 1 μ g/ml anti-CD28 or were un-stimulated in the presence of anti-CD28 for 48 hours at 37°C. After stimulation cells were aspirated and plates extensively washed in PBS containing 0.05% Tween-20. Plates were then incubated for 2 hours at room temperature with biotinylated anti-IL-4 (BVD6-24GB) or anti-IFN γ (XMG 1.2) (Pharmingen). Subsequently, plates were washed as before and then incubated with Streptavidin-peroxidase for 45 minutes at room temperature. Plates were then washed and the reaction was developed with 3-3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma Aldrich) as per manufacturer's guidelines. Plates were washed in dH $_2$ O and air-dried before spots were counted using the AID ELISPOT Reader System and AID software version 3.5 (Autoimmune Diagnostika, Germany). Counts were expressed as Spot-Forming Units (SFUs) per 4×10^5 splenocytes.

2.8.3 Cytokine ELISA

Intracellular cytokines were also detected by ELISA (Enzyme-Linked Immuno-Sorbent Assays). In brief, 4×10^5 splenocytes were plated per well in NUNC Maxisorb flat-bottomed 96-well plates (Thermo Fisher Scientific). Cells were re-stimulated in a total volume of 200 μ l with either anti-CD3 at 10 μ g/well which was pre-coated overnight in PBS, somatic NES at 10 μ g/well or heat-killed STm at 1×10^5 organisms per well. Control wells contained media only. Cells were incubated for 72 hours at 37°C in a humidified 5% CO $_2$ incubator. After incubation the supernatants were harvested and stored at -20°C. ELISAs were performed on these supernatants.

The composition of buffers used for Cytokine ELISAs is provided in Appendix A. Briefly, NUNC Maxisorp 96-well plates were coated overnight at 4°C with either 1 µg/ml purified anti-mouse IFN γ (R4-6A2), IL-10 (JES5-16E3) (both BD Biosciences) or IL-13 (38213) (R&D Systems, Germany) capture antibody in coating buffer. Following incubation plates were washed four times in wash buffer and then incubated overnight at 4°C in blocking buffer. Three-fold dilutions of the samples were prepared in dilution buffer ranging from a 1/3 dilution to a 1/27 dilution, while recombinant mouse standards were diluted two-fold from either 100 ng/ml for IFN- γ or 200 ng/ml for both IL-13 and IL-10. After blocking, plates were washed as before and then incubated with the diluted standards and samples overnight. Following incubation, plates were washed and bound cytokines were probed with corresponding biotinylated rat anti-mouse monoclonal antibodies (XMG1.2 for IFN- γ , SXC-1 for IL-10 and 38213 for IL-13) for 2 hours at 37°C. Subsequent to further washing, plates were incubated for 1 hour at 37°C with alkaline-phosphatase conjugated streptavidin (AP) at 1:1000. Plates were then washed and probed with the ELISA substrate solution. The developing reaction was stopped by adding 25 µl of 1M NaOH and the absorbance was measured at 492 nm (against the reference measurement at 405nm) on a VersaMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA, U.S.A).

2.9 Cell Sorting

2.9.1 Generation of Chimeras

Chimeras were generated by adoptive transfer of sorted cell populations or total splenocytes. For total splenocytes, spleens from OTII Boy J or SM1 mice were pooled and splenocytes were isolated as described. The proportion of antigen-specific cells was determined from the total number of splenocytes by FACS. 10 million cells were usually transferred intraperitoneally into age and sex matched recipients (such as Rag1 deficient or WT animals) in a volume of 200 µl. On some occasions, PEC cells from mice previously immunised with porins were labelled (as described in Table 2.3) and B1b cells were purified to >98% purity by Roger Bird using a MoFlo high speed cell sorter (Dako, Ely, UK). Sorted cells were

harvested and washed at 375 x g for 4 minutes at 4°C. The cell density was then determined and 2×10^5 cells were transferred intraperitoneally into B cell-deficient mice. Chimeras were usually immunised or infected 24 hours post-transfer and transferred cells identified by FACS at time of sacrifice.

2.9.2 Cell Sorting for Real-Time RT-PCR

Splenocytes from SM1-Rag1 deficient chimeras, SM1-OTII Boy J chimeras, SM1-WT chimeras, WT mice, T-bet deficient and IL-4R α deficient mice immunised with sFliC or infected with STm, were isolated to enable purification of CD4 T cells. Purified CD4 T cells were then divided into effector and naïve populations on the basis of CD62L expression. In brief, splenocytes were labelled as described, except the entire population of recovered cells were stained. Cells were then re-suspended in 500 μ l 10% FCS RPMI and were sorted into naïve CD3+CD4+CD62L^{hi} and effector CD3+CD4+CD62L^{lo} populations by Roger Bird using a MoFlo high speed cell sorter.

SM1 cells from chimeras were defined as CD4+V β 2+, these were the only lymphocytes present in SM1-Rag1 deficient chimeras, or CD45.2+CD90.1+ in SM1-OTII Boy J chimeras. OTII Boy J cells were defined as CD45.1+ in OTII-WT chimeras. Sorts typically recovered 2×10^5 cells at >98% purity. Post sort populations were checked for purity and were immediately harvested, washed and frozen at -80°C.

2.10 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used to assess antigen-specific antibodies in the blood pre and post-infection. Blood was collected at times of sacrifice from each animal and serum separated from clotted blood by centrifugation at 6,000 x g for 5 minutes. Serum was stored at -20°C until required.

2.10.1 ELISA Antigens

Antibody-specificity was tested against various antigens including flagellin, purified porins and LPS which were described earlier. Here we describe the preparation of antigens specifically for ELISA.

2.10.1.1 Total STm Homogenate

STm lysate was prepared by harvesting on overnight culture of STm at 6,000 x g for 5 minutes. The cells were then re-suspended in PBS containing 0.01% NaN₃ and were disrupted through vigorous agitation, using a mini bead-beater (Biospec Products) and 0.1 mm zirconia-silicon glass-beads. The concentration of the resulting homogenate was quantified using the BCA assay (Appendix C).

2.10.1.2 Total Outer Membrane Proteins

Total OMP preparations were prepared by 2% Triton X-100 extraction as described (Henderson et al., 1997). Briefly, cells were harvested at an OD_{600 nm} of 1.0 by centrifugation at 10,000 x g for 10 minutes at 4°C. Cells were washed in 10 mM Tris-HCl (pH 7.4) under the same conditions and re-suspended in this buffer containing 2 mM PMSF (Roche). Cells were then disrupted using a French pressure cell at 20,000 psi and harvested at 6,000 x g for 10 minutes at 4°C to remove unbroken cells. The lysate was spun at 30,000 x g for 90 minutes at 4°C and pelleted cell envelopes were incubated in 10 mM Tris-HCl containing 2% Triton X-100 for 15 minutes at room temperature. This was then spun at 30,000 x g as before and the soluble OMPs were washed extensively in 10 mM Tris-HCl to remove the detergent. The total OMP preparation was re-suspended in this buffer and protein concentration was determined using the BCA Assay (Appendix C) before storage at -20°C.

2.10.1.3 Somatic NES Antigen

For re-stimulation assays and antibody ELISAs *N. brasiliensis* excretory-secretory antigen (NES) was prepared. Briefly, L3 stage larvae were harvested in 0.65% NaCl and re-suspended in a final volume of 25 ml. Larvae were washed in dH₂O containing 250 ng/ml fungizone followed by 5 washes in cold filter-sterilised PBS containing 1000 U/ml penicillin (Invitrogen) and 1 mg/ml streptomycin (Invitrogen), with 30 minute waiting periods between each wash step to allow worms to settle. The PBS was aspirated and larvae were snap-frozen in liquid nitrogen before being finely homogenized. The resulting homogenate was diluted 1:10 in PBS and spun at 6,000 x g for 20 minutes at 4°C. Supernatants were

collected and filtered using a 0.22 µm syringe filter (Millipore Corporation). The concentration of the supernatant was quantified using the BCA Assay (Appendix C) and then aliquoted and stored at -80°C.

2.10.2 Antibody Detection

Relative serological antibody titres were assessed for specificity against these antigens by ELISA. Briefly, NUNC Maxisorp flat-bottomed 96 well plates (Thermo Fisher Scientific) were coated overnight at 4°C with the antigen of interest at 5 µg/ml in carbonate coating buffer (Appendix A). Additionally, to detect serum IgM in chimeric mice, plates were coated with 10 µg/ml Rat anti-mouse IgM (MCA199, AbD Serotec, Oxford, UK). After coating, plates were blocked for 1 hour at 37°C in blocking buffer, and then washed in wash buffer. Sera and secondary antibodies were diluted stepwise in dilution buffer starting at 1:10 or 1:20 and plates were incubated as before. After incubation, plates were washed and probed with AP-conjugated goat anti-mouse IgM (1:2000) or IgG, IgG1 and IgG2a (1:1000) secondary antibodies (Southern Biotech, AL, USA). Plates were then washed in wash buffer and signal detected using Sigma-Fast p-Nitrophenylphosphate (pNPP) tablets as per manufacturer's guidelines. Plates were incubated with the substrate at 37°C until desired positivity was obtained. Plates were read at 405 nm using the softmax pro programme on a Emax Precision Microplate Reader (Molecular Devices).

2.11 Histology

2.11.1 Immunohistochemistry (IHC)

IHC was carried out on spleens which were slowly frozen in liquid Nitrogen and stored at -80°C until required. Prior to sectioning, spleens were mounted onto a chuck through a bed of OCT TissueTek compound (Dako, Glostrup, Denmark) and 5 µm sections were obtained on a cryostat (Bright Instruments, Huntington UK) and mounted onto four-spot slides (CA Hendley Essex Ltd, Essex UK). Sections were left to dry for one hour before fixation for 20 minutes at 4°C in acetone, and dried before storing at -20°C in polythene bags.

IHC was carried out as described (Toellner et al., 1996). In brief, slides were brought up to room temperature and hydrated in Tris buffer (pH 7.6) (Appendix A). Sections were subsequently kept in this buffer throughout. Primary antibodies were added at the optimum dilution for 1 hour and then washed twice for 5 minutes in fresh buffer. Secondary antibodies were initially adsorbed with normal mouse serum which was diluted 1:10 prior to addition to the slides. Slides were incubated with secondary antibodies for 45 minutes. All antibodies used are outlined in Table 2.4 and were prepared in Tris buffer at pH 7.6. Slides were then washed as before and streptavidin-complex with alkaline phosphatase (AP) was added (Dako, Glostrup, Denmark) for 30 minutes. Horse-Radish Peroxidase (HRP) and AP reagents were developed sequentially using DAB and naphthol AS-MX phosphate with Fast Blue salt, respectively (Appendix A). After development slides were washed in Tris buffer (pH 7.6) and then dH₂O. 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 Image J.

IHC was used to ascertain the area of the follicle occupied by GC and quantify cell populations. For GCs, sections of spleen were stained for PNA and IgD. Using a graticule the number of intercepts that fell on GC (PNA+), as well as the total number of intercepts that covered the entire follicle were counted, and from this the percentage of grid intercepts occupied by GCs was calculated. Individual cells were quantified per square millimetre using a point counting technique.

2.11.2 Periodic Acid Schiffs (PAS) Staining

For mice infected with *N. brasiliensis* a portion of the jejunum was retained for PAS staining to view goblet cells and assess intestinal mucus production. The jejunum was stored in 4% phosphate buffered formalin before embedding in paraffin wax. Sections for histology were generated at 5-7 µm and stained with Periodic Acid Schiffs (PAS). The embedding, sectioning and staining was carried out by the

Department of Histology, Groote Schuur Hospital, Cape Town and is described in Appendix A1.

Photographs were taken as described.

Table 2.4: Antibodies used for Immunohistology

Table shows primary and secondary antibodies used for histological staining of spleen tissues.

Target	Isotype	Dilution	Source
Primary Antibodies			
CD3	Rat anti-mouse	1:500	Serotech, Oxford, UK
IgD	Sheep anti-mouse	1:1000	The Binding Site, Birmingham
IgM	Rat anti-mouse	1:600	Serotech, Oxford, UK
IgG1	Rat anti-mouse	1:400	Serotech, Oxford, UK
IgG2a	Rat anti-mouse	1:300	Serotech, Oxford, UK
PNA	Biotin	1:100	Vector Laboratories
CD138	Rat anti-mouse	1:200	BD Biosciences
Secondary Antibodies			
Rabbit anti-Rat Biotin		1:600	Dako
Donkey anti-Sheep PX		1:50	Dako

2.12 Real-Time RT-PCR

2.12.1 Extraction of mRNA and Reverse Transcription

RNA was purified from cell populations or spleen tissue using the RNeasy Mini Kit (Qiagen, UK) in accordance with the manufacturer's guidelines. RNA was eluted in water and reverse transcribed using 1 µg OligodT₁₂₋₁₈ and Superscript III (Invitrogen) in a cDNA Hybaid PCR machine at 42°C for 60 minutes before RNA polymerase was heat inactivated at 90°C for 10 minutes. Each reaction was made in a final volume of 30 µl containing the following components: 2 µl dH₂O, 8 µl 5xfirst strand buffer, 4 µl 0.1 M DTT, 2 µl dNTPs (10 mM), 1 µl RNase Inhibitor (40 units/µl) and 1 µl Superscript III (200 units/µl). The amplified cDNA was made up to 100 µl with RNase free-water and stored at -20°C until use.

2.12.2 Real Time-PCR

Relative quantification of specific cDNA species to β -actin message was carried out using standard conditions for the TaqMan PCR on the Stratagene MxPro 3000 PCR machine using 2x PCR Master Mix (Applied Biosystems, Warrington, U.K). Reactions were carried out in a 96-well optical reaction PCR plate in a final volume of 25 μ l. For each reaction a forward primer, reverse primer, and probe specific to the gene of interest was added at the optimum concentration (Table 2.5), into the same reaction, a forward primer, reverse primer, and probe specific to the house-keeping gene β -actin was added. Probes for the house-keeping and target gene were labelled with VIC and FAM, respectively. TaqMan probes and primers were designed using Primer Express computer software (Applied Biosystems) and synthesised by Eurogentec. Each reaction also contained 2 μ l cDNA. The plate was sealed using a MicroAmp™ Clear Adhesive Film before the reaction was run. Relative quantification of signal per cell or tissue was achieved as described (Cunningham et al., 2002). In brief, thresholds within the logarithmic phase of the PCR for the house-keeping gene and the target gene were set and the cycle number at which the threshold was reached (C_T) was determined. The C_T for the target gene was subtracted from the C_T for the house-keeping gene and the relative amount was calculated as $2^{-\Delta C_T}$.

Table 2.5: Primer and probe sequences used for RT-PCR

Gene	Forward primer	Reverse primer	Probe
β -Actin	CGTGAAAAGATGACCCAGATCA	TGGTACGACCAGAGGCATACAG	TCAACACCCCAGCCATGTACGT AGCC
IFN γ	TCTTCTTGGATATCTGGAGGAACTG	GAGATAATCTGGCTCTGCAGGATT	TTCATGTCACCATCCTT-
GATA-3	CCACCCATTACCACCTATCC	CACACACTCCCTGCCTTCTGT	TCGAGGCCCAAGGCACGATCC
IL-4	GATCATCGGCATTTTGAACGA	AGGACGTTTGGCACATCCAT	TGCATGGCGTCCCTTCTCCTGT G
T-bet	CCAAAGGATTCCGGGAGAA	CCCCAAGCAGTTGACAGTT	CTCGTATCAACAGATGCGTACA TGGACTCAAA

2.13 Functional Studies

2.13.1 Motility Assay

Swimming of STm in the presence of anti-sFliC specific sera was assessed using semi-solid agar containing 0.3% agar. Semi-solid agar was mixed with high-titre, complement inactivated anti-sFliC specific sera or naïve sera at a 1:300 dilution. STm from an overnight culture that had just reached OD_{600 nm} of 1.4 was injected into the agar. The swim zones diameters (distance of migration away from the point of inoculation) were measured after overnight incubation at room temperature.

2.13.2 Western Blotting

The detailed methods used for protein analysis, including western blotting are described in Appendix C. Here we describe specific blots.

2.13.2.1 Assessment of Flagella Shedding

Western blot analysis was used to detect the presence of flagella on the surface of STm following incubation with anti-sFliC specific sera. STm and FliC-locked STm (only express FliC) from an overnight culture which had just reached OD_{600 nm} of 1.4 was harvested and washed at 6000 x g for 5 minutes at 4°C. Pelleted cells were re-suspended in PBS and incubated for 1 hour at room temperature under gentle agitation with complement inactivated sera from naïve or sFliC-immunised WT mice at a 1:300 dilution. Following incubation, cells were harvested, washed and run on a SDS-PAGE gel. Proteins were transferred to a Polyvinylidene Fluoride (PVDF) membrane which was sequentially probed with an anti-FliC specific monoclonal antibody and HRP-conjugated goat-anti mouse IgG at 1:100. Signal was developed using Supersignal Chemiluminescence (Thermo Fisher Scientific).

2.13.2.2 Assessment of Antibody Binding to OMPs

Western blotting was used to assess the specificity of anti-porin specific antibody against OMP preparations of STm. In brief, OMP preparations (Section 2.10.2) isolated from STm or strains of STm bearing selective deletions of specific OMPs, were separated by SDS-PAGE and transferred to PVDF membranes. Proteins were probed with sera from naive T-cell deficient mice and T-cell deficient mice immunised with porins for 7 days at 1:50 and 1:150, respectively. Subsequently, HRP-conjugated goat-anti-mouse IgM secondary antibodies were added and signal developed as described.

2.14 Statistical Analysis

Data are presented as bar charts where bars show the arithmetic mean (usually from 4-6 mice per groups) and error bars represent the standard deviation of the arithmetic mean. Both the mean and standard deviation are presented on a log scale throughout. Statistical analysis was conducted using GraphPad PRISM version 5.01 where the median was compared between groups and significance was accepted where $P \leq 0.05$. Significant differences were determined using the Mann-Whitney non-parametric sum of ranks test. Experiments were repeated at least once to ensure consistency between the results. Numbers of experiments are indicated in the figure legends.

3.0: Flagellin induces a primary Th2 response yet promotes T-bet regulated Th1 clearance of STm upon challenge

3.1 Introduction

Innate immunity controls early infection with STm before adaptive immunity clears the infection, in a CD4 T cell dependent manner requiring IFN γ (Mastroeni et al., 1999; Mastroeni et al., 1996; Mittrucker et al., 2000). Identifying key potential antigenic targets against STm and assessing their influence on the immune system is necessary for vaccine design. Surface exposed antigens are ideal candidates and include the flagellar proteins, FliC and FljB, which are the focus of this study. These highly conserved proteins are powerful adjuvants (Bates et al., 2009; McSorley et al., 2002b) and important targets for the immune response to STm. Flagellin is recognised by PRR and can drive potent and sustained antigen-specific CD4 T cell responses (Cookson and Bevan, 1997; McSorley et al., 2000). Although immunity to STm after systemic immunisation with flagellin has been described previously (Harada et al., 2002; McSorley et al., 2002a; Strindelius et al., 2004), the characteristics of the CD4 T cell response to flagellin remain relatively unknown.

We and others have previously shown flagellin drives T and B cell responses with a strong Th2 bias to itself and co-administered antigens (Cunningham et al., 2004a; Didierlaurent et al., 2004). However, the direction of antibody switching to flagellin when attached to the surface of STm reflects Th1 responses (Cunningham et al., 2004a). This capacity of flagellin to drive directional switching to IgG1 or IgG2a depending on the context in which it is initially encountered in vivo (Cunningham et al., 2004a), makes flagellin a good model to study the mechanisms operating in vivo during the onset of T cell priming and T-dependent (TD) antibody switching. In addition, whether this bi-directional switching induced by flagellin is reflected in the T cell response raises questions as to how the immune system responds in two distinct manners. This prompted assessment of how the immune response to flagellin informs on the correlates of immunity. The immune response to flagella when given alone using soluble (s)

recombinant flagellin (sFliC) or when attached to the bacterial surface was assessed which illustrates that antigen-context *in vivo* is important in directing subsequent T and B cell responses. These studies were extended to examine whether flagellin immunisation can protect against a subsequent systemic STm infection. The model of infection used throughout this study is first described before providing in-depth analysis of responses to flagellin.

3.1.1 Model of Infection

Since the severity of NTS is associated with systemic infection (Graham et al., 2000), to induce a synchronised disseminated infection we typically use an intraperitoneal route, and all the data presented in this thesis has been generated following intraperitoneal immunisation of mice with STm, unless stated otherwise. Other routes of infection exist for STm, such as intravenous infection, but intravenous administration does not allow a full assessment of the role of antibody to protection to be made. Subcutaneous infections, such as in the foot are typically restricted to only the draining lymph node, especially in the early stages of infection, and the kinetics of dissemination are both varied and vastly delayed.

Intraperitoneal infection allows a compromise to be made. It induces a low grade bacteraemia which resembles the bacteraemia in NTS. Furthermore, this model has advantages of allowing protective T cell and antibody responses to be examined, as well as ensuring responses after dissemination can be assessed. There are disadvantages to this model, including the failure to translocate across epithelial cells and the subsequent impaired ability to induce mucosal antibody responses, which are protective in ST (Salazar-Gonzalez et al., 2004) as demonstrated by oral infection. However, oral infection with attenuated strains of STm is not applicable to susceptible mouse strains, and factors such as gastric acid may restrict colonisation. We are aware of the limitations of this and are cautious in how data obtained from oral infection studies is interpreted.

3.2 T cells are important at day 18 for clearance of STm

The known immune correlates of protection against STm were examined to ascertain the kinetics of the response, with particular emphasis on identifying when CD4 T cells are important. WT C57BL6/J mice were infected intraperitoneally with 5×10^5 STm and bacterial numbers were quantified from the spleen at days 4, 7, 20, 28 and 35 post-infection to establish a time-course of bacterial clearance (Fig 3.1A). This demonstrated that bacterial numbers were comparable at days 4 and 7 post-STm infection, but fell at day 20 and continued to decline thereafter. By day 35 the average bacterial load was a hundred organisms.

The contribution of the different components of the adaptive immune system was then assessed. WT mice and mice lacking B and T cells (Rag1-deficient) were infected with 5×10^5 STm for 7 or 18 days (Fig 3.1B). This revealed the bacterial burden in the spleen and liver at day 7 post infection was comparable between WT and Rag1-deficient mice. However, by day 18 post-infection WT mice had significantly reduced splenic and liver bacterial numbers relative to day 7, whilst bacterial numbers in Rag1-deficient mice largely remained unchanged, highlighting the importance of adaptive immunity for resolution of infection. As an additional control the contribution of T and B cells during STm infection was examined. Since T cells are critical for the resolution of STm (Mastroeni and Menager, 2003) WT, TCR-deficient ($\beta\delta$ TCR-deficient) and B cell deficient (IgH-deficient) mice were infected intraperitoneally with 5×10^5 STm. As expected, quantification of the bacterial load in the spleen at day 7 post-infection found no differences between the groups (Fig 3.1C). At day 18 post-infection both WT and B cell deficient mice had significantly lower bacterial numbers relative to day 7, and they were comparable to each other, whilst numbers in TCR-deficient mice were similar to day 7. These data demonstrate that during a systemic STm infection T cells are needed by day 18 onwards for resolution of infection as is in agreement with previous studies (Hess et al., 1996; Mastroeni et al., 1999).

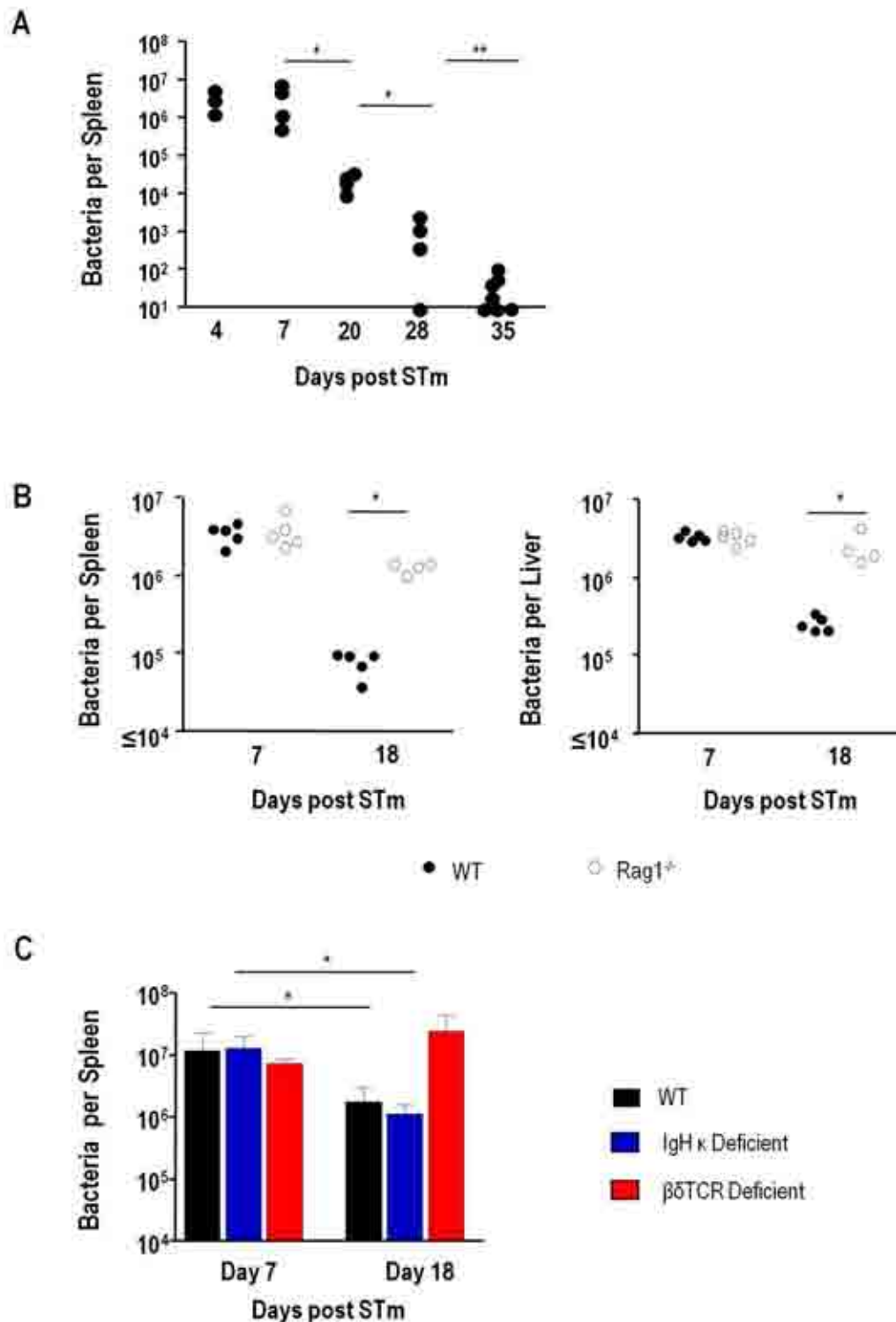


Figure 3.1

Primary infection with STm requires innate immunity for early control but adaptive elements for resolution: (A) WT mice were infected with 5×10^5 STm for 4-35 days. Spleens were isolated and bacterial CFU enumerated at sacrifice at the defined time-points. (B) WT and Rag1^{+/-} mice were infected with 5×10^5 STm for 7 or 18 days and bacterial CFU quantified from the spleen (left panel) and liver (right panel) at sacrifice. (C) WT, IgH^{-/-} (B cell deficient) and βδTCR^{-/-} (T cell deficient) mice were infected as in (B) and bacterial CFU quantified from the spleen. Groups contained 4-7 mice. ** $p < 0.01$ * $p < 0.05$

3.3 Immunisation with flagellin promotes a Th2 response

3.3.1 Mice immunised with sFliC induce IL-4 mRNA and protein

Previous studies have shown that responses to flagellin directs polarisation of B cells to IgG1, indicative of Th2 activity (Cunningham et al., 2004a) whereas when attached to the surface of STm, the anti-flagellin B cell response to IgG2a reflects Th1 activity. The properties of the CD4 T cell response to sFliC, prepared as described in section 2.2.3.1 were examined. The purity of the protein is illustrated in Fig 2.1 which demonstrates a dominating band at 55 kDa and a smaller species directly beneath this following his-tag and affinity chromatography purification. Both bands correspond to the expected molecular mass of flagellin (55 kDa). The identity was further confirmed by immunoblotting which revealed that both bands were recognised by an anti-FliC monoclonal antibody (data not shown).

To examine the CD4 T cell response to sFliC WT mice were immunised with 20 µg sFliC or 5×10^5 STm intraperitoneally. After 3 days splenic CD3⁺CD4⁺ T cells were sorted into naïve (CD62L^{hi}) and effector (CD62L^{lo}) populations. Gene expression was then assessed by real-time RT-PCR (Fig 3.2). Expression of the Th2 associated transcription factor GATA-3 (Farrar et al., 2001) and cytokine IL-4 (Mosmann et al., 1986) were strongly induced in mice immunised with sFliC relative to STm-infected mice, with the signal predominantly from the effector cell population rather than the naïve population. In contrast, induction of the global Th1 regulator T-bet (Szabo et al., 2000) and cytokine IFN γ was mostly detected in mice immunised with STm, with greatest expression found in the effector population.

This experiment was repeated using flagellin-specific CD4 T cells (SM1) (McSorley et al., 2002a). SM1 T cells were adoptively transferred into OTII Boy J mice (CD45.1) and chimeras were immunised with 20 µg sFliC or infected with 5×10^5 STm intraperitoneally (Fig 3.3A). Antigen-specific T cells were sorted at day 3 post-immunisation using CD45.2 as a marker and further sub-divided into naïve and effector populations (Fig 3.3B). As observed in WT CD4 T cells, T-bet and IFN γ mRNA was mostly induced in

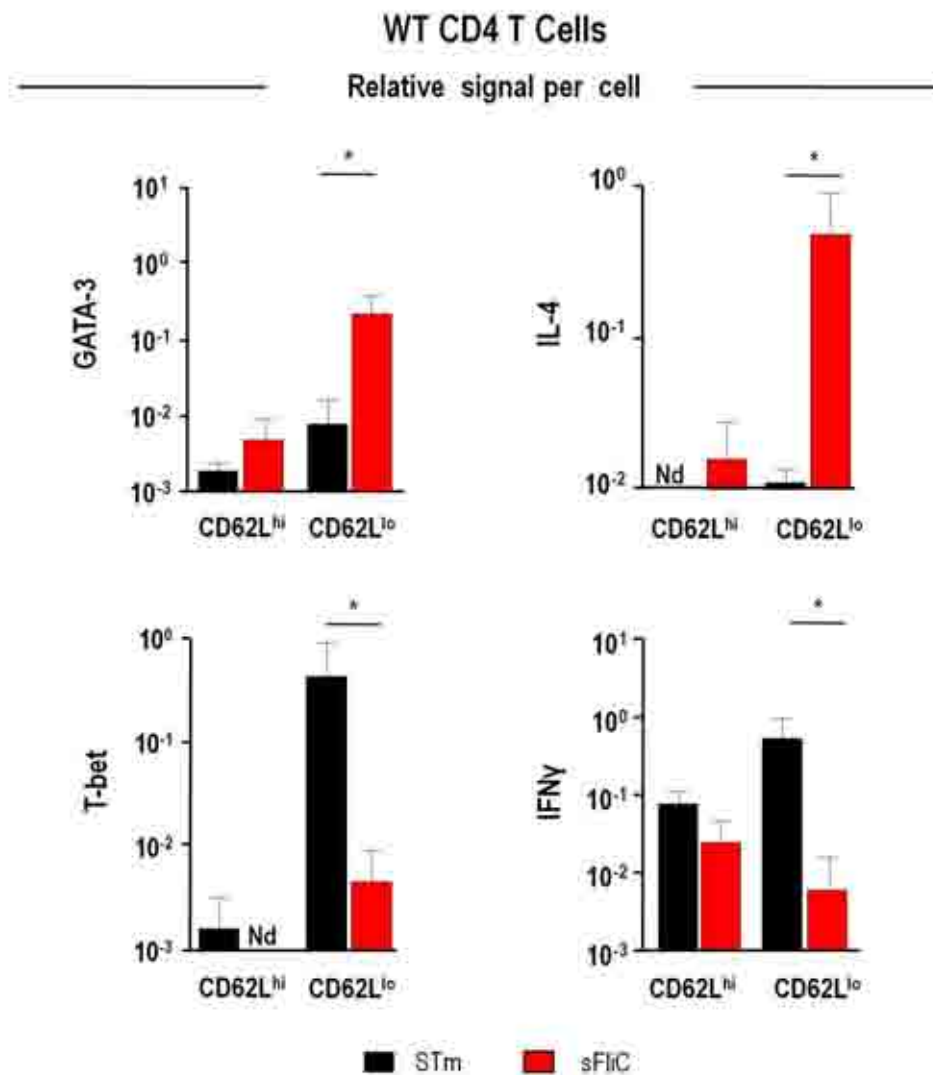
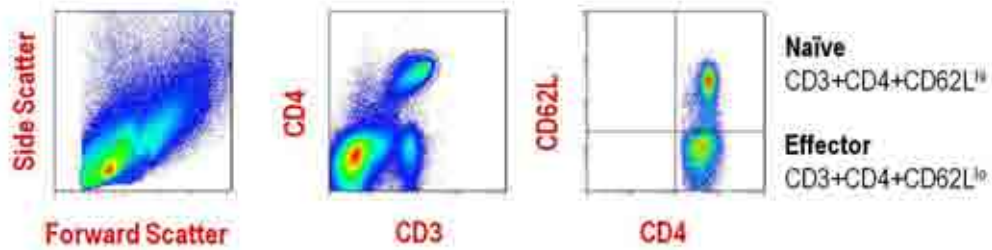


Figure 3.2

T cell responses to flagellin reflect Th2 activity in WT CD4 T cells: Splenocytes from WT mice immunised intraperitoneally with 20 µg sFliC or 5x10⁵ STm for 3 days were sorted into CD3⁺CD4⁺CD62L^{hi} and CD3⁺CD4⁺CD62L^{lo} populations by FACS. Expression of GATA-3, IL-4, T-bet and IFNγ was quantified by real-time RT-PCR from these sorted CD4 T cells. Data is representative of two independent experiments and 4 mice per group. Nd = Not-detected *p<0.05

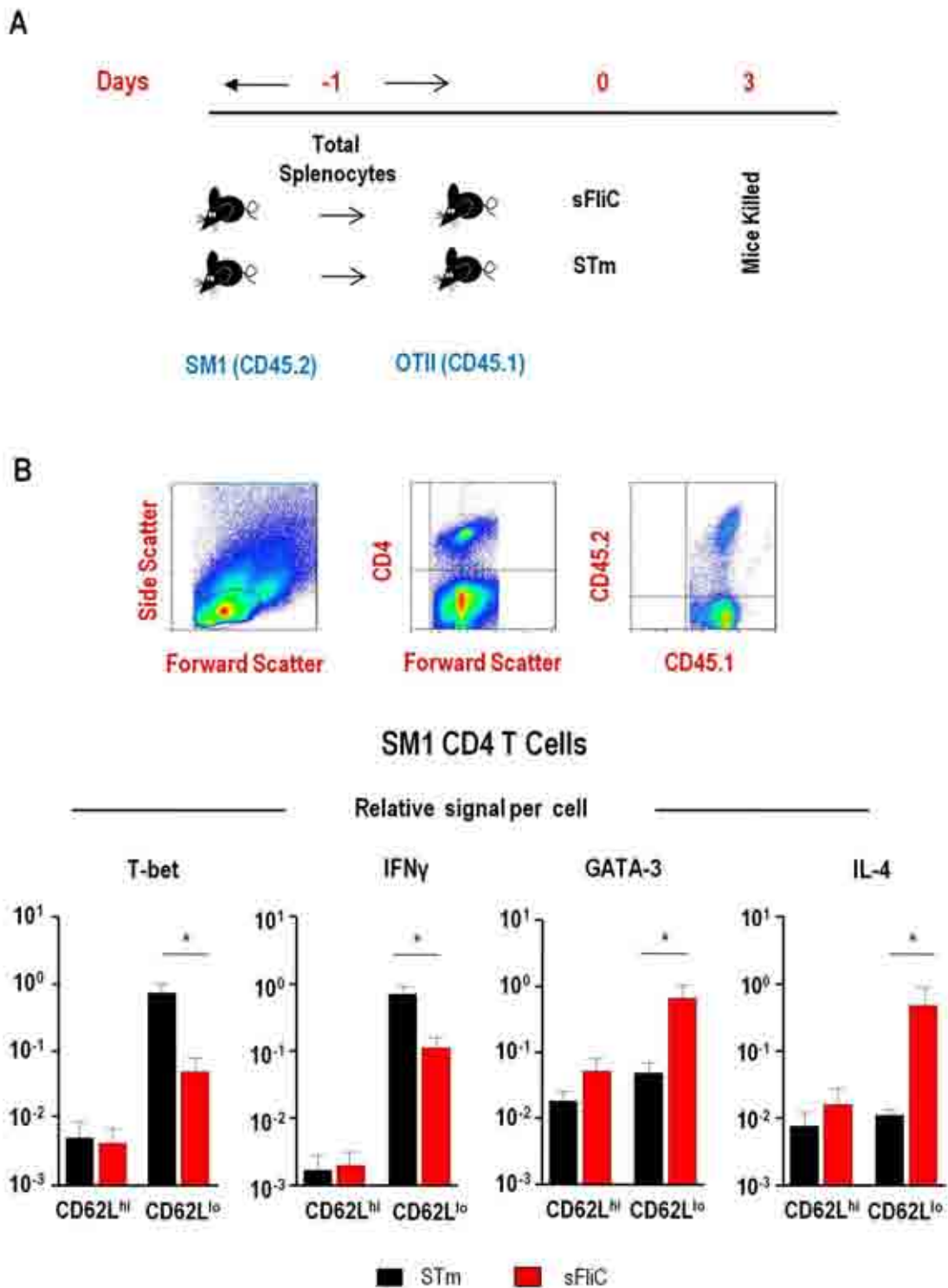


Figure 3.3

T cell responses to flagellin reflect Th2 activity in antigen-specific CD4 T cells. (A) Total splenocytes from flagellin-specific (SM1) mice were transferred intraperitoneally into OTII BoyJ mice. Chimeras were immunised intraperitoneally 24 hours later with 20 μ g sFluC or 5×10^5 STm for 3 days. (B) Splenic SM1 CD4⁺ T cells were sorted into CD62L^{hi} and CD62L^{lo} populations by FACS and expression of T-bet, IFN γ , GATA-3 and IL-4 was quantified by real-time RT-PCR from these sorted populations. Data is representative of two independent experiments and 4 mice per group. * $p < 0.05$.

effector (CD62L^{lo}) SM1 T cells sorted from STm-infected chimeras. GATA-3 and IL-4 mRNA was mostly detected from effector SM1 T cells after sFliC-immunisation.

To confirm that the IL-4 mRNA induced reflected protein production secreted IL-4 was evaluated in re-stimulated T cells by ELISPOT (Fig 3.4). SM1-Rag1 deficient chimeras were generated by adoptive transfer of SM1 cells into Rag1-deficient mice intraperitoneally. Chimeras were either non-immunised (NI), immunised with 20 µg sFliC or infected with 5x10⁵ STm alongside WT mice. Splenocytes were isolated 7 days post-immunisation and re-stimulated in vitro with sFliC for 48 hours in an anti-IL-4 pre-coated ELISPOT plate, and the number of IL-4 Spot-Forming Cells (SFC) quantified. Splenocytes from both WT mice and chimeras immunised with sFliC, had a marked increase in the number of IL-4 SFC relative to NI mice and mice infected with STm. Therefore, the number of IL-4 protein-producing cells increase upon immunisation with sFliC which is reflected at the protein level and in changes in mRNA expression.

3.3.2 Mice immunised with sFliC fail to induce IFN γ

Production of the Th1 cytokine IFN γ was then examined. WT mice were either NI or immunised intraperitoneally with 20 µg sFliC or infected with 5x10⁵ STm. Splenocytes were isolated 7 days post-immunisation and re-stimulated in vitro with anti-CD3 or sFliC in the presence of anti-CD28, and IFN γ production by CD4 T cells was measured by intracellular FACS (Fig 3.5). Anti-CD3 stimulation revealed sFliC immunisation unlike STm, failed to induce IFN γ in WT CD4 T cells, as levels were equivalent to NI mice in terms of both the proportion and total CD4 T cells. IFN γ production in mice infected with STm was almost entirely induced in effector (CD62L^{lo}) CD4 T cells, agreeing with a previous study (Gaspal et al., 2008). To examine antigen-specific responses, cells were re-stimulated with sFliC which reflected these findings, but levels of IFN γ from STm-infected mice were half that observed after anti-CD3 stimulation. This reflects previous studies that flagellin is a dominant antigen in the CD4 T cell response to STm, as almost half the activated T cells isolated from STm-infected mice were responding to

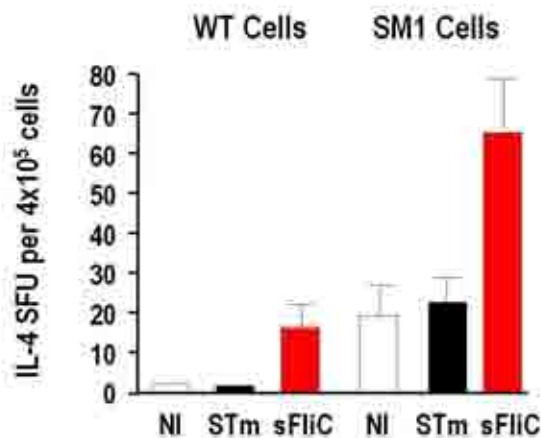
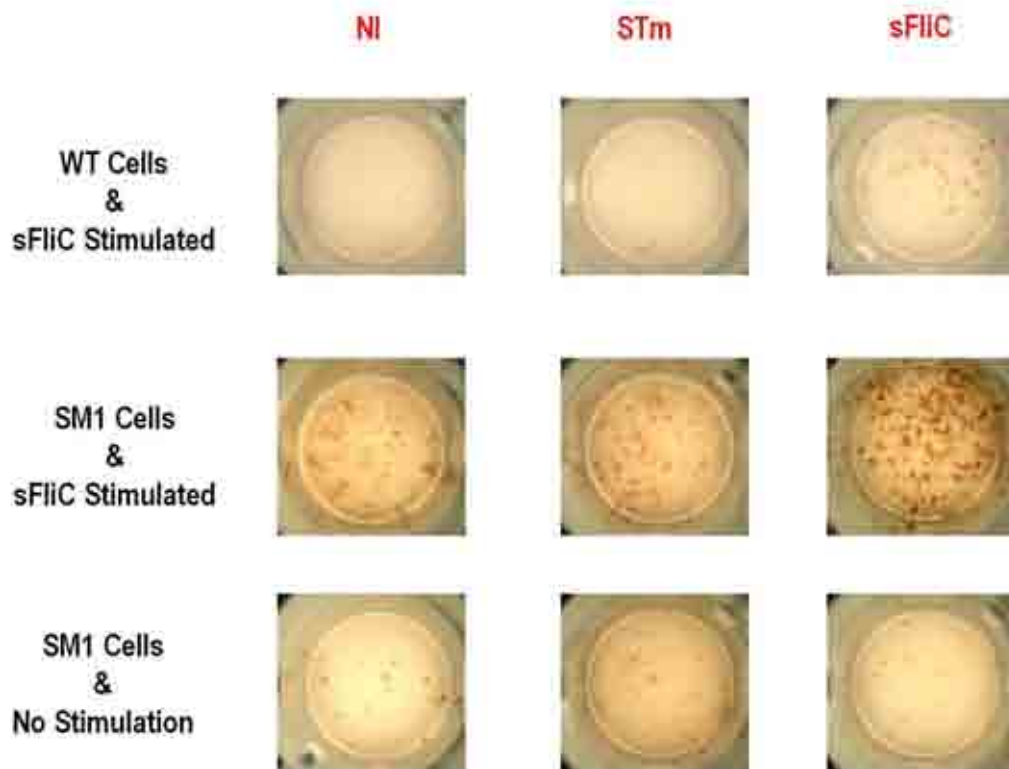


Figure 3.4

Immunisation with flagellin induces IL-4 protein as detected by ELISPOT: Total splenocytes from SM1 mice were adoptively transferred intraperitoneally into Rag1-deficient mice. Chimeras were immunised intraperitoneally 24 hours later with 20 μ g sFlIC or 5×10^5 STm for 7 days alongside WT mice. At sacrifice splenocytes were isolated from these mice alongside non-immunised counterparts. 4×10^5 splenocytes were re-stimulated for 48 hours with sFlIC or left non-stimulated before numbers of IL-4 SFC were enumerated by ELISPOT. Data is representative of 2 independent experiments and has been generated in conjunction with Dr. A. Flores-Langarica.

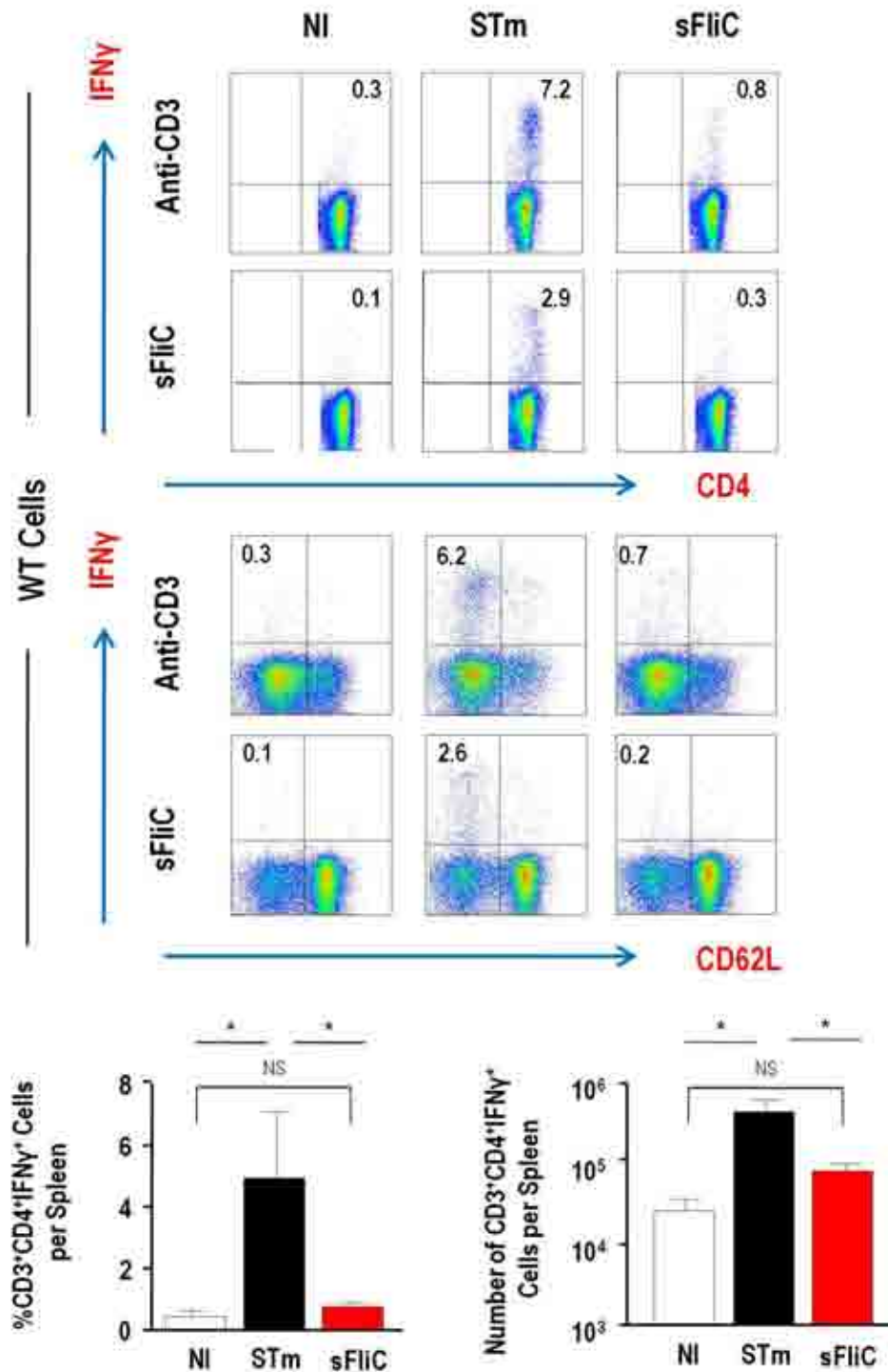


Figure 3.5

Immunisation with flagellin does not induce IFN γ in WT CD4 T cells unlike STm: WT mice were immunised intraperitoneally with 5×10^5 STm or 20 μ g sFliC. Splenocytes were isolated from these mice alongside non-immunised mice at day 7 post-immunisation. 5×10^6 splenocytes were stimulated in vitro for 6 hours and the proportion of IFN γ quantified by FACS. The first and second rows show IFN γ in total CD4 T cells after re-stimulation with anti-CD3 or sFliC respectively. The third and fourth rows show IFN γ expression in effector CD62L^{lo} CD4 T cells after re-stimulation with anti-CD3 or sFliC respectively. Graphs show IFN γ expression in total CD4 T cells after anti-CD3 stimulation. This data has been generated in conjunction with Dr. A. Flores-Langarica and is representative of at least two independent experiments with 4 mice per group. * $p < 0.05$ and NS = Non-significant.

flagellin (Alaniz et al., 2006; McSorley et al., 2000). Responses were then examined in SM1 T cells following sFliC immunisation using a similar experimental design in SM1-WT chimeras which were generated by adoptive transfer of SM1 cells into WT mice (Fig 3.6). As expected, this showed a marked induction of IFN γ in chimeras immunised with STm, but little IFN γ in SM1 T cells after sFliC immunisation. The levels of IFN γ induced in SM1-WT chimeras were similar regardless of the stimulating conditions. Therefore, almost all of the activated SM1 T cells were responding to flagellin.

To address if the low proportion of IFN γ -producing CD4 T cells induced after sFliC immunisation reflected levels induced to other Th2 antigens, responses were assessed after immunisation with the model Th2 antigen alum-precipitated ovalbumin in WT and OVA-specific CD4 T cells. WT-OTII chimeras were generated by adoptive transfer of splenocytes from transgenic OVA-specific CD4 T cells (OTII T cells) into WT mice (Fig 3.7). Chimeras received, 24 hours post-transfer, 5×10^5 of an OVA-expressing strain of STm (Sal OVA) or 50 μ g alum-precipitated ovalbumin. Splenocytes were isolated 7 days post-immunisation and were re-stimulated in-vitro with anti-CD3 in the presence of anti-CD28. IFN γ production was assessed in antigen specific CD45.1 cells which were sorted from host CD45.2 cells by FACS. Immunisation with alum-precipitated ovalbumin failed to induce IFN γ relative to mice infected with Sal OVA as reflected in the fraction and number of CD4 T cells. Therefore, similar levels of IFN γ were induced in WT or OTII CD4 T cells by alum-precipitated ovalbumin as observed in WT or SM1 CD4 T cells in response to sFliC.

3.3.3 Mice immunised with polymeric flagella fail to induce IFN γ

To test if the Th2 features observed after sFliC immunisation would be maintained when using the native polymeric antigen, responses were examined in WT mice immunised with 20 μ g polymeric flagella filaments isolated from the surface of STm or infected with 5×10^5 STm (Fig 3.8). Splenocytes were isolated 7 days post-immunisation and were re-stimulated in vitro with anti-CD3 or sFliC in the presence of anti-CD28 and IFN γ production was measured by intracellular FACS. This revealed mice

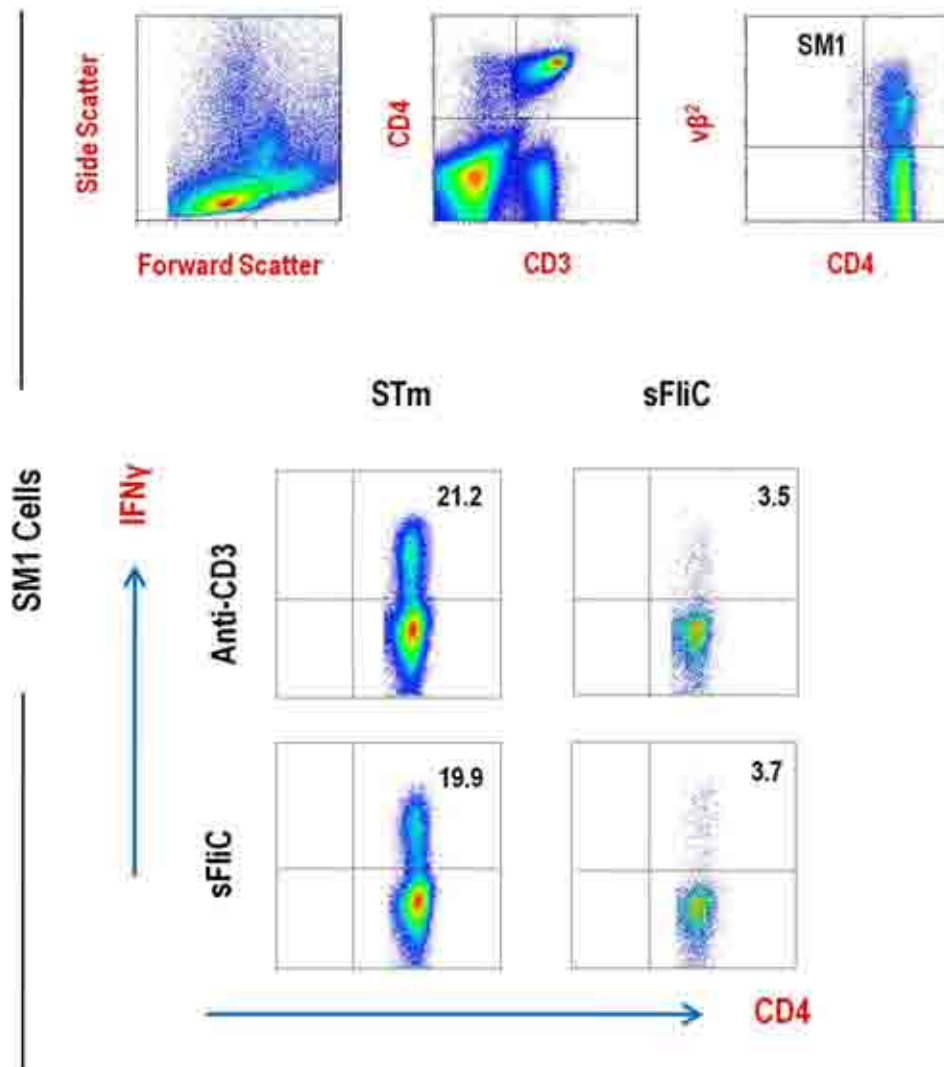


Figure 3.6

Immunisation with flagellin fails to induce IFN γ in SM1 cells: Chimeras were generated by adoptive transfer of total splenocytes intraperitoneally from flagellin-specific SM1 mice into WT mice. Chimeras were immunised intraperitoneally 24 hours later with 5×10^5 STm or $20 \mu\text{g}$ sFliC. Splenocytes were isolated 7 days post-immunisation and 5×10^6 cells were re-stimulated in vitro for 6 hours. Splenic CD4 $^+$ T cells were gated from the lymphocyte population and IFN γ expression in SM1 CD4 T cells quantified by FACS after anti-CD3 (first row) or sFliC (second row) re-stimulation in the presence of anti-CD28. This data has been generated in conjunction with Dr. A. Flores-Langarica and is representative of at least two independent experiments with 4 mice per group.

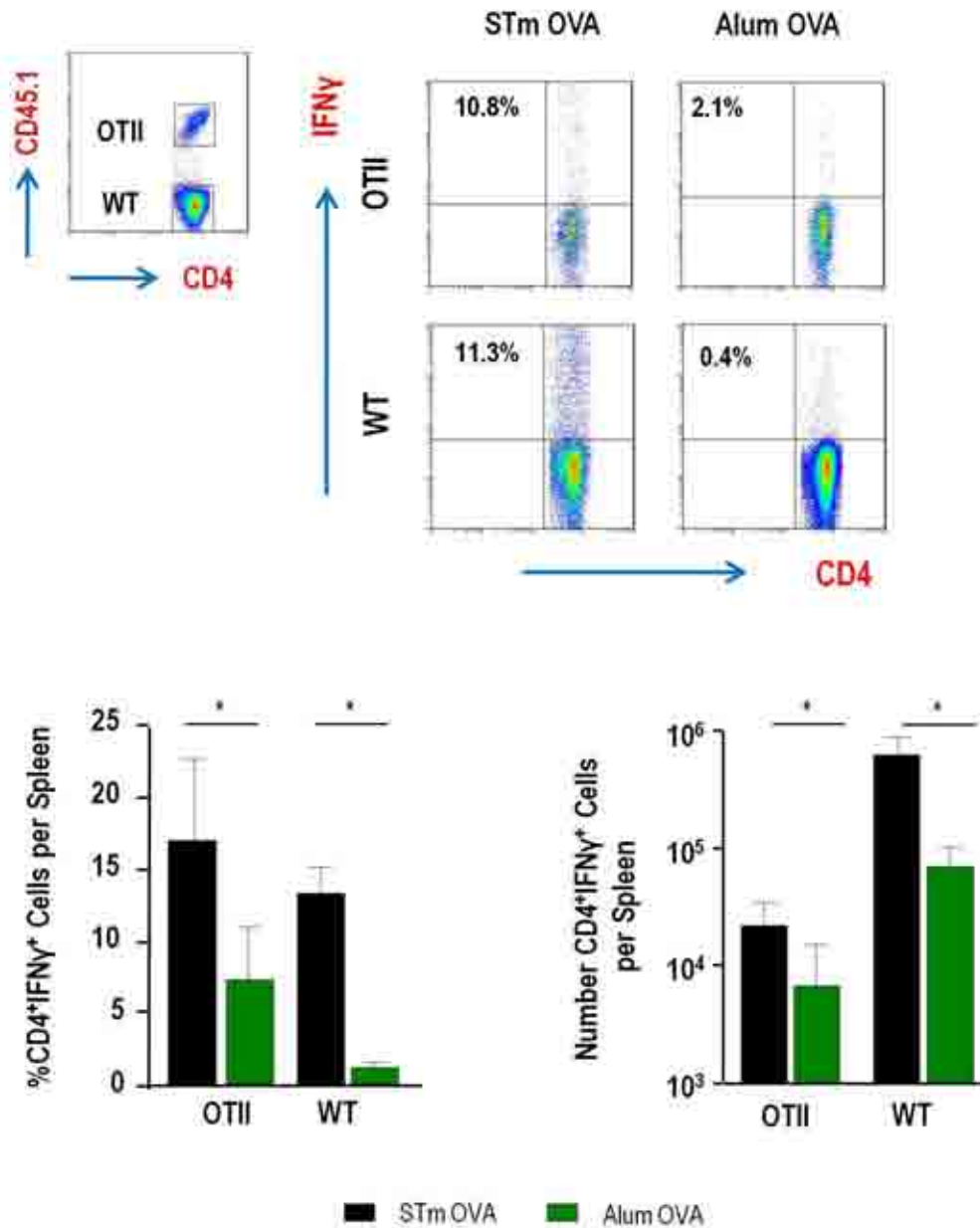


Figure 3.7

Flagellin and alum-precipitated ovalbumin fail to induce IFN γ : Splenocytes from OTII BoyJ mice were adoptively transferred intraperitoneally into WT mice and chimeras immunised intraperitoneally the following day with 5×10^5 STm expressing ovalbumin (STm OVA) or 50 μ g alum-precipitated OVA. 7 days post-immunisation 5×10^6 splenocytes were re-stimulated in-vitro for 6 hours with anti-CD3 in the presence of CD28 and IFN γ was quantified by FACS in OTII (top row) and WT CD4 T cells (bottom row). Graphs shows proportion and number of CD4⁺ T cells producing IFN γ . Data is representative of groups of 4 mice and at least two independent experiments. * P<0.05

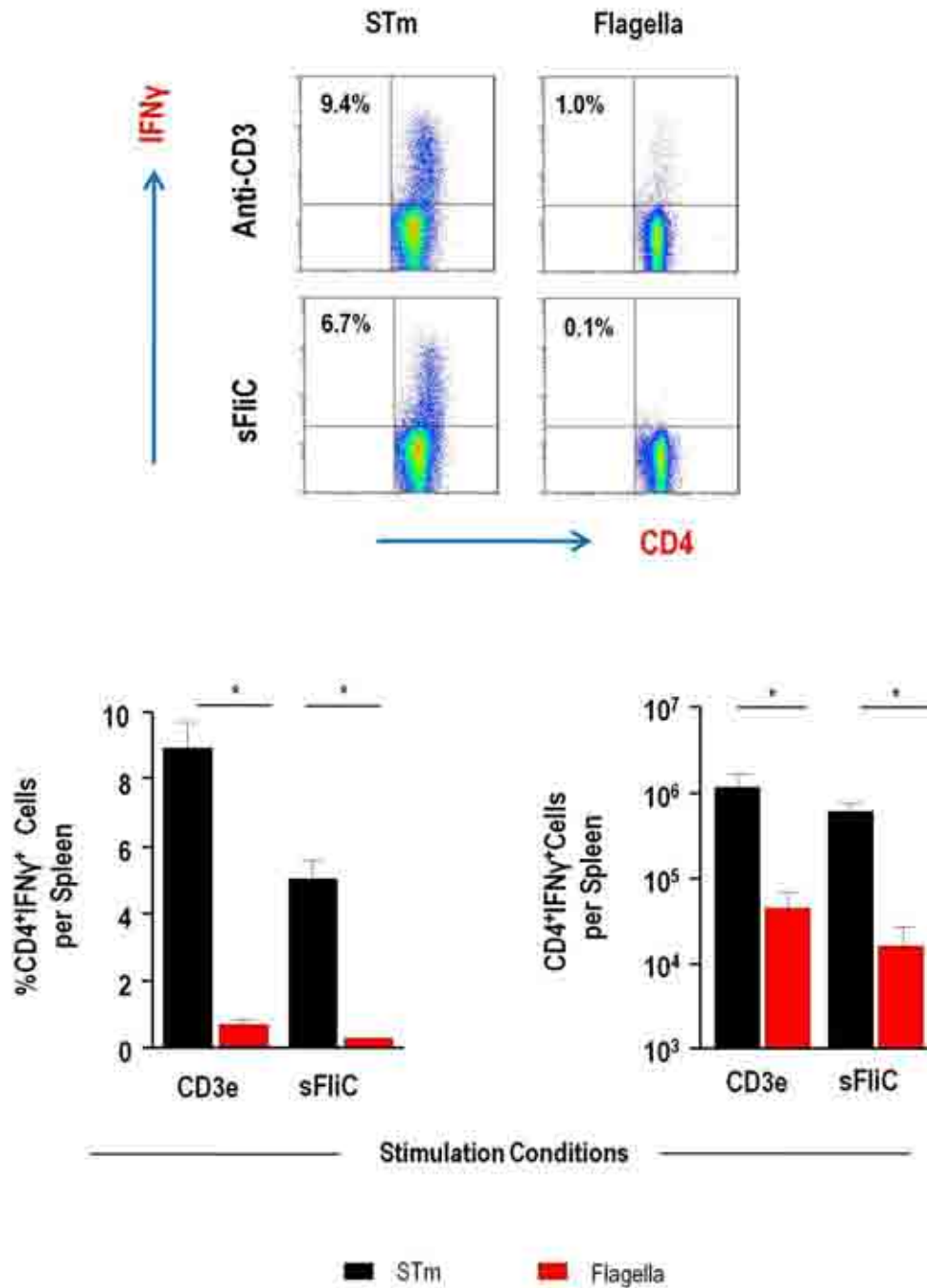


Figure 3.8

Failure to induce IFN γ is not influenced by the conformation of flagellin: WT mice were immunised intraperitoneally with 5×10^5 STm or 20 μ g polymeric, surface-isolated flagella. Splenocytes were isolated 7 days post-immunisation and 5×10^6 splenocytes were re-stimulated in-vitro for 6 hours. IFN γ was quantified by FACS after anti-CD3 (top) or sFliC (bottom) re-stimulation in the presence of anti-CD28. Data is representative of groups of 4 mice and at least two independent experiments. * P < 0.05

immunised with polymeric flagella filaments also failed to induce detectable levels of IFN γ relative to mice infected with STm. This suggested that in terms of IFN γ production, CD4 T cell responses in mice immunised with the polymeric isolate was similar to mice immunised with the monomeric protein. Therefore, the source (surface isolated or a recombinant protein) and conformation (polymeric or monomeric) of flagellin is not responsible for the failure to produce IFN γ .

3.3.4 Immunisation with sFliC fails to induce T-bet

Although IFN γ is the Th1 type cytokine, not all Th1 cells produce this cytokine therefore, expression of the global Th1 regulator T-bet (Szabo et al., 2000) was examined. SM1-WT chimeras were generated as described previously. Chimeras were infected 24 hours post-transfer with 5×10^5 STm or immunised with 20 μ g sFliC intraperitoneally. Splenocytes were isolated 7 days post-immunisation from these chimeras alongside NI chimeras and stimulated with anti-CD3 or sFliC in the presence of anti-CD28, and intracellular T-bet expression was quantified in SM1 CD4 T cells by FACS (Fig 3.9). This revealed mice immunised with STm induced expression of T-bet from effector CD4 T cells, but T-bet expression was not seen in NI mice or mice immunised with sFliC. This lack of T-bet expression after sFliC immunisation reflected the failure to induce IFN γ production.

3.3.5 The sFliC-Th2 response is stable in the absence of IL-4R α signalling.

The stability of this sFliC-driven Th2 response was then assessed using IL-4R α -deficient mice. WT and IL-4R α deficient mice were immunised with sFliC for 4 days and CD3⁺CD4⁺ splenocytes were sorted into effector (CD62L^{lo}) and naïve (CD62L^{hi}) populations. Real-time RT-PCR was then used to measure mRNA expression of GATA-3 and IL-4 (Fig 3.10A). This showed that sFliC selectively upregulated expression of GATA-3 and IL-4 mRNA in the effector CD4 T cell population, and the response was equivalent between WT and IL-4R α deficient mice. To assess whether T-bet was required for the induction of IL-4 after sFliC immunisation the experiment was repeated in WT and T-bet deficient mice

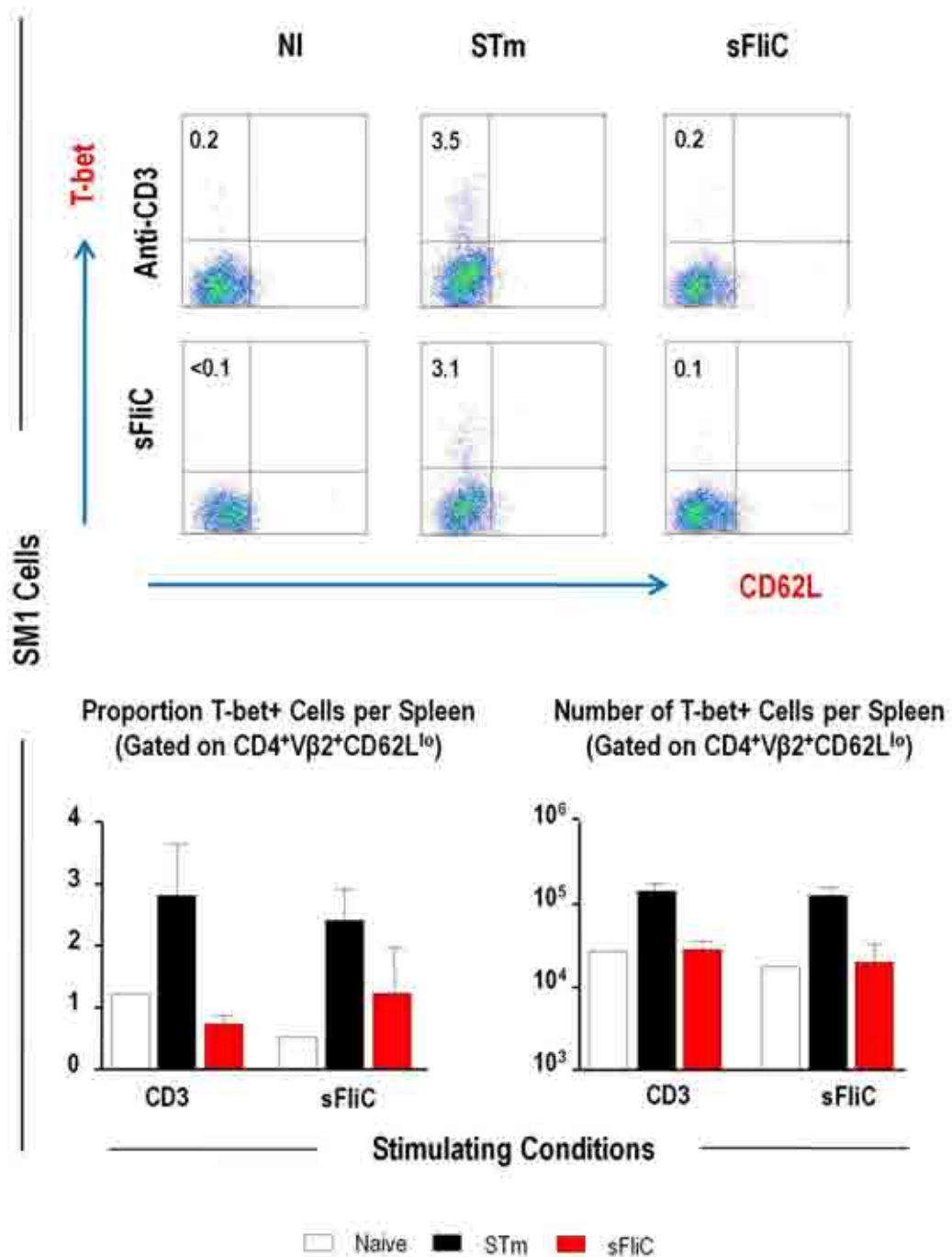
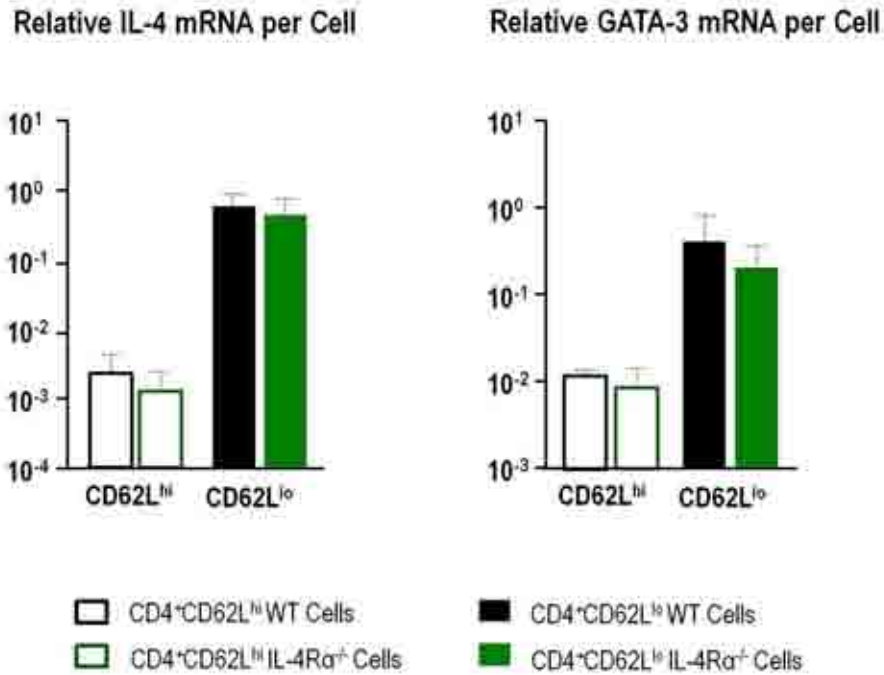


Figure 3.9

Immunisation with flagellin does not induce the global Th1 regulator T-bet: Representative FACS plots of T-bet expression in SM1 T cells from SM1-WT chimeras. Chimeras were generated by adoptive transfer of total splenocytes (intraperitoneally) from SM1 mice into WT mice. They were infected intraperitoneally 24 hours later with 5×10^5 STm or immunised with $20 \mu\text{g}$ sFliC. Splenocytes were isolated 7 days post-immunisation and 5×10^6 cells were re-stimulated in-vitro for 6 hours. T-bet expression was quantified in splenic SM1 CD4⁺CD62L^{lo} T cells after anti-CD3 (top row) or sFliC (bottom row) re-stimulation in the presence of CD28. This data has been generated in conjunction with Dr. A. Flores-Langarica and is representative of at least 2 independent experiments with 4 mice per group.

A



B

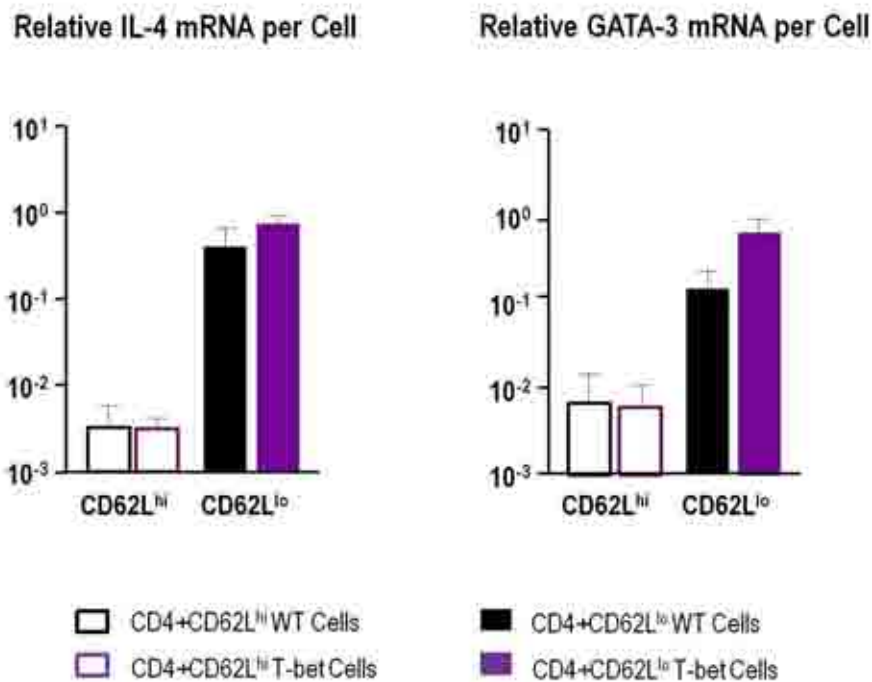


Figure 3.10

Flagellin drives a stable Th2 response: (A) WT (black) and IL-4Rα deficient (green) or (B) WT and T-bet deficient (purple) mice were immunised intraperitoneally with 20 μg sFlC for 4 days. Splenocytes were isolated and CD4⁺ T cells were sorted into naïve CD62L^{hi} (open bars) and effector CD62L^{lo} (closed bars) populations. Expression of the Th2 markers: GATA-3 and IL-4 was quantified by real-time RT-PCR. Data is representative of 4 mice per group.

(Fig 3.10B). This revealed that both IL-4 and GATA-3 mRNA was strongly induced from effector CD4 T cells and was comparable between the mice.

Previously, it was demonstrated that immunisation with soluble flagellin failed to induce switching to IgG2a. The data presented here show that sFliC drives a CD4 T cell response that reflects Th2 switching in vivo. This is not influenced by whether flagellin is presented in a monomeric or polymeric form or whether it is administered as a recombinant protein or isolated from the surface of STm. Furthermore, this study highlights that this potent sFliC driven Th2 response is stable in the absence of IL-4 and IL-13 signalling through IL4-R α , and does not require T-bet for its induction.

3.4 Co-immunisation of sFliC and STm

3.4.1 Soluble FliC can suppress IFN γ induced by STm

Since sFliC and STm can induce potent Th2 and Th1 responses respectively, the response when both antigens were given together was examined. SM1-WT chimeras were generated as described earlier which were infected 24 hours post-transfer with 5×10^5 STm or immunised with 20 μ g sFliC, or co-immunised with both. Splenocytes were isolated 7 days post-immunisation and re-stimulated in-vitro with anti-CD3 in the presence of anti-CD28, and the proportion of IFN γ in endogenous (WT) and antigen-specific (SM1) CD4 T cell populations was quantified by FACS (Fig 3.11). As expected, immunisation with STm induced IFN γ , but sFliC did not. Surprisingly, chimeras which were co-immunised with sFliC and STm, had a significantly lower proportion of IFN γ CD4 T cells than chimeras which were only infected with STm. This pattern of IFN γ was similar in WT and SM1 CD4 T cells, and thus, not an intrinsic property of transgenic SM1 T cells.

To assess whether this reflected a general suppression of Th1 responses by Th2 antigens and thus, a common property of Th2 immunogens, the experiment was repeated using WT-OTII chimeras which were generated as previously earlier. Chimeras were infected 24 hours post-transfer with 5×10^5 STm

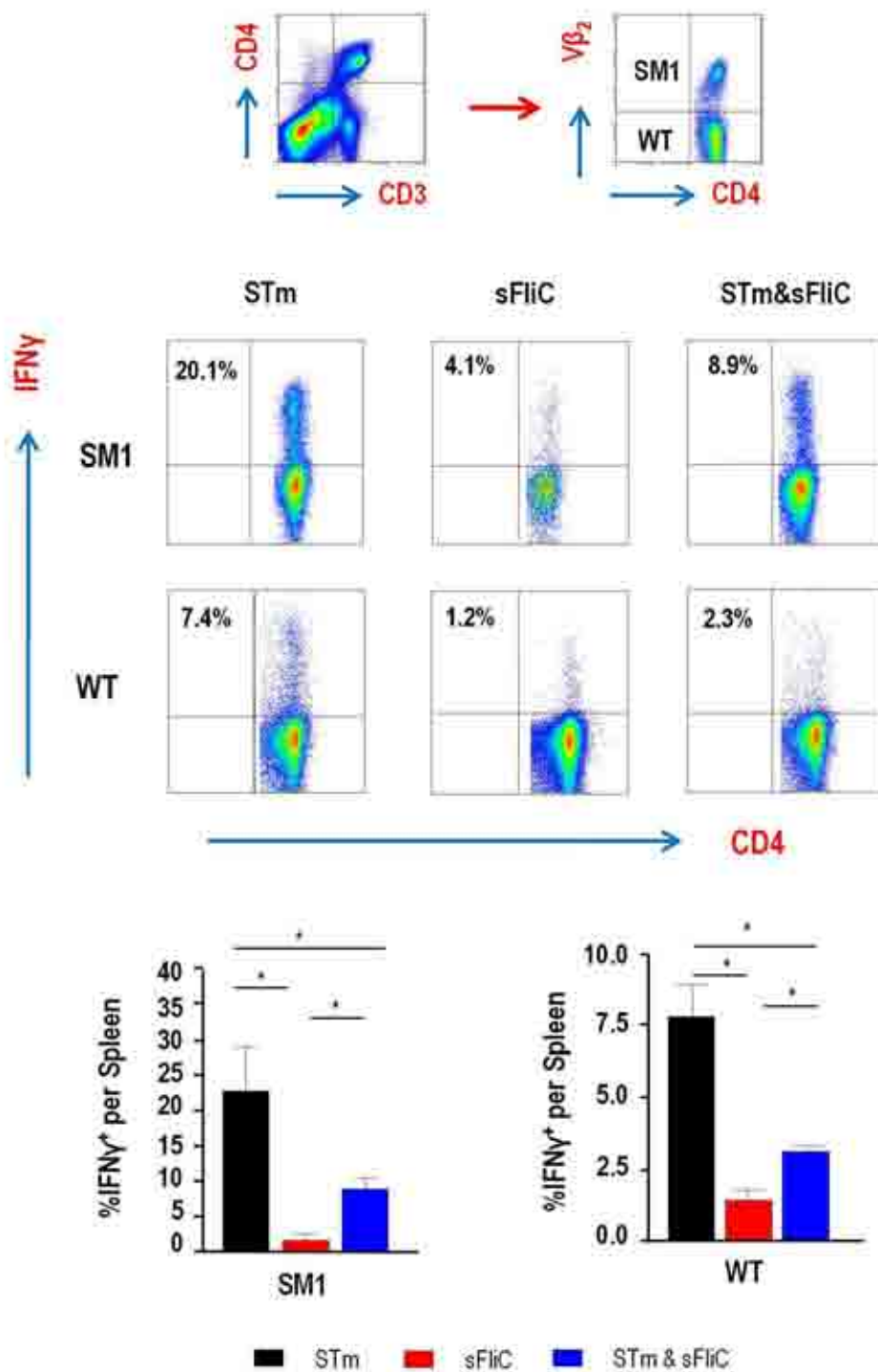


Figure 3.11

Flagellin immunisation suppresses IFN γ induced in response to STm: Splenocytes from flagellin-specific SM1 mice were adoptively transferred (intraperitoneally) into WT mice and chimeras immunised the following day with either 5×10^5 STm, $20 \mu\text{g}$ sFliC or both intraperitoneally. Splenocytes were isolated at day 5 post-immunisation and 5×10^6 cells were stimulated in-vitro with anti-CD3 in the presence of anti-CD28 for 6 hours. SM1 (top row) and endogenous (bottom row) CD4 T cells were then gated from the lymphocyte population and IFN γ expression quantified by intracellular FACS. Plots and graphs are representative of 4 mice per group and three experiments. * $P < 0.05$

OVA or immunised with 50 µg alum precipitated ovalbumin, or co-immunised with both and responses were assessed 7 days post-immunisation (Fig 3.12). The suppression of IFN γ observed in SM1-WT chimeras co-immunised with STm and sFliC was not observed in WT or OTII CD4 T cells isolated from mice which were co-immunised with STm OVA and alum-precipitated ovalbumin, indicating this was not a general phenomenon. Thus, immunisation with sFliC suppresses the Th1-induced IFN γ in response to STm.

3.4.2 IFN γ suppression in response to sFliC is independent of IL-10

One way the IFN γ response may be suppressed was through a general suppression of Th1 responses by means of IL-10 (Bai et al., 1997). This cytokine is induced by both STm and sFliC (data not shown) and can be produced by numerous innate and adaptive cell types. To address the role of IL-10 the CD4 T cell response to sFliC in WT and IL-10 deficient mice immunised with 20 µg sFliC was first assessed. Quantification of total CD4 T cell populations in the spleen 7 days post-immunisation revealed a lower proportion of CD4 T cells in IL-10 deficient mice relative to WT mice, but similar numbers (3.13A) whilst little differences were seen in the activation status of these CD4 T cells (Fig 3.13B).

Responses in IL-10 deficient mice which were either infected with 5×10^5 STm, immunised with 20 µg sFliC, or co-immunised with STm and sFliC were then examined. Assessment of the splenic CD4 T cell response 7 days post-immunisation revealed mice immunised with sFliC had a greater proportion, but not number of CD4 T cells relative to mice immunised with STm or STm and sFliC (Fig 3.14A). Analysis of CD4 T cell activation demonstrated sFliC immunised mice had a lower proportion of effector T cells than mice infected with STm, but the proportion was equivalent to co-immunised mice, whilst numbers were broadly equivalent between the three groups (Fig 3.14B). Lastly, splenocytes were stimulated *in vitro* with anti-CD3 in the presence of anti-CD28 and intracellular IFN γ was quantified by FACS (Fig 3.14C). This demonstrated the proportion and number of IFN γ producing CD4 T cells was significantly lower in co-immunised mice, relative to mice only infected with STm, reflecting earlier observations in

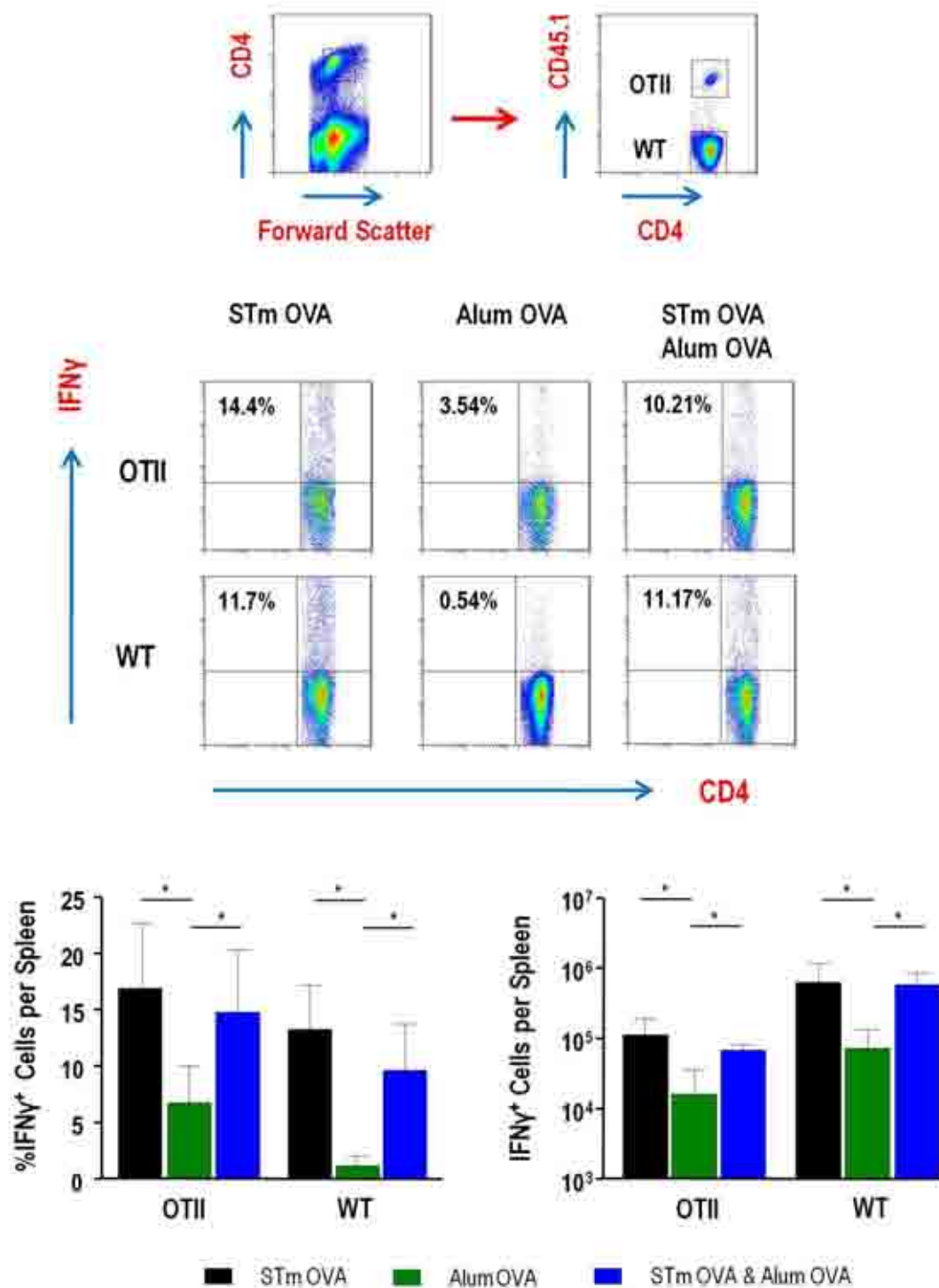


Figure 3.12

Alum^{pp1} ovalbumin does not suppress IFN γ induced in response to STm upon co-immunisation: Splenocytes from transgenic OTII BoyJ mice were transferred intraperitoneally into WT mice and chimeras immunised the following day with either 5×10^5 STm OVA, $50 \mu\text{g}$ Alum^{pp1} ovalbumin or both intraperitoneally. Splenocytes were isolated 7 days post-immunisation and 5×10^6 cells were stimulated in-vitro with anti-CD3 in the presence of anti-CD28 for 6 hours. Splenic OTII (top row) and endogenous (bottom row) CD4 T cells were then gated from the lymphocyte population and IFN γ expression quantified by intracellular FACS. Plots and graphs are representative of 4 mice per group and at least two independent experiments. * $P < 0.05$

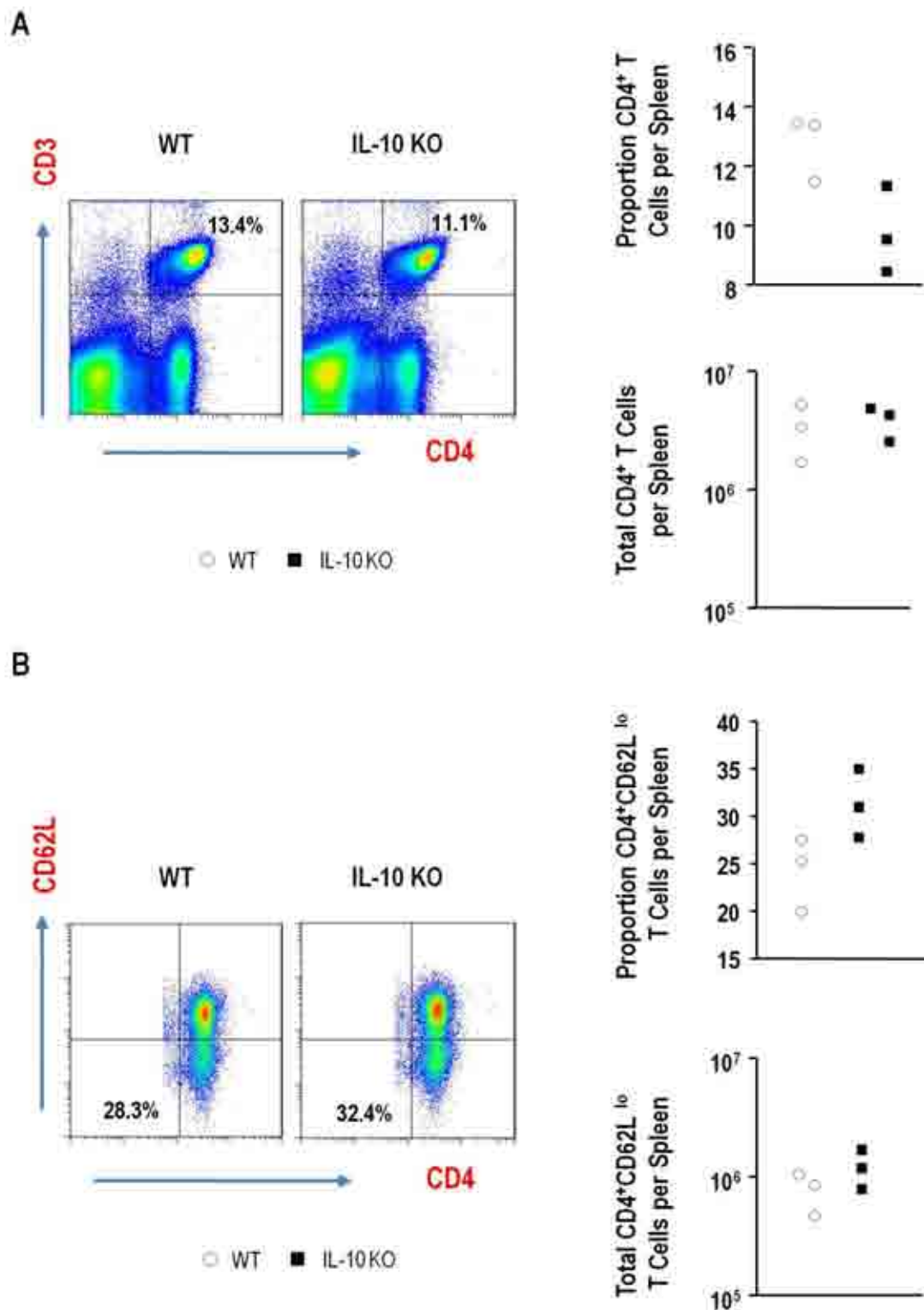


Figure 3.13

IL-10 does not influence the CD4 T cell response to sFliC; WT and IL-10 deficient mice were immunised intraperitoneally with 20 μ g sFliC. Splenocytes were isolated 7 days post-immunisation and CD4 T cells gated from the lymphocyte population (not shown). The proportion and total number of (A) CD4 T cells and (B) effector CD62L^{lo} CD4 T cells were quantified by FACS.

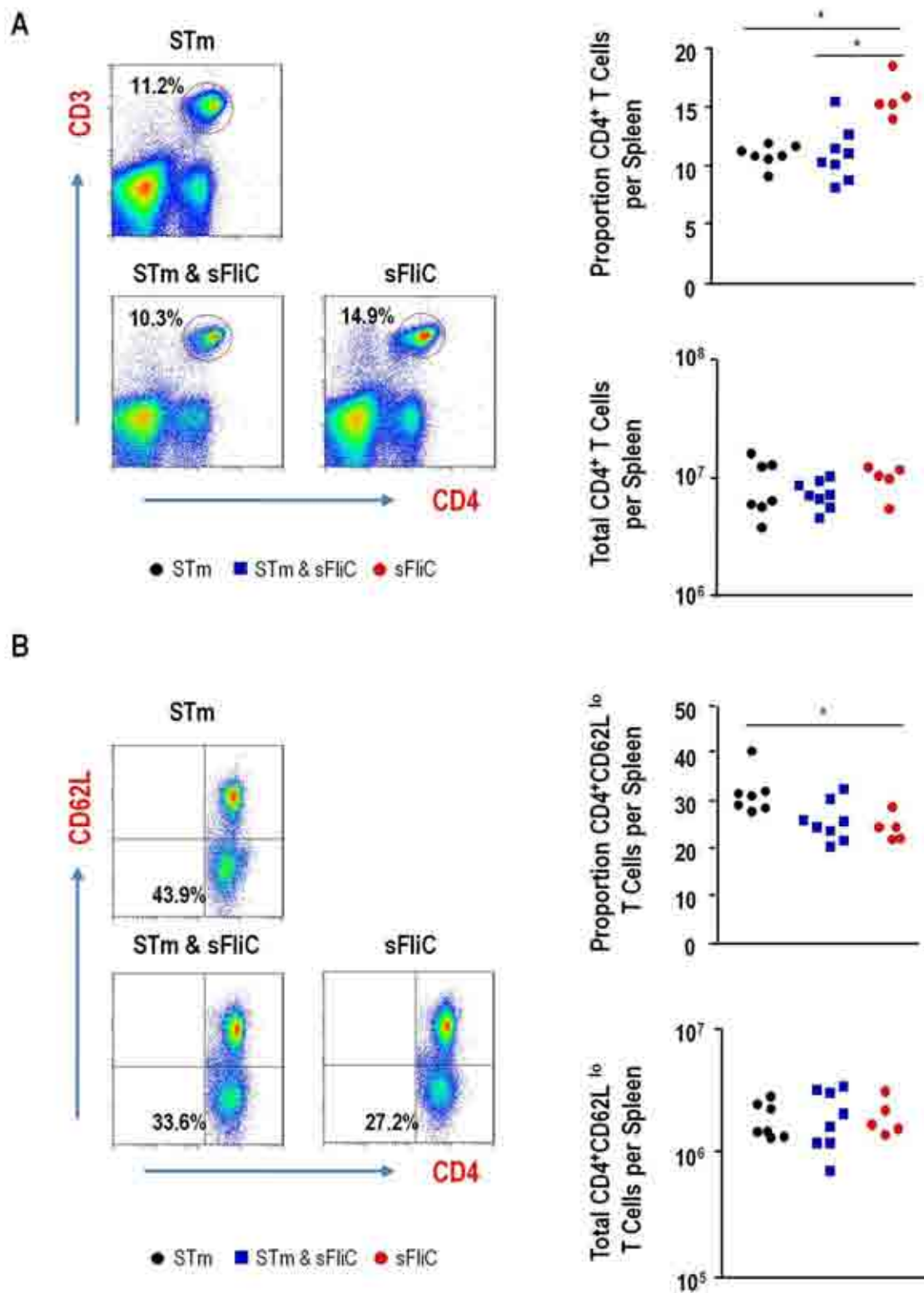


Figure 3.14

IL-10 does not influence the CD4 T cell response in STm and sFlIC co-immunised mice: IL-10 deficient mice were immunised intraperitoneally with 5×10^5 STm or $20 \mu\text{g}$ sFlIC or both. Splenocytes were isolated 7 days post-immunisation and CD4 T cells gated from the lymphocyte population (not shown). The proportion and number of (A) CD4 T cells and (B) effector CD62L^{lo} T cells were quantified by FACS. * $P < 0.05$

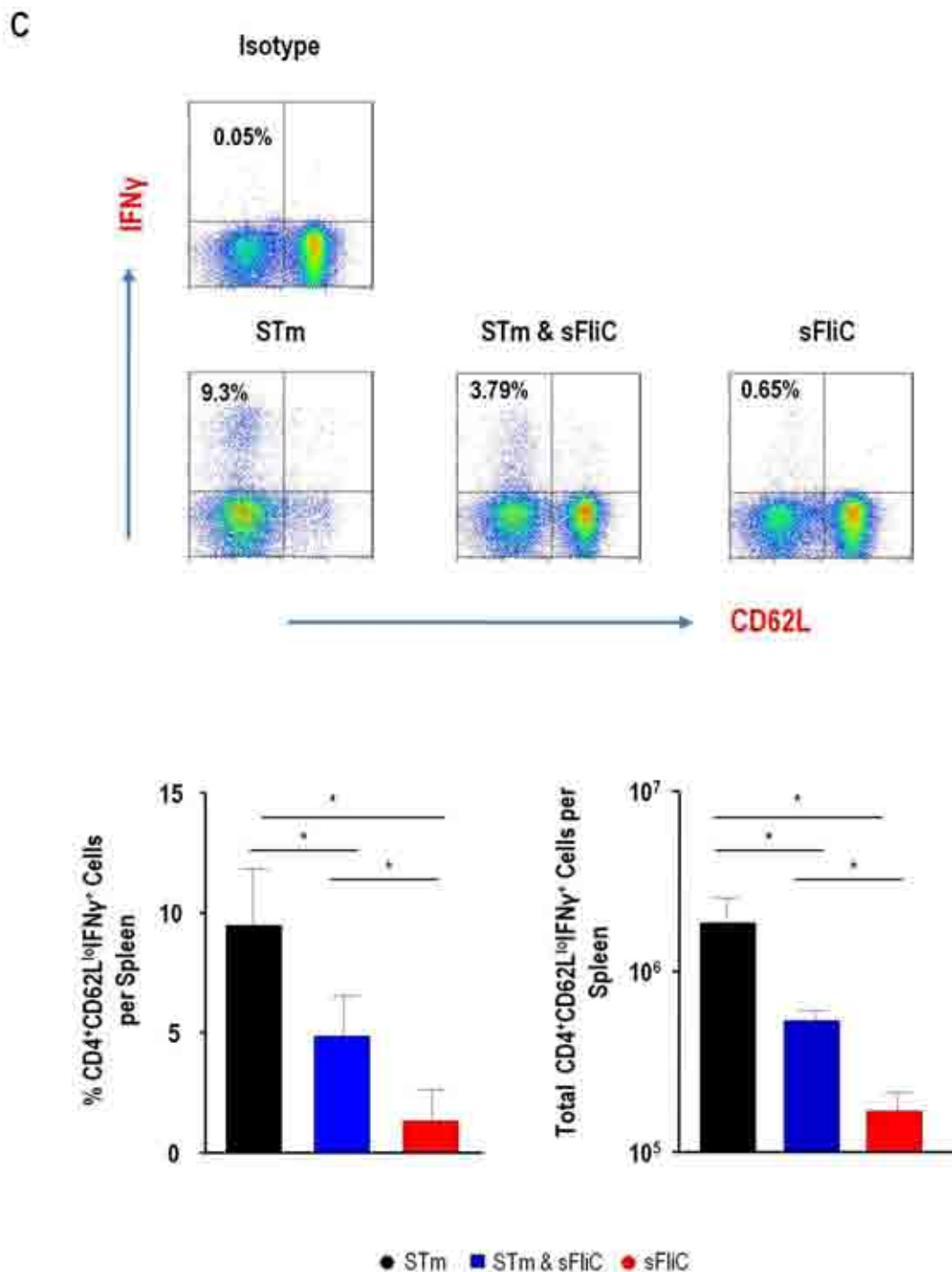


Figure 3.14

IL-10 does not regulate the suppression of IFN γ in STm and sFliC co-immunised mice: IL-10 deficient mice were immunised intraperitoneally with 5×10^5 STm or 20 μ g sFliC or both. (C) Splenocytes were isolated 7 days post-immunisation and 5×10^6 cells were re-stimulated in-vitro with sFliC in the presence of anti-CD28 for 6 hours. CD4⁺CD62L^{lo} T cells were then gated from the lymphocyte population and expression of IFN γ was quantified by intracellular FACS. Representative FACS plots are shown. This data has been generated in conjunction with Dr. A. Flores-Langarica and represents at least two independent experiments with 3-5 mice per group. * P < 0.05

WT mice. This suggested that the suppression of Th1 responses to STm upon co-immunisation with sFliC was independent of IL-10.

3.4.3 sFliC can suppress IFN γ during secondary responses to STm

Since resolution of STm requires Th1 responses and sFliC is capable of suppressing this response in primary infection models upon co-administration with STm, the impact of priming with STm and sFliC on the subsequent response to STm challenge was addressed. NI WT mice, and WT mice infected with 5×10^5 STm or co-immunised with 5×10^5 STm and 20 μ g FliC were subsequently challenged on day 35 with 5×10^6 STm (Fig 3.15A). Quantification of the splenic bacterial burden 5 days post-challenge revealed, both STm-primed and STm and sFliC co-primed mice had a significantly lower bacterial burden relative to non-primed mice. Interestingly, STm and sFliC co-primed mice consistently had a higher bacterial load than STm-primed mice (Fig 3.15).

Quantification of the CD4 T cell response in the spleen revealed the proportion of CD4 T cells was enhanced in both STm-primed and STm and sFliC co-primed mice relative to non-primed counterparts (Fig 3.15B). In the main, there were no differences in total CD4 and effector CD4 T cell responses between STm-primed and STm and sFliC co-primed mice. To further dissect the CD4 T cell response splenocytes were FACS sorted into naïve and effector CD4 populations for assessment of mRNA expression by real-time RT-PCR (Fig 3.16A). The relative expression of T-bet and IFN γ revealed the signal was largely detected from effector CD4 T cells, consistent with previous experiments (Fig 3.2). Interestingly, the relative expression of both T-bet and IFN γ was significantly lower in STm and sFliC co-primed mice in comparison to STm-primed mice, and was similar to non-primed mice. Analysis of relative IL-4 mRNA revealed no differences between non-primed and primed mice.

As an additional control, IFN γ was assessed by intracellular FACS following anti-CD3 re-stimulation in the presence of anti-CD28 (Fig 3.16B). This revealed CD4 T cells from STm-primed and STm and sFliC

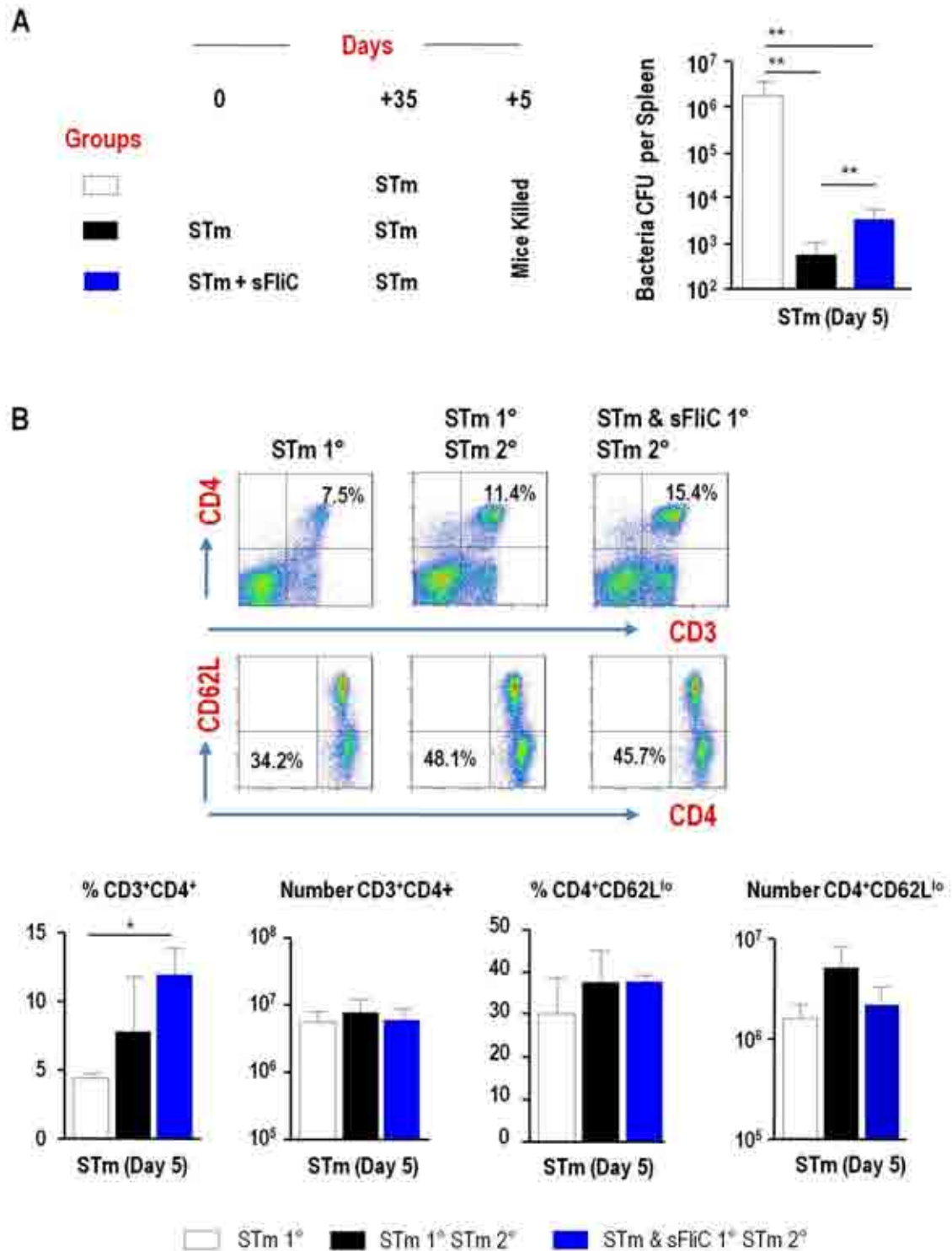


Figure 3.15

Co-immunisation and infection with STm and sFliC negatively influences protection afforded by Salmonella upon challenge: WT mice were immunised with 5×10^5 STm or STm and $20 \mu\text{g}$ sFliC for 35 days before subsequent challenge with 5×10^8 STm alongside non-immunised mice. (A) Splenic bacterial numbers were enumerated 5 days post-STm challenge. (B) Splenocytes were isolated and CD4⁺ (top row) and CD4⁺CD62L^{lo} (bottom row) T cell responses were quantified by FACS. Data is representative of 4-8 mice per group and two independent experiments. All immunisations were intraperitoneal. * $P < 0.05$ ** $P < 0.01$

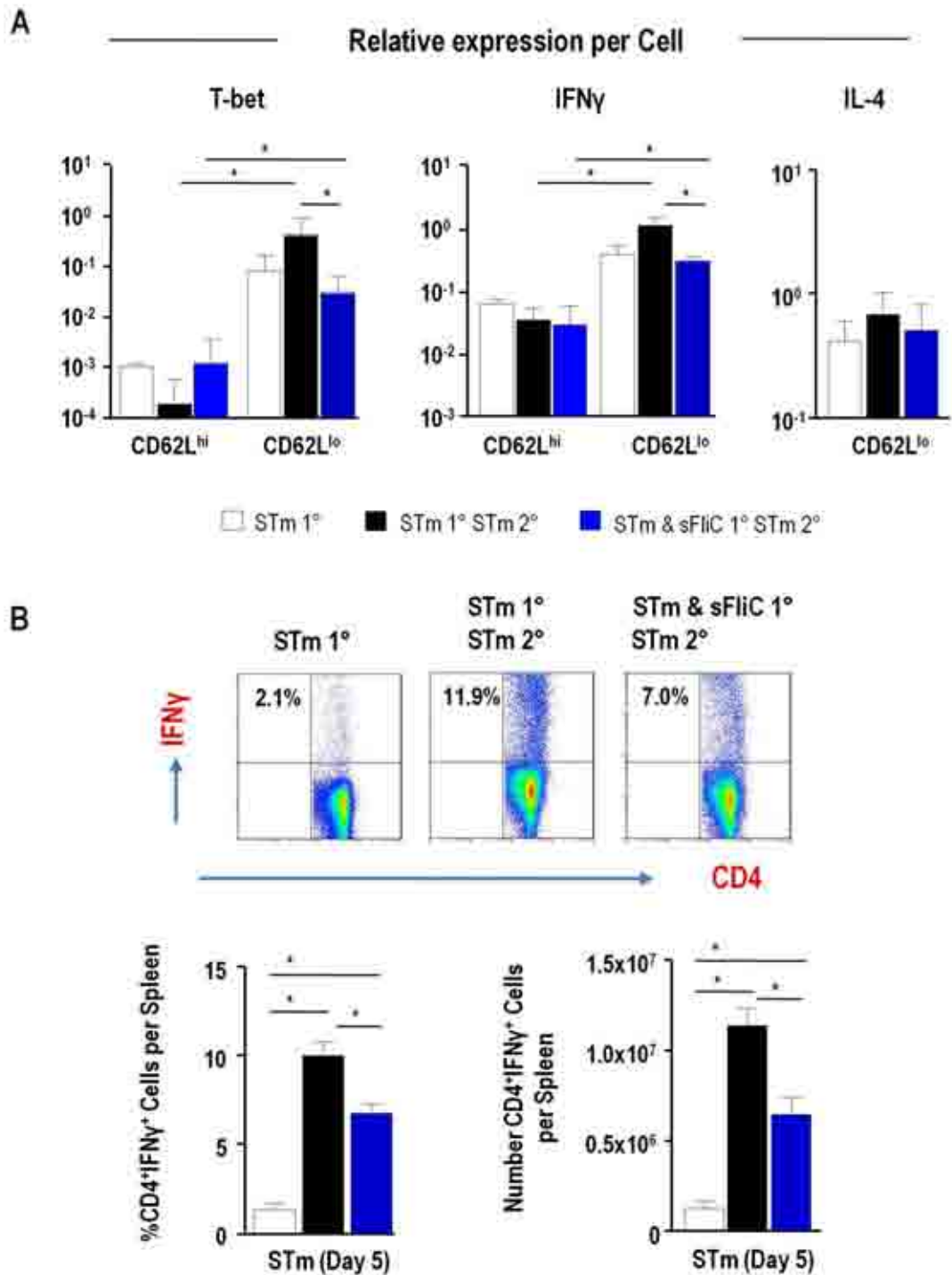


Figure 3.16

Co-immunisation with STm and sFliC suppresses the IFN γ response to STm upon challenge: (A) WT mice were immunised as described in Figure 3.15A and splenic CD4⁺ T cells were sorted into naïve (CD62L^{hi}) and effector (CD62L^{lo}) populations by FACS 5 days post-STm challenge. The relative expression of T-bet, IFN γ and IL-4 was measured by RT-PCR. (B) IFN γ was measured by FACS in these mice following in-vitro re-stimulation of 5×10^5 splenocytes for 6 hours with anti-CD3 in conjunction with Dr. A Flores-Langarica. Data is representative of 4-8 mice per group and two independent experiments. All immunisations were intraperitoneal *P<0.05

co-primed mice induced significantly more IFN γ compared to non-primed mice. Furthermore, as observed by RT-PCR the expression of IFN γ was significantly lower in STm and sFliC co-primed mice relative to STm-primed mice which was apparent both as a proportion and total numbers.

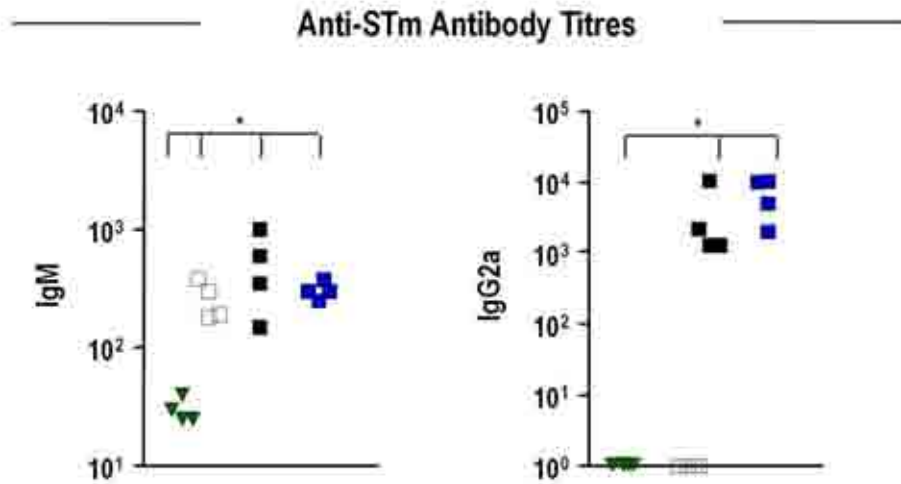
Finally, antibody responses in these mice post-challenge were assessed. Assessment of anti-STm and anti-sFliC IgM titres revealed no differences between the three groups (Fig 3.17). As expected, anti-STm IgG2a was only detected in STm-primed and STm and sFliC co-primed mice and titres were comparable, whilst anti-sFliC IgG2a and IgG1 antibody titres were only detected in STm and sFliC co-primed mice. This suggested, the impact of co-immunisation with STm and sFliC did not appear to impinge upon antibody switching.

These data show that sFliC can suppress IFN γ induced in response to primary and secondary STm infections upon co-administration. This IFN γ suppression by sFliC is not typical of co-administration of Th2 antigens and STm, but it is an intrinsic property of sFliC as shown by experiments using STm-OVA. Furthermore, this study shows that this suppression of IFN γ by sFliC is independent of IL-10 expression. Additionally, it demonstrates that co-immunisation with sFliC and STm can negatively affect the benefit afforded by STm immunisation upon challenge. This was independent of B cells and IL-4 expression, but was driven through this suppression of the Th1 response upon co-immunisation with sFliC and STm.

3.5 Immunisation with sFliC protects at discrete stages of a systemic STm infection

Since sFliC induces potent Th2 responses which can suppress IFN γ upon co-administration with STm negatively impacting on early clearance of STm from the tissues, whether priming with sFliC by itself would influence a subsequent systemic STm challenge in a vaccine model was investigated. WT mice were immunised intraperitoneally with 20 μ g sFliC and on day 35 they were challenged with 5×10^5 STm for 5, 18 or 35 days alongside non-primed WT mice (Fig 3.18A). Quantification of splenic and liver

A



B

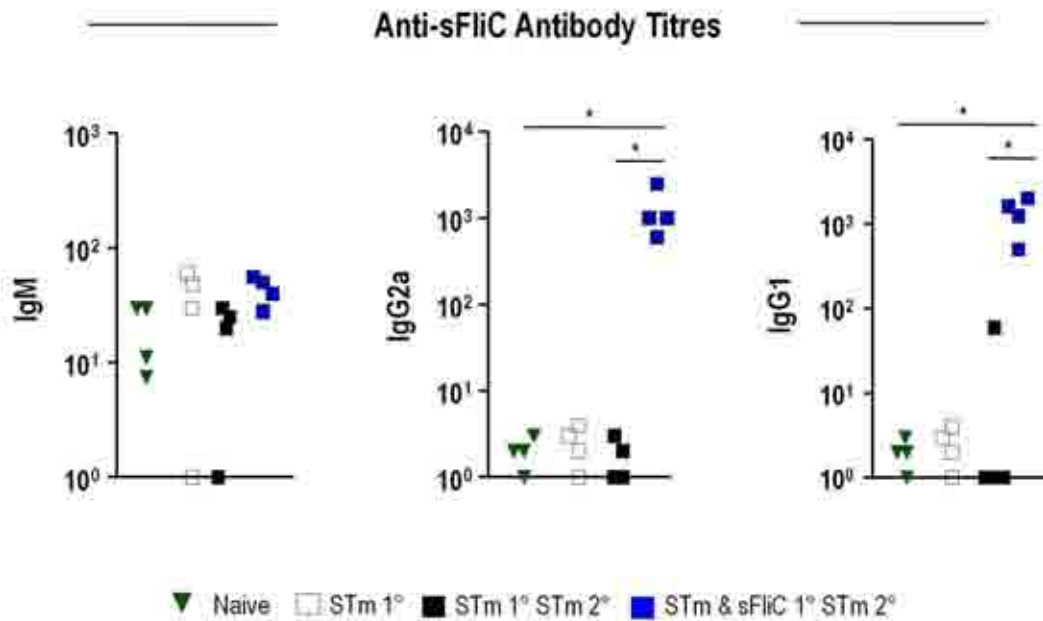


Figure 3.17

Co-immunisation with STm and sFlIC does not influence the anti-STm antibody response: (A) Anti-STm antibody titres for IgM and IgG2a and (B) anti-sFlIC antibody titres for IgM, IgG2a and IgG1 were quantified from non-immunised WT mice or WT mice immunised intraperitoneally for 35 days with 5×10^5 STm or STm and 20 μ g sFlIC before challenge with 5×10^5 STm for 5 days. Data is representative of 4 mice per group and two independent experiments. * $P < 0.05$

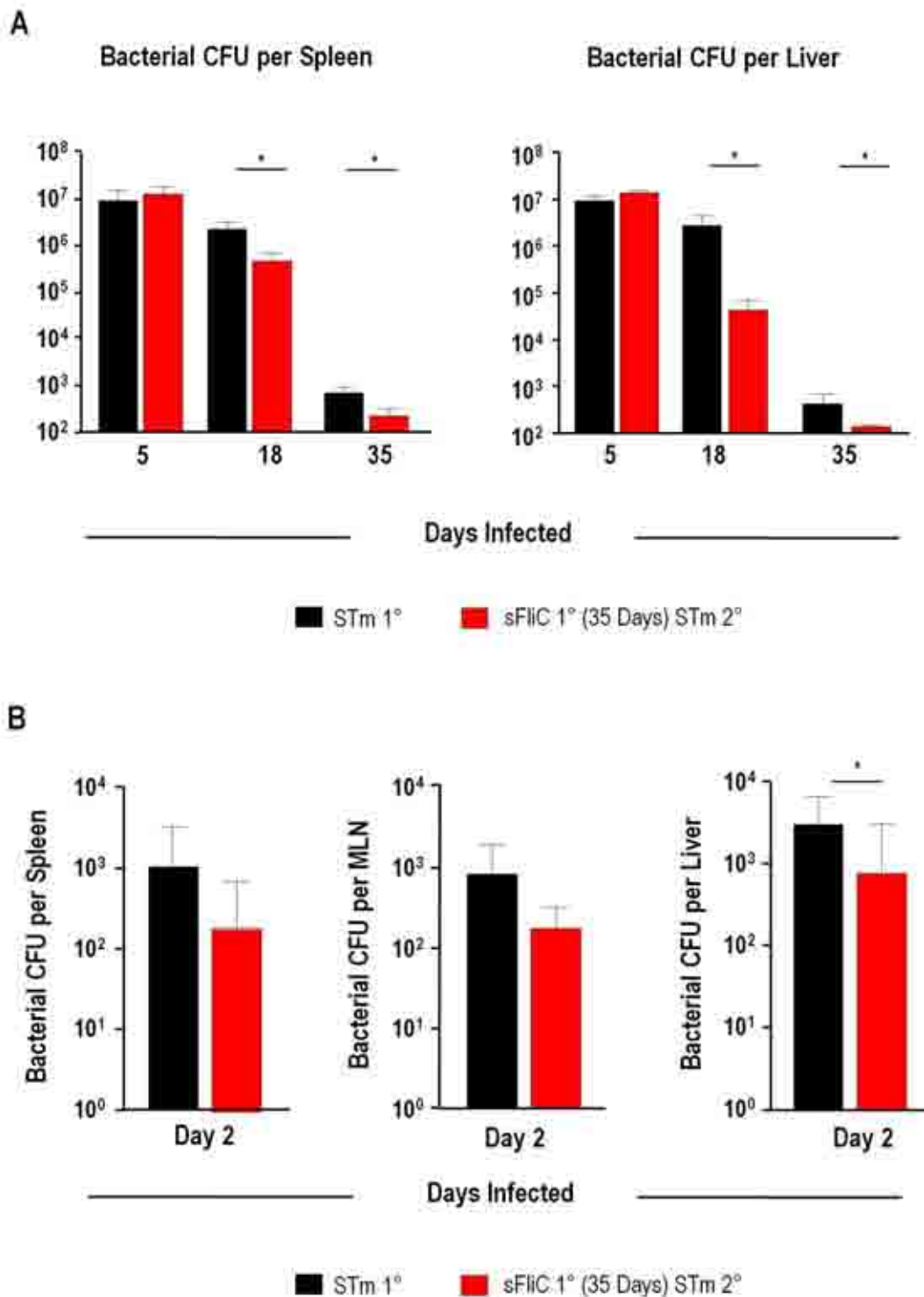


Figure 3.18

Priming with flagellin protects at discrete stages of infection: (A) Non-primed or sFliC-primed (35 days) WT mice were infected intraperitoneally with 5×10^5 STm for 5, 18 or 35 days and bacterial CFU were enumerated from the spleen and liver. (B) Non-primed or sFliC-primed (35 days) WT mice were infected orally with 1×10^9 STm (SL3261) for 2 days and bacterial CFU were enumerated from the spleen, MLN and Liver. Data is representative of at least 4 mice per group and two independent experiments. * $P < 0.05$

bacterial numbers revealed non-primed and sFliC-primed mice had a comparable bacterial load at day 5. In contrast, by day 18 and 35 sFliC-primed groups had significantly lower bacterial numbers relative to non-primed groups. Whether sFliC immunisation promoted mucosal immunity was also investigated. WT mice were immunised intraperitoneally with 20 µg sFliC and on day 35 mice were orally challenged with 10⁹ STm (SL3261) for 2 days. Bacterial numbers were enumerated from the spleen, mesenteric lymph nodes and liver (Fig 3.18B). This revealed that there was a small trend towards lower colonisation in these tissues in sFliC-primed mice relative to non-primed mice, but this did not reach significance despite the large group sizes (at least 7 mice per group), except for in the liver, where the difference just reached significance.

These data demonstrate that sFliC immunisation does not confer early benefits in controlling systemic STm infection. Nevertheless, despite inducing potent Th2 features which can suppress IFN γ in primary and secondary responses to STm, immunisation with sFliC accelerates bacterial clearance after the first week of a subsequent infection with STm.

3.6 Antibody to sFliC fails to control systemic STm infection

3.6.1 Antibody to sFliC can impair the motility of STm

Previous studies have shown that immunisation with heat-killed STm or subunit vaccines against STm can reduce systemic infection through antibody-mediated mechanisms, which are effective by day 5 after infection (Chapter four) (Gil-Cruz et al., 2009). Antibody in STm infections is likely to act to inhibit bacterial colonisation rather than clear the organism from infected tissues. The lack of early protection after infection of sFliC-immunised mice suggested that anti-sFliC antibody may not inhibit bacterial colonisation. To address this, properties of the anti-sFliC antibody response in sFliC-immunised mice were investigated.

WT mice were immunised intraperitoneally with 20 µg sFliC or 20 µg surface-isolated flagella for 35 days and serum anti-sFliC IgG1 and IgG2a antibody titres were assessed by ELISA (Fig 3.19A). This showed that both sFliC-immunised and flagella-immunised mice induced a potent IgG1 response relative to IgG2a at day 35 post-immunisation. Functional properties of the anti-sFliC antibody response were then investigated, including assessment of whether anti-sFliC antibody could cause loss of flagella from the bacterial surface. Flagellated FliC-locked STm (which only express FliC) and STm (which can express both FliC and FljB) were adsorbed with complement-inactivated (CI) sera from NI WT mice or WT immunised twice with sFliC for 14 days. Following incubation, bacteria were harvested and analysed by immunoblot analysis (Fig 3.19B). This demonstrated the binding of an anti-FliC monoclonal antibody to flagella attached to FliC-locked STm which had been absorbed with either NI or sFliC-specific sera. Furthermore, the immunoblot of STm adsorbed with sFliC-specific sera revealed binding of the anti-FliC monoclonal. This suggested that flagella were not shed from the bacterial surface upon binding of anti-sFliC antibody.

Whether anti-sFliC antibody could impair the motility of STm was then examined. CI sera from NI or sFliC-immunised WT mice was mixed with semi-solid agar prior to inoculation with STm, and the effect of antibody on bacterial motility was assessed by measuring the colony radius from the point of inoculation (Fig 3.19C). This demonstrated that bacterial swimming through agar containing sFliC-specific antibody was reduced relative to swimming in agar containing sera from NI mice. Thus, sFliC promotes antibody responses that can limit bacterial motility.

3.6.2 Antibody to sFliC does not impair early colonisation with STm

The influence of sFliC-immunisation on inducing protective antibody responses to STm was then assessed. WT and IgH-deficient mice were NI or immunised intraperitoneally with 20 µg sFliC and on day 35 they were subsequently challenged with 5×10^5 STm, and responses were evaluated at 5 or 18 days post-challenge (Fig 3.20A). Quantification of the splenic and liver bacterial burden revealed both

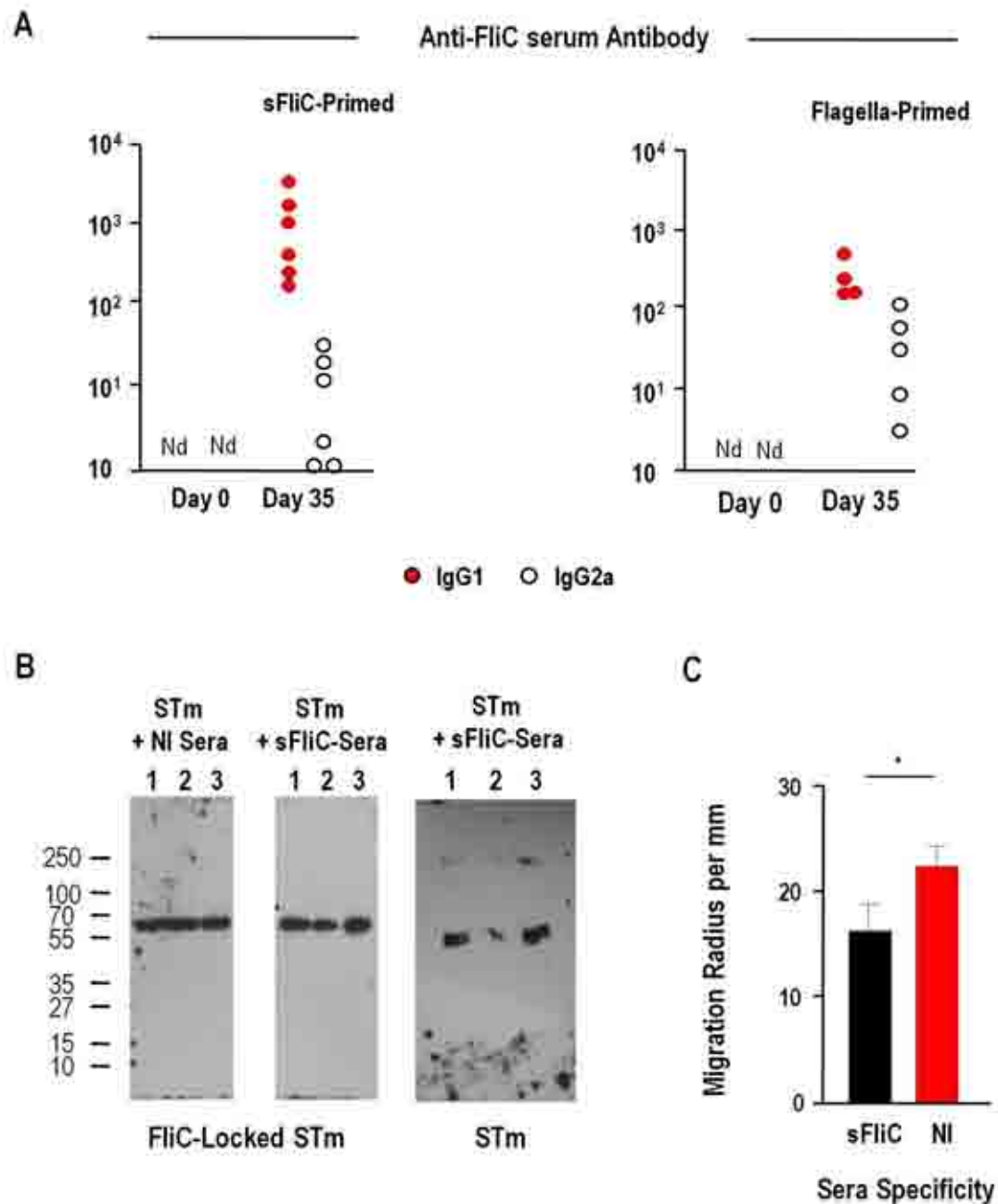
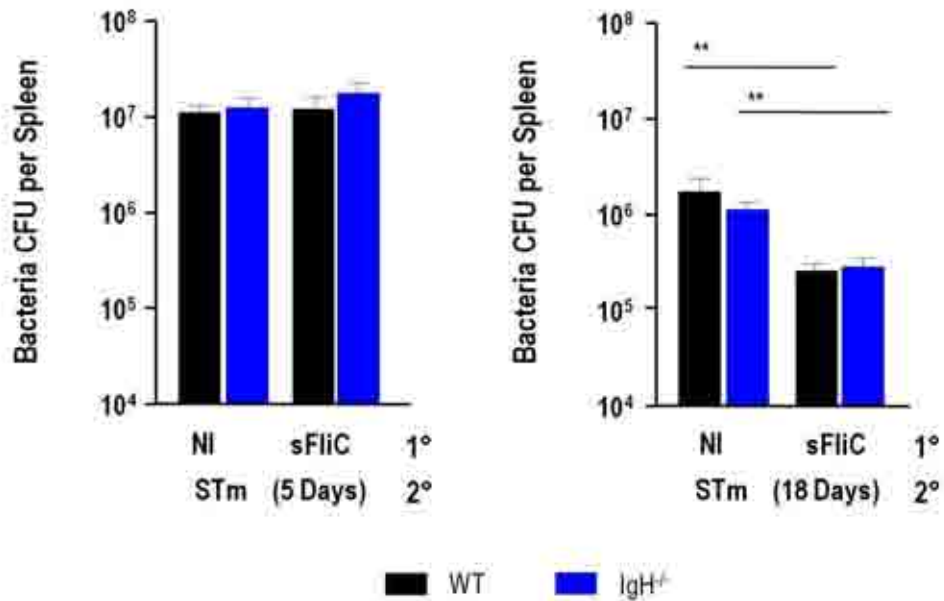


Figure 3.19

Antibody to flagellin can restrict STm motility through agar: (A) WT mice were immunised intraperitoneally with 20 μ g sFliC (left panel) or surface-isolated flagella (right panel) for 35 days and serum anti-sFliC IgG1 and IgG2a titres were assessed by ELISA. Data representative of at least 4 mice per group and two experiments. (B) Immunoblot showing binding of a monoclonal anti-FliC antibody to flagella on FliC-locked STm (left and centre) and STm (right) adsorbed with CI sera from either NI or sFliC-immunised WT mice. STm was separated by SDS-PAGE, transferred to a PVDF membrane and probed sequentially with an anti-FliC monoclonal and HRP-conjugated anti-IgG antibody. The bands show the approximate Mr of the flagellin monomer. Three different sera were tested in each case and sera was added at 1:300. (C) Motility of STm was measured after overnight incubation at room temperature in semi-solid agar containing either CI sera from a NI WT animal or a sFliC-immunised WT animal. 4 different sera were tested for each group. NI = Non-immunised. CI = Complement inactivated * $P < 0.05$, ** $P < 0.01$

A



B

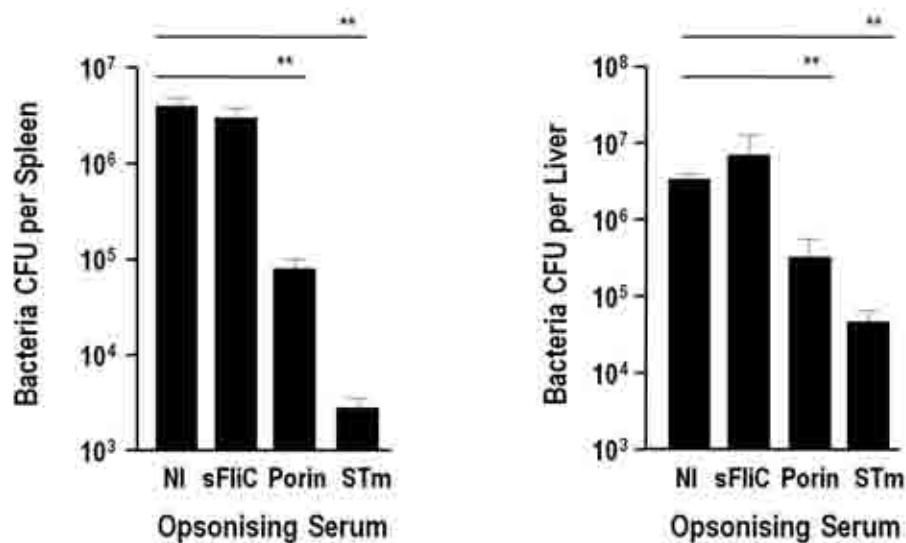


Figure 3.20:

Flagellin induces potent antibody responses which fail to impair STm infection: (A) NI and sFliC-immunised WT and B cell deficient ($IgH^{-/-}$) mice were challenged on day 35 with 5×10^5 STm and splenic bacterial numbers were enumerated at day 5 (left panel) and day 18 (right panel) post-STm challenge. (B) WT mice were infected for 5 days with 5×10^5 STm opsonised with complement inactivated sera from either NI mice, STm-infected (day 35) mice, porin-immunised (day 14) mice or FliC-primed and boosted (day 14) mice. Splenic (left panel) and liver (right panel) bacterial numbers were enumerated (representative of two repeats). All immunisations were intraperitoneal. * $P < 0.05$, ** $P < 0.01$

sFliC-primed WT and IgH-deficient mice had a significantly lower bacterial load relative to non-primed counterparts at day 18, reflecting earlier observations. However, in the main no differences were seen between WT and IgH-deficient mice at both time-points.

To confirm that antibody to sFliC was dispensable in this model, STm was opsonised immediately prior to infection with antibody from mice immunised with different antigens. STm was incubated with CI sera from either NI mice, mice primed and boosted with sFliC for 14 days, mice immunised with STm porins for 14 days which we show later can confer protection (Chapter 4), or mice infected with STm for 35 days. WT mice were then infected and bacterial numbers were quantified from the spleen and liver 5 days post-infection (Fig 3.20B). Mice immunised with STm-opsonised with porin-specific or STm-specific sera had a significantly lower bacterial load relative to mice immunised with STm opsonised with NI or sFliC-specific sera, which were comparable. Thus, opsonisation of STm with sFliC-specific antibody could not impair the capacity of STm to subsequently colonise mice.

3.6.3 Antibody to sFliC impairs early infection when STm cannot phase-switch

Lastly, whether the lack of benefit afforded by sFliC-specific antibody was due to the capacity of STm to phase-switch their flagellin expression between FljB and FliC was investigated. WT mice were immunised intraperitoneally with 20 µg sFliC and at day 35 they were infected with 5×10^5 STm that can express both phases of flagellin, or only FljB (FljB-locked), or only FliC (FliC-locked). Quantification of the bacterial burden in the spleen and liver at day 5 post-infection revealed, mice infected with FliC-locked STm had a 10-fold lower bacterial load relative to mice infected with either STm or FljB-locked STm, which were equivalent (Fig 3.21A). Thus, some of the capacity to avoid early antibody-mediated clearance was associated with the ability of STm to phase-switch.

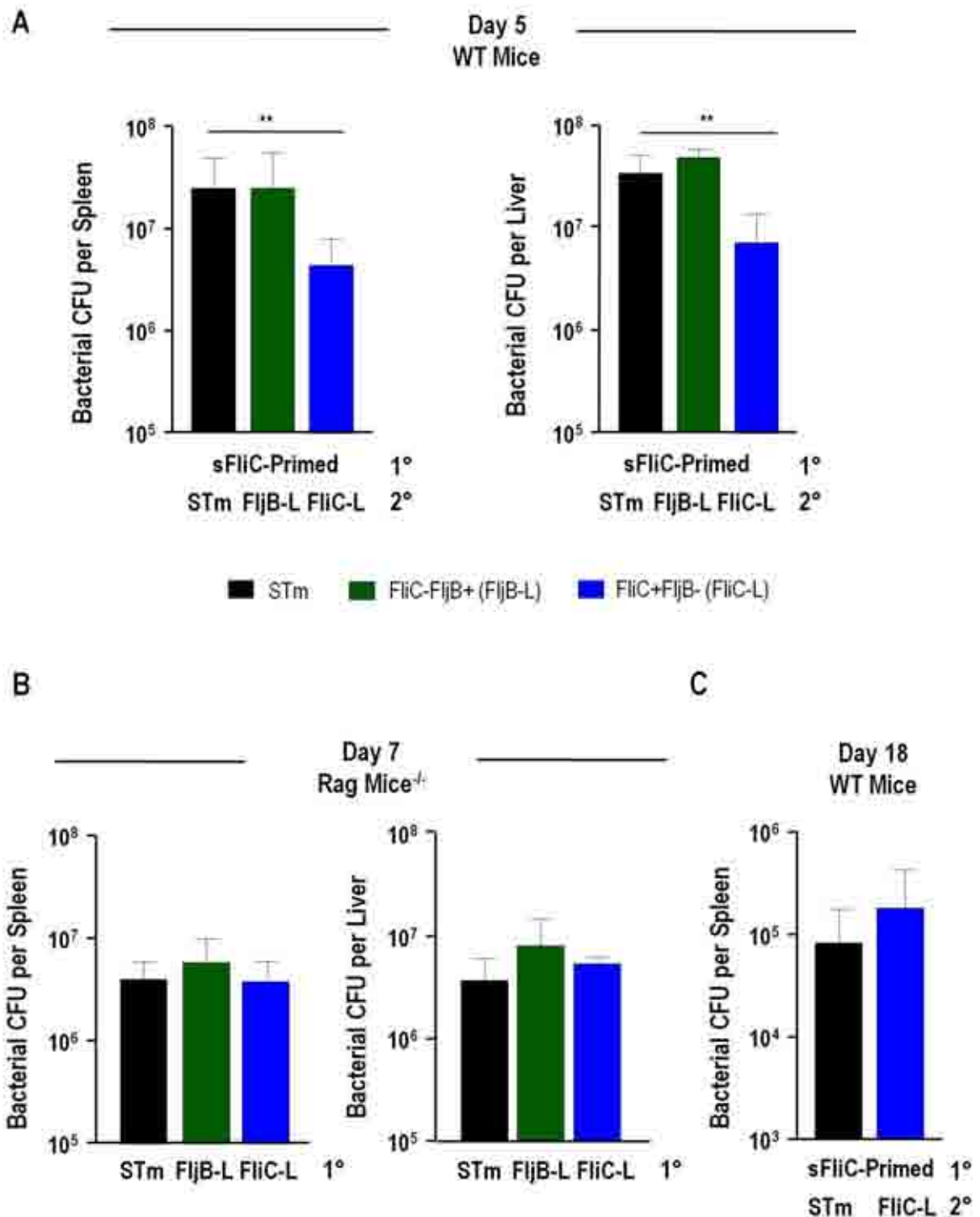


Figure 3.21:

Anti-sFliC antibody impairs early colonisation when STm phase-switching is compromised: (A) sFliC-immunised WT mice were challenged on day 35 with either 5×10^6 STm, FliB-locked STm (FliB-L) or FliC-locked STm (FliC-L) and splenic (left panel) and liver (right panel) bacterial numbers were quantified 5 days post-infection. (B) Rag1^{-/-} mice were infected with either 5×10^5 STm, FliC-L STm or FliB-L STm for 7 days and splenic (left panel) and liver (right panel) bacterial CFU assessed. (C) WT mice were immunised with 20 μ g sFliC and subsequently challenged on day 35 with either 5×10^6 STm or FliC-L STm. Splenic bacterial numbers were assessed 18 days post-infection. All immunisations were intraperitoneal. **P=<0.01

To exclude the possibility that FliC-locked STm were intrinsically more susceptible to killing by innate mechanisms, naïve Rag1 deficient mice were infected with these different STm strains and bacterial numbers were enumerated in the spleen and liver 7 days post-infection (Fig 3.21B). This showed that all strains colonised similarly. Lastly, we assessed whether this benefit seen in sFliC-immunised mice upon subsequent infection with FliC-locked STm was sustained. WT mice were immunised with 20 µg sFliC and at day 35 they were challenged with either STm or FliC-locked STm (Fig 3.21C). Quantification of the splenic bacterial burden at day 18 post-infection found no differences between sFliC-immunised mice infected with STm or FliC-locked STm. This suggests, the early impairment in STm colonisation was not sustained later during infection, irrespective of whether the ability of STm to phase-switch its flagellin genes was compromised.

Flagellin varies significantly between serovars, and antibody to flagellin is used to type these serovars through the Kauffman-White scheme. The data presented here demonstrate that immunisation with sFliC or flagella induces a potent and sustained antibody response that can impair bacterial motility, but not early colonisation, partly through the ability of STm to phase-switch its flagellin genes.

3.7 Enhanced clearance of STm in sFliC-immunised mice correlates with augmented IFN γ production.

3.7.1 Benefit afforded by sFliC immunisation is CD4 T cell dependent

Immunisation with sFliC reduced bacterial numbers at discrete stages after STm challenge (from day 18) (Fig 3.18A) which was independent of B cells. Earlier we demonstrated that T cells were not important for controlling STm infection in the first week of infection but were necessary after this (Fig 3.1), as in agreement with other studies (Hess et al., 1996; Hormaeche et al., 1990; Nauciel, 1990). Therefore, we sought to investigate whether the protection conferred by sFliC immunisation on day 18 after a subsequent STm infection was mediated through T cells.

Before this, whether the benefit afforded by sFliC immunisation upon subsequent STm challenge was due to a direct effect by sFliC on the innate immune system was assessed. Rag1 deficient mice were immunised intraperitoneally with 20 µg sFliC and on day 7 they were challenged with 5×10^5 STm alongside naïve counterparts, and responses were assessed at days 7 and 18 (Fig 3.22A). Quantification of the bacterial load in the spleen and liver found no differences between non-primed and sFliC-primed mice at both time-points. Thus, lymphocytes are required for the protection seen in sFliC-immunised mice.

To assess the importance of T cells in sFliC-immunised mice, WT and TCR-deficient mice were immunised intraperitoneally with 20 µg sFliC and on day 35 mice were challenged with 5×10^5 STm alongside NI counterparts, and responses were assessed at days 7 and 18 post-infection (Fig 3.22B). As expected, quantification of bacterial numbers on day 7 revealed both WT and TCR-deficient mice had equivalent bacterial numbers, irrespective of sFliC immunisation, in both the spleen and liver. However, at day 18 WT mice had significantly fewer bacteria than TCR-deficient mice regardless of sFliC immunisation. As expected, at day 18 sFliC-primed WT mice had a lower bacterial load than non-primed WT mice whilst sFliC immunisation did not influence bacterial numbers in TCR-deficient mice. These data demonstrate that the benefit afforded by sFliC-immunisation at day 18 upon subsequent STm infection is dependent on T cells.

3.7.2 The benefit of sFliC immunisation upon STm challenge correlates with augmentation of IFN γ

As the clearance of STm is associated with Th1 priming the influence of sFliC-priming on the Th1 response to STm was assessed. NI WT mice and WT mice primed with 20 µg sFliC were challenged with 5×10^5 STm on day 35 and CD4 T cell responses were assessed by FACS at days 5, 18 and 35 post-infection (Fig 3.23). This revealed the proportion of splenic CD4 T cells and effector (CD62L^{lo}) CD4 T cells in non-primed and sFliC-primed mice were comparable to each other at the individual time points

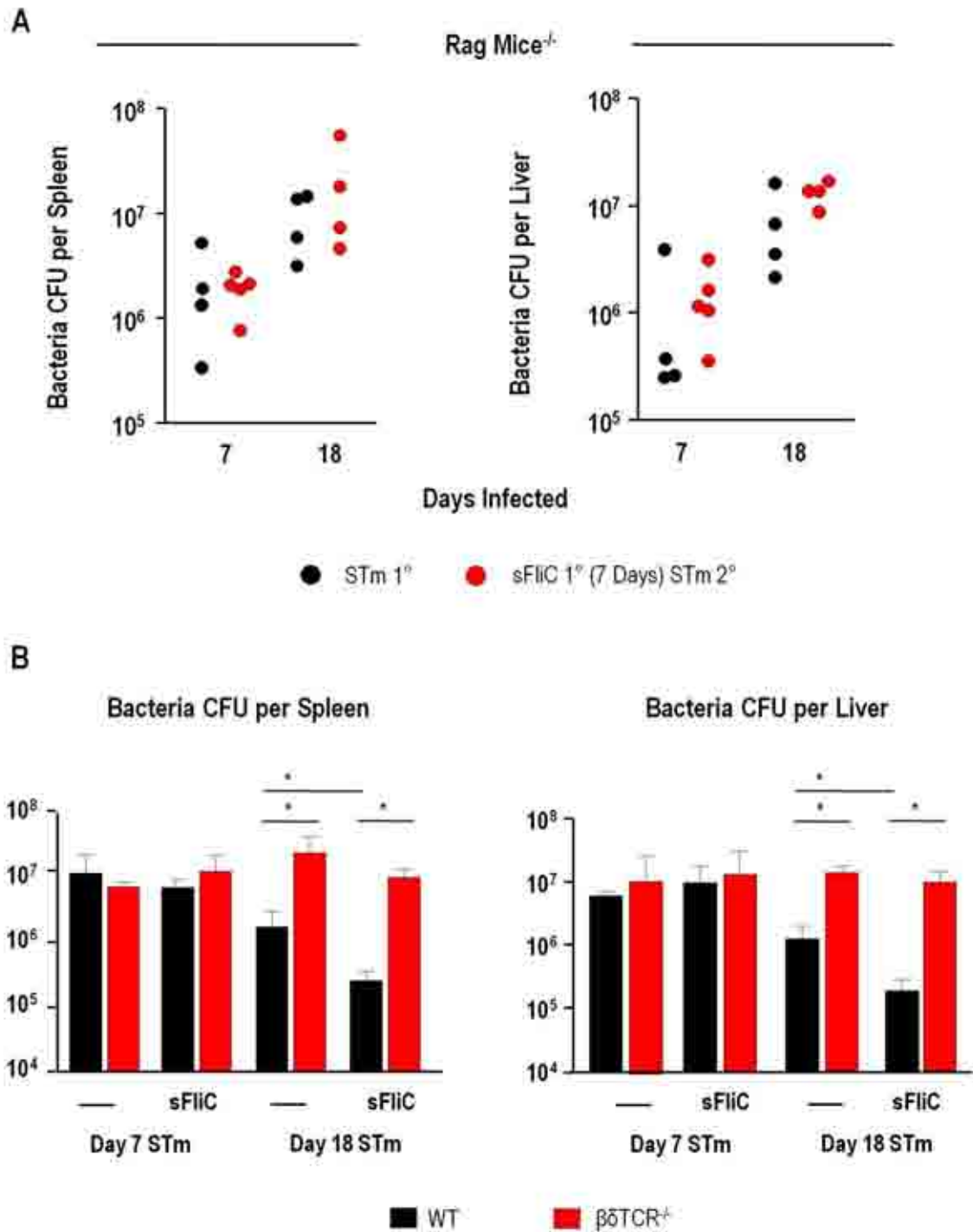


Figure 3. 22

The benefit afforded by sFliC immunisation requires T cells: (A) NI and sFliC-immunised (20 μg) Rag1-deficient mice were challenged on day 7 with 5×10^5 STm. Splenic (left panel) and liver (right panel) bacterial numbers were quantified at day 7 and 18 post-infection. (B) NI and sFliC-immunised (20 μg) WT and $\beta 2\text{-m}^{-/-}$ mice were challenged on day 35 with 5×10^5 STm. Splenic (left panel) and liver (right panel) bacterial numbers were quantified at day 7 and 18 post-infection. Data is representative of four mice per group and two separate experiments. All immunisations were intraperitoneal. NI = Non-immunised * $P < 0.05$, ** $P < 0.01$

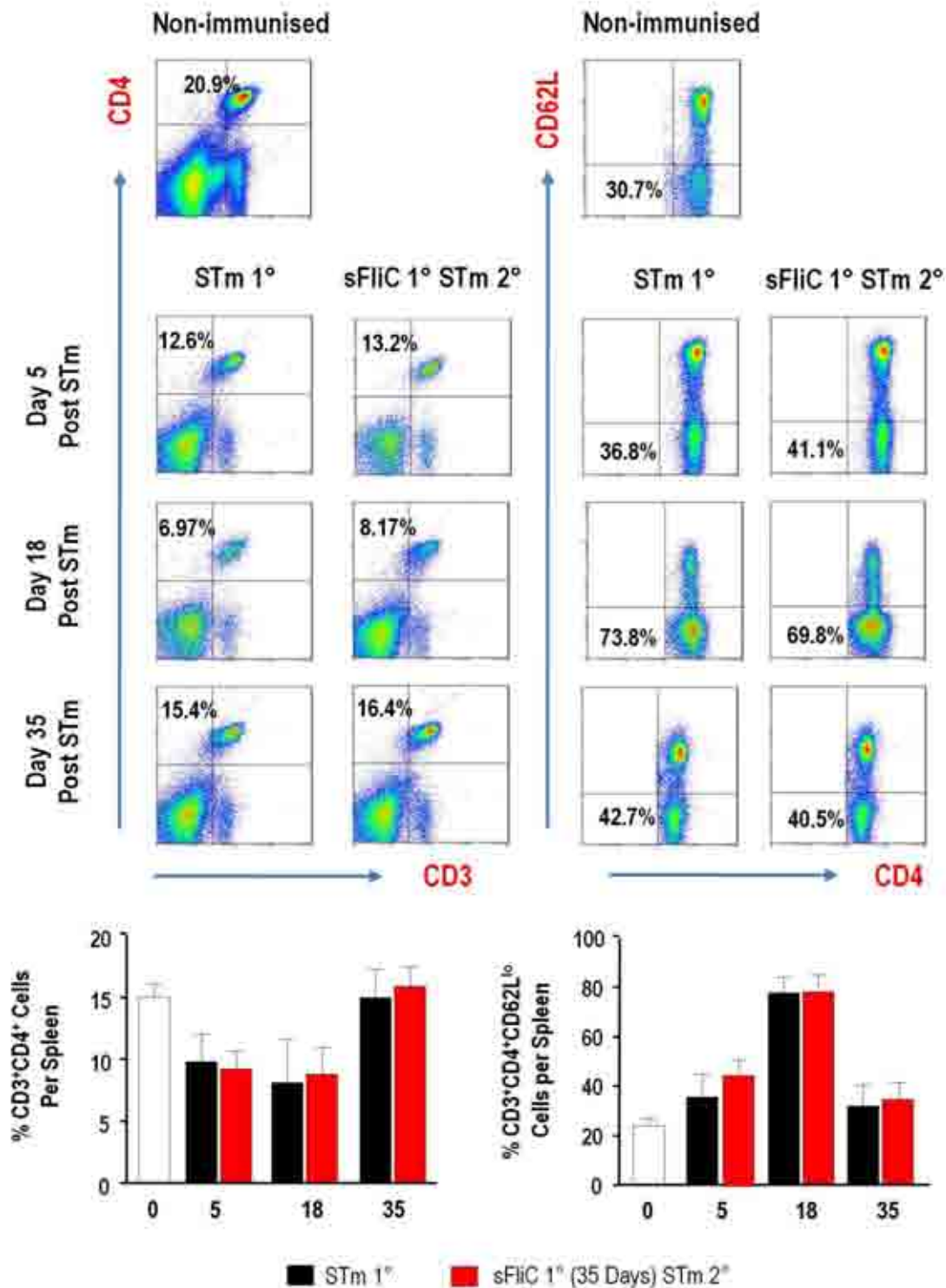


Figure 3.23

Flagellin immunisation does not influence the frequency of effector CD4⁺ T cells upon STm challenge: NI and sFluC-immunised (20 µg) WT mice were challenged on day 35 with 5x10⁵ STm and splenic CD4 T cell responses were assessed by FACS at days 5, 18 and 35 post-challenge. Columns one and two show CD4 T cell responses in non-primed and sFluC-primed mice respectively. Columns three and four show effector CD4⁺CD62L^{lo} T cell responses in non-primed and sFluC-primed mice respectively. Data is representative of 4 mice per group and two independent experiments. All immunisations were intraperitoneal. *P<0.05 ** P<0.01

assessed post-STm infection. Subsequently, the influence of sFliC immunisation on IFN γ production by CD4 T cells during subsequent STm challenge was examined. Splenocytes were re-stimulated in vitro with anti-CD3 in the presence of anti-CD28 and IFN γ production was quantified by intracellular FACS (Fig 3.24). This revealed that at day 5, IFN γ expression was significantly lower in sFliC-primed mice relative to non-primed mice, and this was reflected both in terms of the proportion and number of CD4 T cells. In contrast, when responses were assessed at day 18 IFN γ expression was significantly greater in sFliC-primed mice relative to non-primed mice and by day 35, when the infection had nearly resolved in both sFliC-primed and non-primed mice, IFN γ expression was comparable between the two groups. As discussed earlier (Fig 3.5) at each time point assessed the signal for IFN γ was almost entirely detected from effector CD4 T cells.

As an additional control secreted protein was assessed by ELISPOT (Fig 3.25). Splenocytes from non-primed and sFliC-primed mice subsequently challenged with STm for 18 days were re-stimulated in vitro with sFliC for 48 hours in an anti-IFN γ pre-coated ELISPOT plate, and the number of IFN γ SFC were enumerated. This demonstrated sFliC-primed mice had more IFN γ SFC relative to both NI and non-primed mice at day 18 post-STm challenge. IL-4 responses were also examined by ELISPOT but were largely undetectable in all groups (Fig 3.25). Together, the ELISPOT and intracellular FACS data suggested that the enhanced clearance of STm observed at day 18 in sFliC-primed mice was associated with increased numbers of IFN γ producing CD4 T cells.

3.7.3 T-bet is essential for the protection provided by sFliC immunisation

In STm infections IFN γ secretion correlates with T-bet induction (Ravindran et al., 2005). As sFliC immunisation promoted accelerated clearance of STm through an enhancement of IFN γ , whether this was regulated by the global Th1 regulator T-bet was investigated, although this would be surprising since sFliC did not induce T-bet expression (Fig 3.9). Initially, the importance of T-bet in the clearance of STm at day 18 was assessed and as a comparison, responses in Th2 defective IL-4R α deficient mice

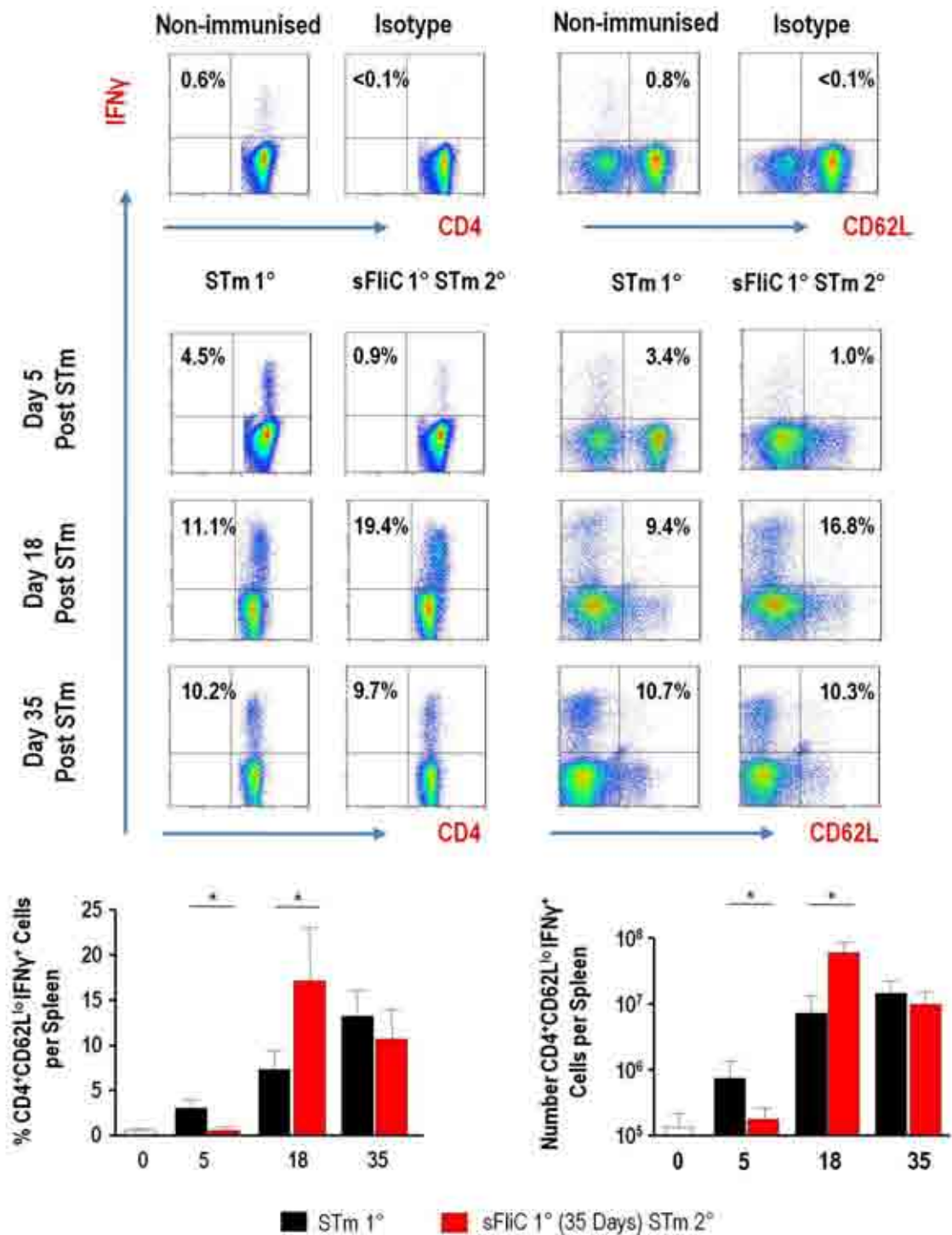


Figure 3.24

Enhanced clearance of STm after sFliC immunisation is associated with an enhanced CD4 IFN γ response: NI and sFliC-immunised (20 μ g) WT mice were challenged on day 35 with 5×10^5 STm and CD4 T cells responses were assessed at days 5, 18 and 35 post-challenge. Splenocytes were isolated and 5×10^6 cells were re-stimulated in-vitro with anti-CD3 for 6 hours. IFN γ was then quantified by FACS: Columns one and two show IFN γ expression in CD4 T cells from non-primed and sFliC-primed mice respectively. Columns three and four show IFN γ expression in effector CD4 $^{+}$ CD62L lo T cells in non-primed and sFliC-primed mice respectively. Data is representative of 4 mice per group and two independent experiments performed in conjunction with Dr. A. Flores-Langarica. *P<<0.05

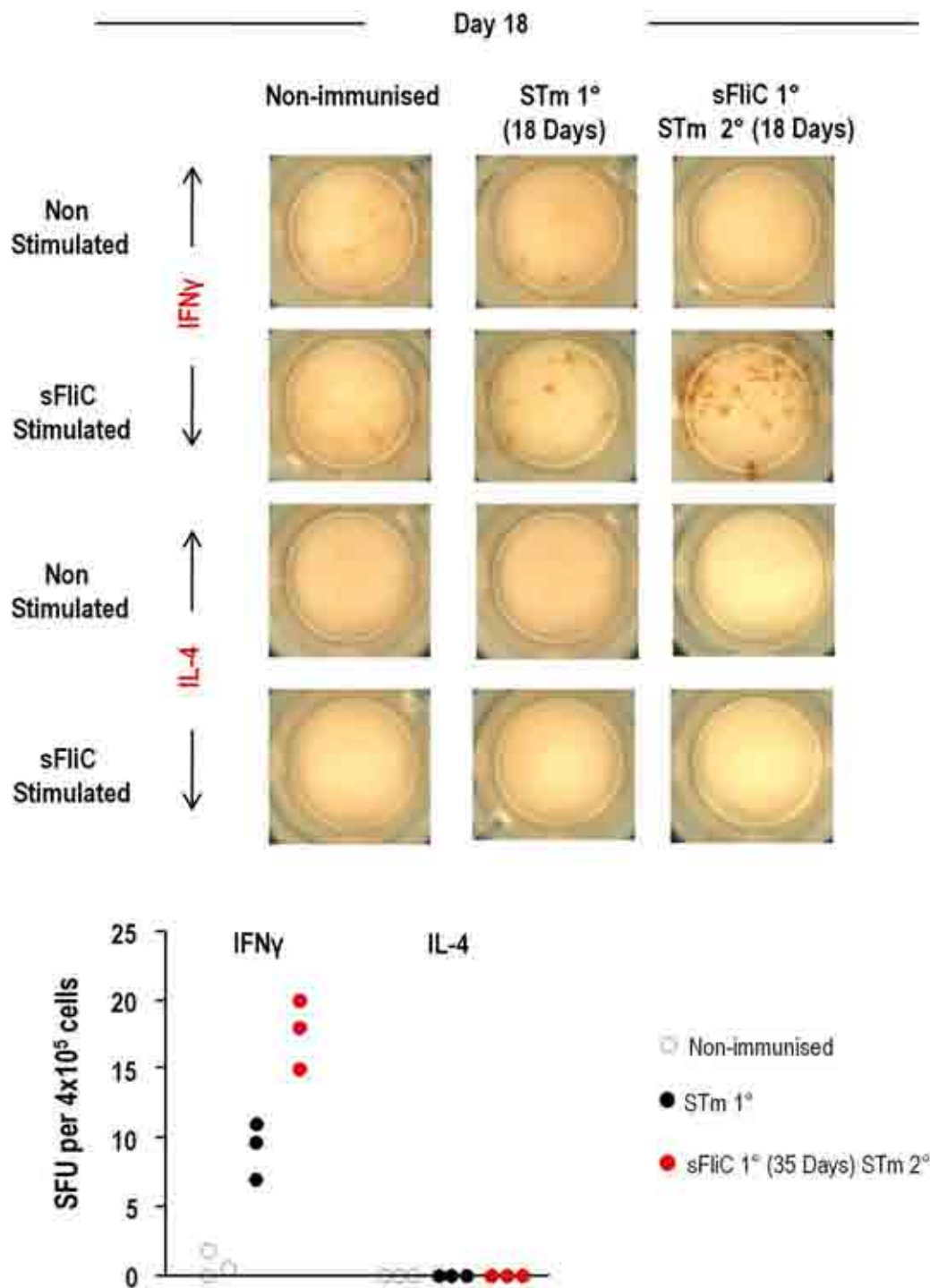


Figure 3.25

IFN γ expression by CD4 T cells is enhanced in sFlIC-primed mice 18 days after subsequent STm challenge and IL-4 is not detected: NI and sFlIC-immunised (20 μ g) WT mice were challenged on day 35 with 5×10^5 STm. Splenocytes were isolated 18 days post-challenge and 4×10^5 cells were re-stimulated for 48 hours with sFlIC or left non-stimulated before numbers of IL-4 and IFN γ SFC were enumerated by ELISPOT. This data has been generated in conjunction with Dr. A Flores-Langarica and is representative of 2 independent experiments.

were included. BALB/c (WT) and IL-4R α deficient mice or C57BL6/J (WT) and T-bet deficient mice were infected with 5×10^5 STm and responses assessed 18 days post-infection (Fig 3.26A). Quantification of bacterial numbers in the spleen and liver showed no differences between WT and IL-4R α -deficient mice, whilst T-bet deficient mice had an appreciably elevated bacterial load relative to WT mice. Thus, T-bet but not IL-4 and IL-13 signalling through IL-4R α is required for the control of primary STm infections.

Quantification of splenic CD4 T cell responses in WT and T-bet deficient mice revealed, the total number of CD4 and effector CD4 T cells were comparable between infected WT and T-bet deficient mice 18 days post-STm infection (Fig 3.26B). However, the proportions were lower in infected T-bet deficient mice relative to infected WT mice. Subsequently, IFN γ CD4 T cell responses were examined by intracellular FACS following anti-CD3 re-stimulation in the presence of CD28 (Fig 3.27). This demonstrated IFN γ was virtually undetectable in splenocytes isolated from T-bet deficient mice with levels comparable to NI WT mice. Thus, the impaired ability to drive IFN γ production in the absence of T-bet signalling correlated with the failure to clear STm from the tissues relative to WT mice, as in agreement with previous studies (Ravindran et al., 2005).

Given the importance of T-bet for the clearance of primary STm infections at day 18, whether T-bet was also important for the benefit afforded by sFliC immunisation upon subsequent STm challenge was assessed. T-bet deficient mice were either NI or immunised with 20 μ g sFliC and subsequently challenged on day 35 with 5×10^5 STm. Quantification of the bacterial burden in the spleen and liver at day 18 post-STm infection revealed immunisation with sFliC did not influence the bacterial load in T-bet deficient mice (Fig 3.28A). This suggested T-bet expression was required for the benefit observed in sFliC-primed mice 18 days post-STm challenge.

The proportion and total number of CD4 and effector CD62L^{lo} CD4 T cell responses were comparable between sFliC-primed and non-primed T-bet deficient mice 18 days post-STm challenge (Fig 3.28B).

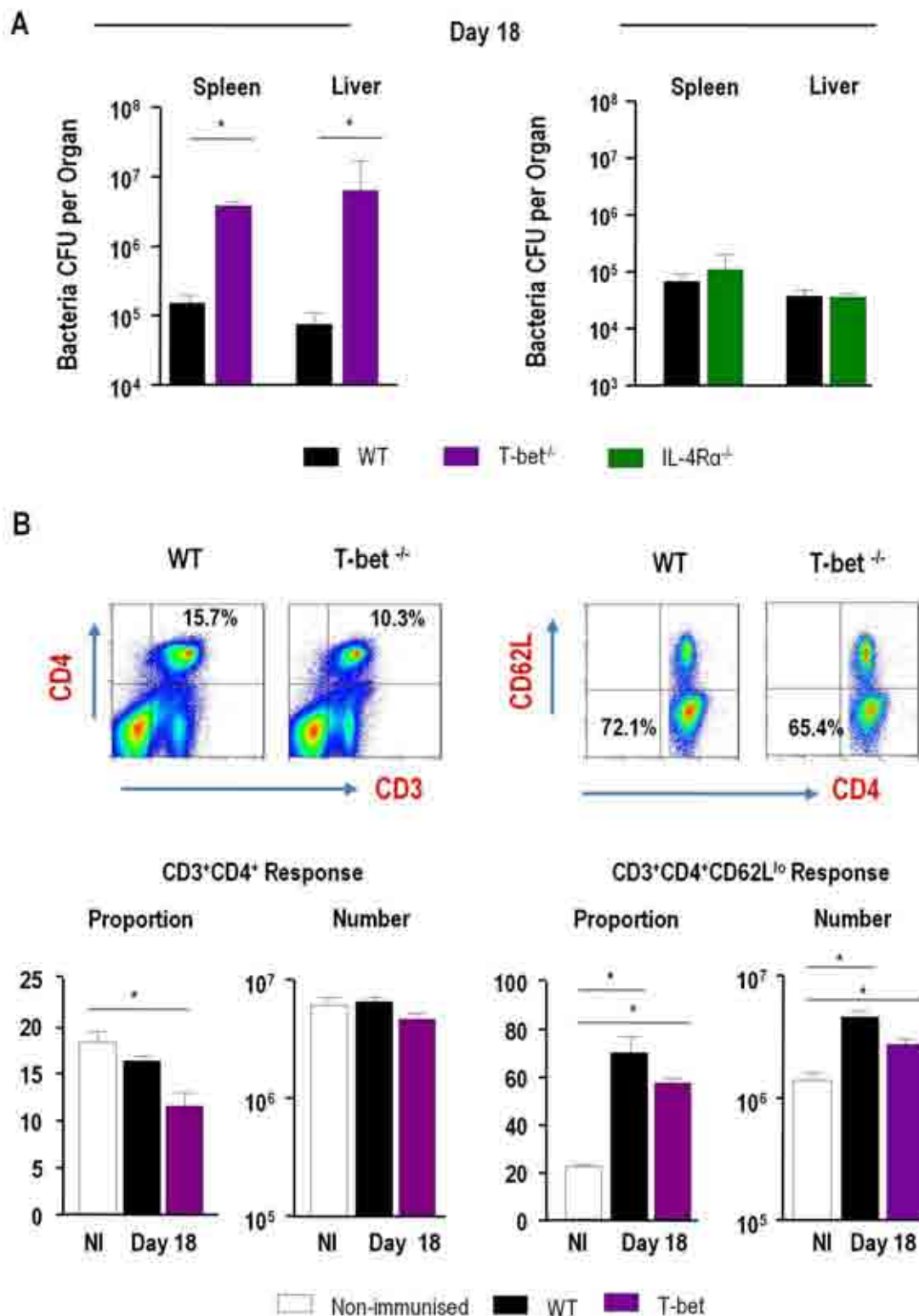


Figure 3. 26

T-bet-deficient mice demonstrate impaired clearance of STm at day 18 post-infection: (A) WT and T-bet deficient (left panel) and WT and IL-4Ra deficient (right panel) mice were infected intraperitoneally with 5×10^5 STm and splenic and liver bacterial numbers were quantified 18 days post-infection. (B) CD4⁺ and CD4⁺CD62L^{lo} T cell responses were assessed by FACS in WT and T-bet deficient mice 18 days post-STm infection. Data is representative of 4 mice per group and two independent experiments. *P<0.05

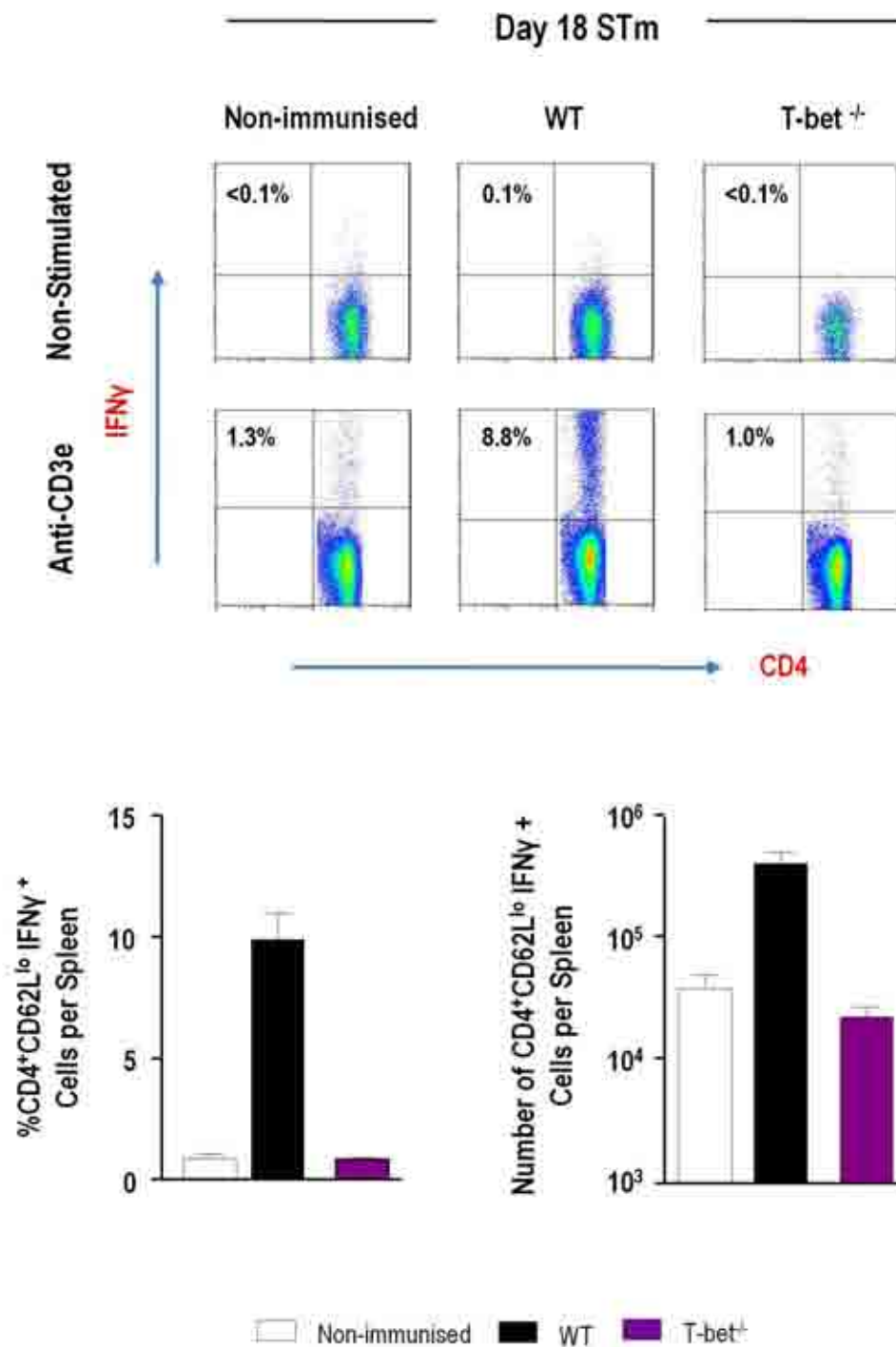


Figure 3. 27

T-bet is required for promoting Th1-mediated clearance of STm: WT and T-bet deficient mice were infected intraperitoneally with 5×10^5 STm and responses were assessed 18 days post-infection. Splenocytes were isolated alongside non-immunised WT mice and 5×10^6 cells were re-stimulated in vitro with anti-CD3 in the presence of anti-CD28 for 6 hours. IFN γ was then quantified by intracellular FACS in CD4⁺CD62L^{lo} T cells. Data is representative of 4 mice per group and two independent experiments. *P<0.05 ** P<0.01

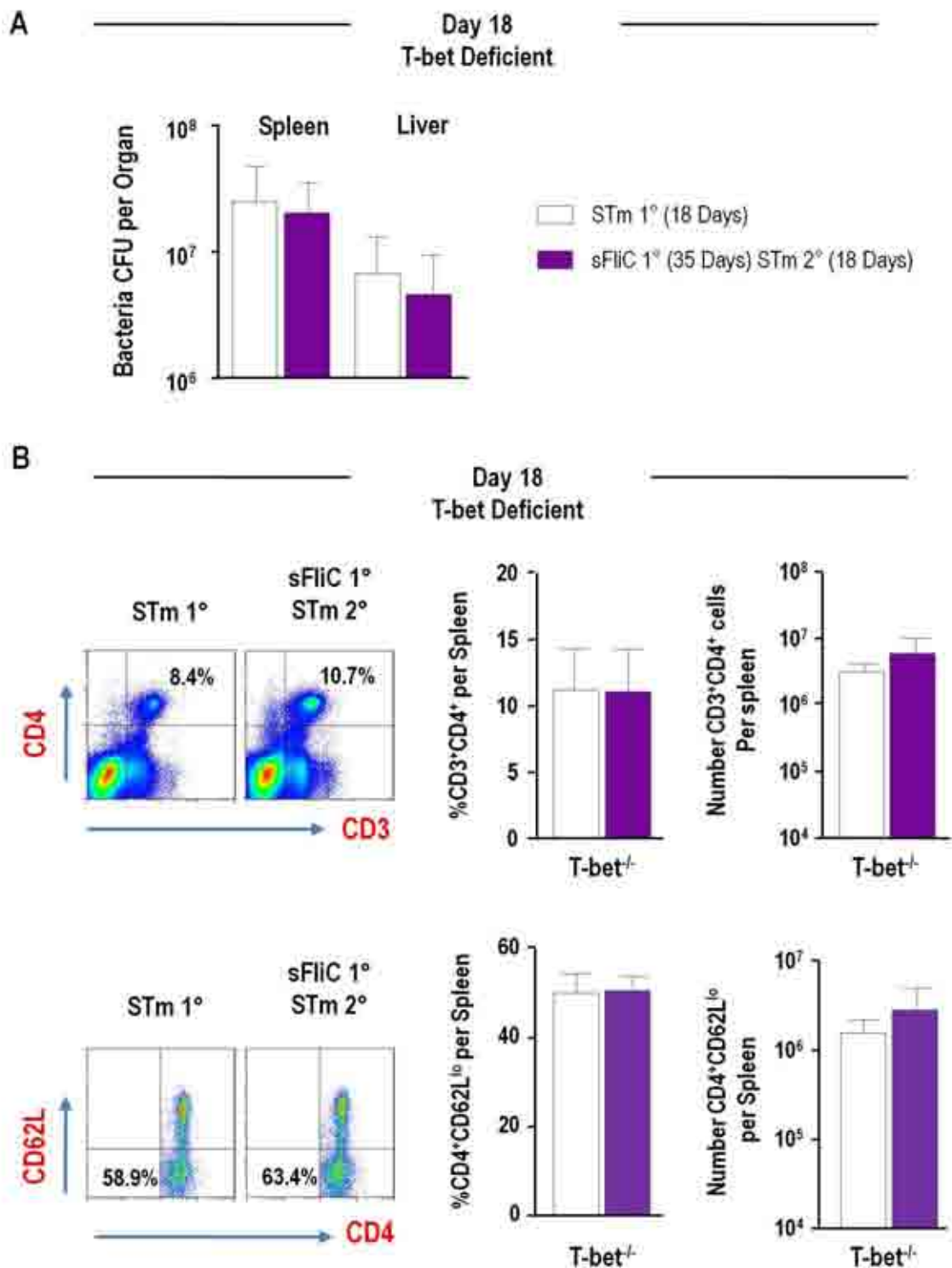


Figure 3. 28

CD4 T cell responses are comparable between non-primed and sFluC-primed T-bet-deficient mice 18 days post-STm challenge: Non-primed and sFluC-primed (20 μ g) T-bet deficient mice were challenged on day 35 with 5×10^5 STm and responses were assessed 18 days post-infection; (A) Splenic and liver bacterial numbers were quantified. (B) Splenic CD4⁺ T cell responses were assessed by FACS. Row 1 shows CD4⁺ T cell responses and Row 2 shows CD4⁺CD62L^{lo} responses. Data is representative of 4 mice per group and two independent experiments. *P<0.05 ** P<0.01

Quantification of IFN γ expression by CD4 T cells following anti-CD3 re-stimulation in the presence of anti-CD28 revealed the absence of IFN γ in both sFliC-primed and non-primed T-bet deficient mice (Fig 3.29). As a control to show that sFliC-primed and non-primed T-bet deficient mice could produce cytokines, cells were also stained for IL-5 which showed the induction of IL-5 in both sFliC-primed and non-primed CD4 T cells. Thus, the loss of the benefit in sFliC-primed mice upon subsequent STm challenge at day 18 correlated with the absence of IFN γ which was dependent on T-bet expression.

3.7.4 T-bet expression is not required for sFliC Priming

Immunisation with sFliC did not induce T-bet (Fig 3.9) yet surprisingly, the benefit afforded by sFliC immunisation upon subsequent STm challenge was T-bet dependent. To ascertain whether this was because T-bet was required for the induction of responses to sFliC, WT and T-bet deficient mice were immunised with 20 μ g sFliC and splenic CD4 T cell responses were assessed 4 days post-immunisation. This revealed the proportion and total number of CD4 T cells and effector CD62L^{lo} T cells were equivalent between WT and T-bet deficient mice (Fig 3.30). In addition, CD4 T cells were FACS sorted from these mice into naïve CD62L^{hi} and effector CD62L^{lo} populations for assessment of mRNA expression by real-time RT-PCR (Fig 3.31A). This revealed the expression of both GATA-3 and IL-4 was equivalent between WT and T-bet deficient mice. This suggests that T-bet expression is not required for induction of the early features of the sFliC-specific CD4 T cell response.

To address whether the antibody response to sFliC was influenced by T-bet signalling, serum anti-sFliC antibody titres were assessed by ELISA in WT and T-bet deficient mice which were either NI or immunised with sFliC for 7 or 35 days (Fig 3.31B). This revealed immunisation with sFliC induced an anti-sFliC specific IgM response by day 7 and as expected, by day 35 both WT and T-bet deficient mice had a significantly lower IgM titre, but the response was equivalent between WT and T-bet deficient mice at both time-points. Assessment of the IgG1 response found both sFliC-immunised WT and T-bet deficient mice induced a modest IgG1 response at day 7 which remained detectable at day 35.

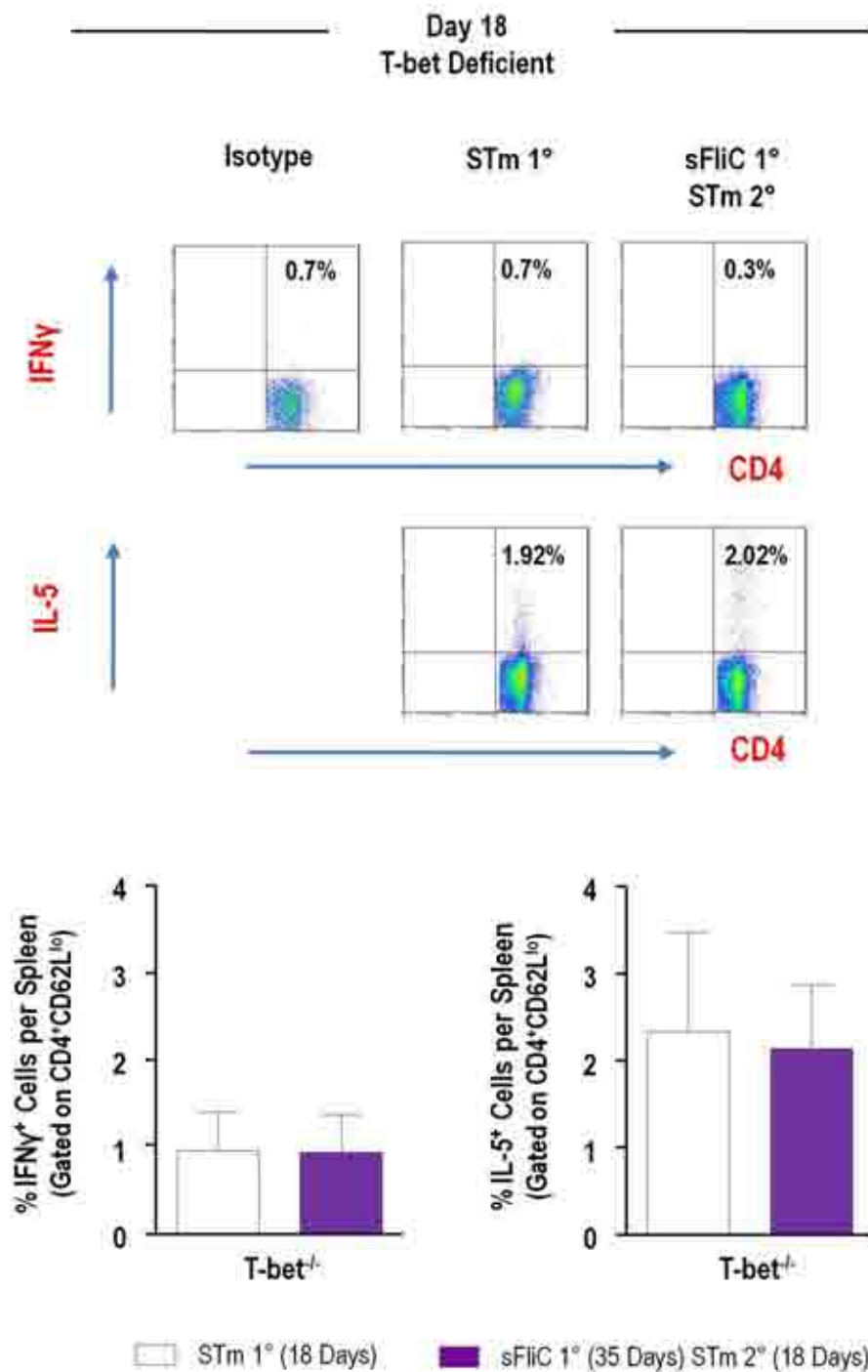


Figure 3. 29

T-bet is required for promoting Th1-mediated clearance after sFliC immunisation: Non-primed and sFliC-primed (20 μ g) T-bet deficient mice were challenged on day 35 with 5×10^5 STm and responses were assessed 18 days post-infection. Splenocytes were isolated and 5×10^6 cells were re-stimulated in-vitro with anti-CD3 in the presence of anti-CD28 for 6 hours. The proportion of IFN γ (top row and left graph) and IL-5 (bottom row and right graph) was then quantified in CD4⁺CD62L^{lo} T cells by intracellular FACS. Data is representative of 4 mice per group and two independent experiments. *P<0.05 ** P<0.01

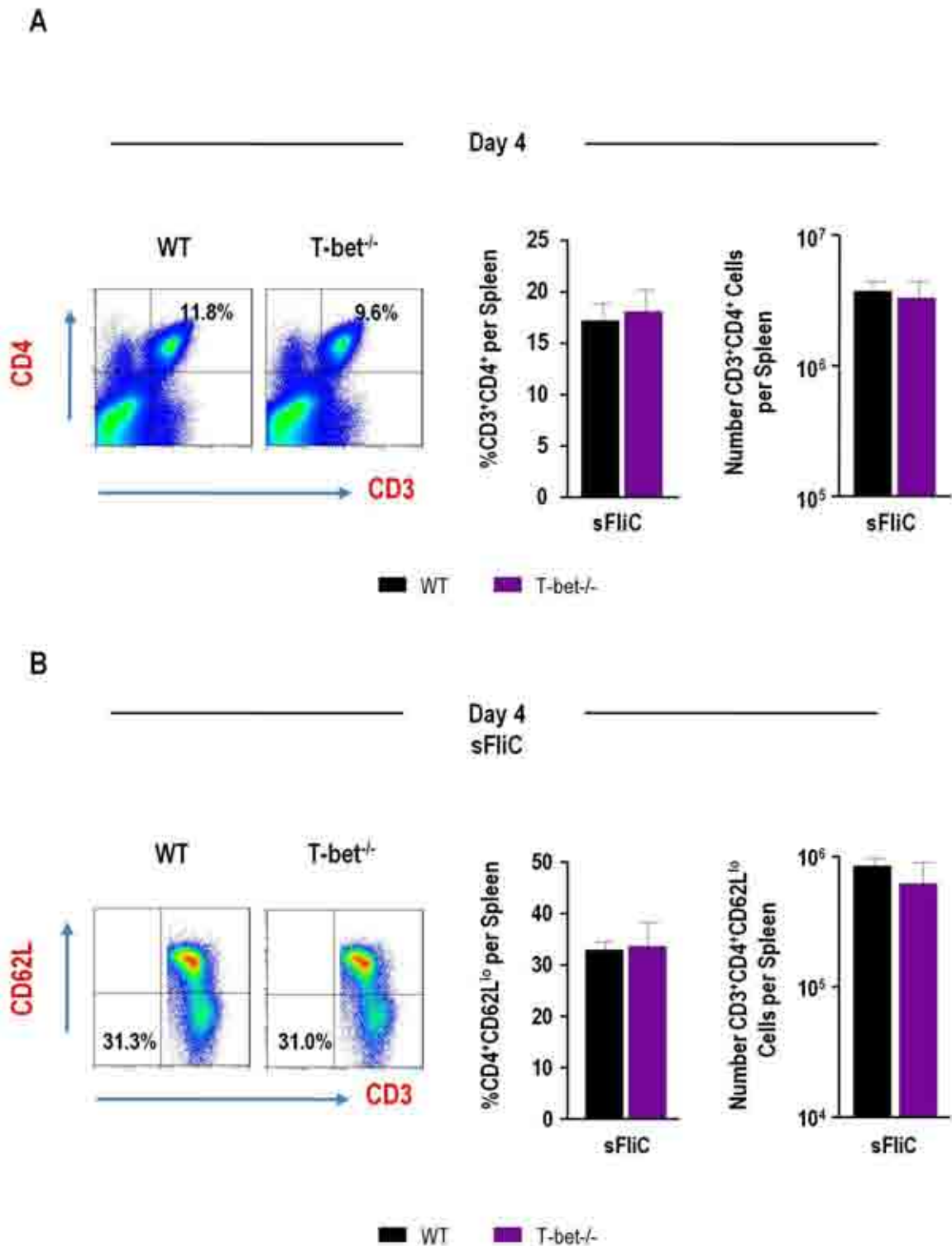


Figure 3. 30

T-bet signalling does not influence total splenic CD4 T cell responses to sFliC: WT and T-bet deficient mice were immunised intraperitoneally with 20 µg sFliC and splenic CD4 T cell responses were assessed 4 days post-immunisation. (A) CD4⁺ and (B) CD4⁺CD62L^{lo} T cell responses were assessed by FACS. Data is representative of 4 mice per group. *P<0.05 **P<0.01

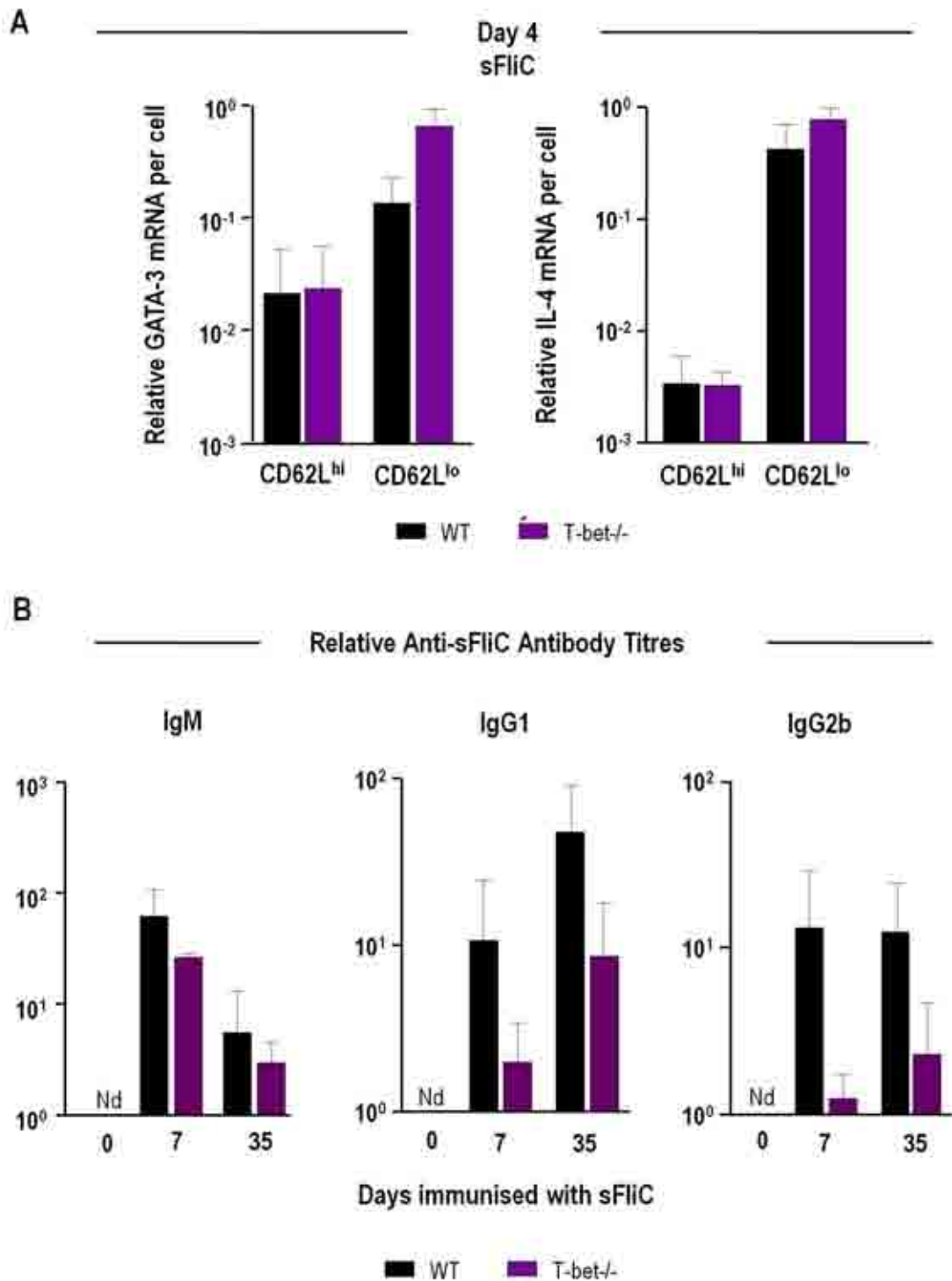


Figure 3.31

T-bet signalling is not required for the early induction of IL-4 after sFliC immunisation but may be important for antibody switching to IgG1: (A) WT and T-bet^{-/-} mice were immunised intraperitoneally with 20 µg sFliC and splenocytes were isolated 4 days post-immunisation and CD4 T cells were sorted into CD62L^{hi} and CD62L^{lo} populations by FACS. Expression of GATA-3 and IL-4 was then quantified by real-time RT-PCR. (B) WT and T-bet^{-/-} mice were immunised intraperitoneally with 20 µg sFliC for 7 or 35 days and serum anti-sFliC IgM, IgG1 and IgG2b titres were assessed by ELISA. Nd= Non-Detected. Data is representative of 4 mice per group. *P<0.05 ** P<0.01

Interestingly, T-bet deficient mice displayed a trend for lower IgG1 titres. This trend was also observed to other antigens such as alum-precipitated ovalbumin (data not shown). Anti-sFliC IgG2b was induced in sFliC-immunised WT and T-bet deficient mice at both days 7 and 35, and as observed with the IgG1 response, this revealed a trend for lower antibody titres in sFliC-immunised T-bet deficient mice relative to WT mice at both time-points. Therefore, T-bet may not be required for the induction or maintenance of Th2 features in response to sFliC immunisation, but may contribute to antibody switching.

These data demonstrate that immunisation with the Th2 antigen sFliC can offer limited protection against subsequent STm challenge, dependent upon when responses are assessed. Protection is independent of B cells but requires T cells at day 18. This protection is associated with augmentation of Th1 responses which is dependent on T-bet expression, suggesting that under certain conditions the Th2 antigen sFliC can promote Th1 responses. Significantly, sFliC requires T-bet expression to promote clearance of STm but it does not require T-bet to induce sFliC-specific Th2 features.

3.8 The loss of flagella does not influence the clearance of STm

Since flagellin is an important target for innate and adaptive immunity, whether loss of flagella could influence the clearance of STm from the tissues and the subsequent response initiated by the host was assessed. WT mice were infected intraperitoneally with 5×10^5 flagellated STm (STm) or STm lacking flagella (aflagellated STm) and primary responses were assessed 18 days later (Fig 3.32A). Quantification of the bacterial burden in the spleen and liver found no differences between mice infected with flagellated or aflagellated STm.

Assessment of splenic CD4 T cell responses revealed the proportion and total number of CD4 and effector CD62L^{lo} CD4 T cells were equivalent between mice infected with the flagellated or aflagellated strain of STm (Fig 3.32B). IFN γ production by CD4 T cells was then assessed following re-stimulation of splenocytes with anti-CD3 in the presence of anti-CD28 (Fig 3.32C). This revealed stimulation with anti-

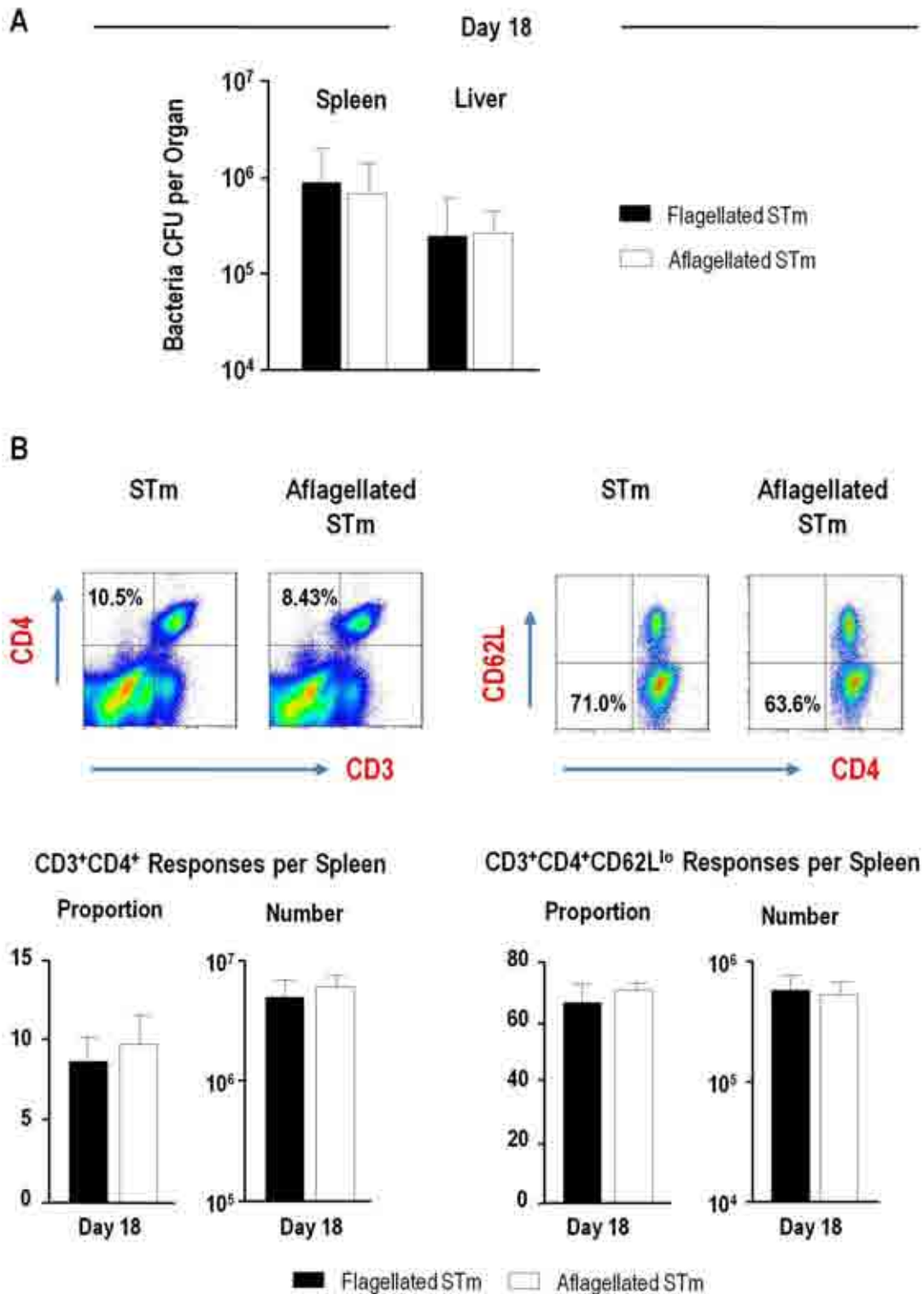


Figure 3. 32

Mice clear aflagellated STm with equal kinetics as flagellated STm as CD4 T cell responses are comparable: WT mice were infected intraperitoneally with 5×10^5 flagellated STm (STm) or aflagellated STm and responses were assessed 18 days post-infection. (A) Splenic and liver bacterial numbers were quantified. (B) Splenocytes were isolated and CD3⁺CD4⁺ and CD3⁺CD4⁺CD62L^{lo} T cell responses were assessed by FACS. Data is representative of 5 mice per group and two independent experiments.

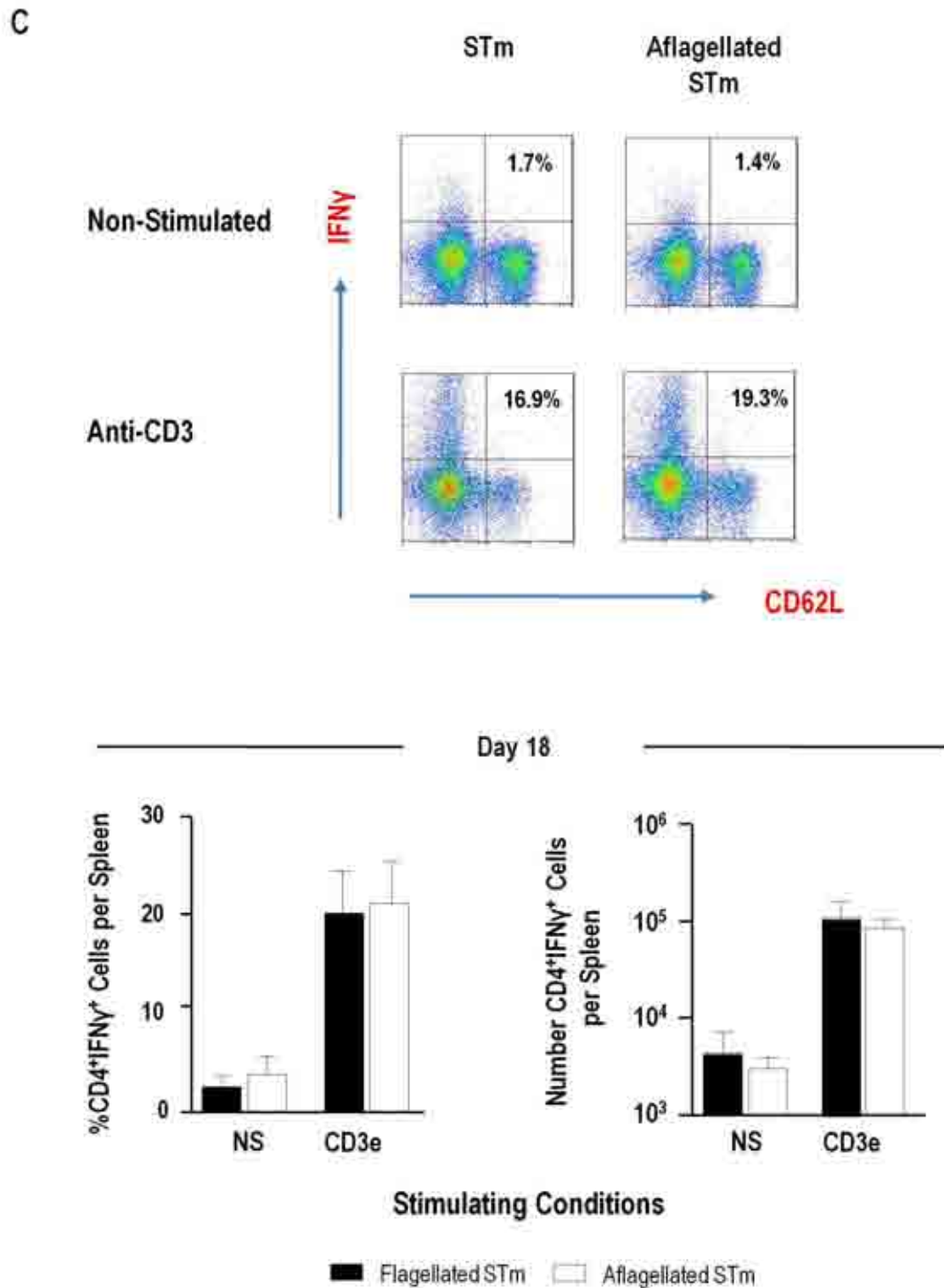


Figure 3.32

Mice infected with aflagellated STm induce comparable IFN γ as mice infected with flagellated STm:
 (C) WT mice were infected intraperitoneally with 5×10^5 flagellated STm (STm) or aflagellated STm and responses were assessed 18 days post-immunisation. Splenocytes were isolated and 5×10^6 cells were re-stimulated in-vitro with or without anti-CD3 in the presence of anti-CD28 for 6 hours. IFN γ was then quantified by intracellular FACS in the CD3⁺CD4⁺CD62L^{lo} T cell population. Data is representative of 5 mice per group and two independent experiments.

CD3 induced IFN γ production relative to non-stimulated splenocytes. However, there were no differences in both the proportion and number of IFN γ producing CD4 T cells between mice infected with the flagellated or aflagellated strain of STm. These data demonstrate that the loss of flagella from the surface of STm does not influence the clearance of STm from the host, or alter the direction of the immune response at day 18 post primary infection.

3.9 Deletion of some of the putative TLR5 ligating motif does not influence sFliC-specific antibody responses

TLR5 recognises an evolutionary conserved site on flagellin (Andersen-Nissen et al., 2005). However, not all bacterial flagellin monomers are able to ligate TLR5 owing to variations in this site. To investigate whether TLR5 ligation of this site was responsible for the properties of sFliC, a sFliC construct was generated in which part of the recognised TLR5 ligation motif was deleted to create Δ TLR5 sFliC as outlined in Chapter Two and Appendix B. Once the construct was generated it was assessed whether an anti-sFliC monoclonal antibody was still able to recognise it by ELISA (data not shown). This demonstrated the monoclonal antibody recognised both sFliC and the mutated Δ TLR5 sFliC protein with comparable avidity, but failed to recognise flagellin isolated from BL21.

To address whether the antibody response to Δ TLR5 sFliC was similar to sFliC and thus, reflective of Th2 responses, WT mice were infected with 5×10^5 STm or immunised with 20 μ g sFliC or 20 μ g Δ TLR5 sFliC and serum anti-sFliC antibody titres were assessed by ELISA 14 days post-immunisation (Fig 3.33A). This demonstrated that IgM was present in all groups but greatest after sFliC. In contrast, the relative titres for both IgG1 and IgG2a were only detectable in mice immunised with sFliC or Δ TLR5 sFliC and titres were comparable. Thus, altering part of the putative TLR5 binding motif in sFliC did not impinge upon sFliC-specific switching to IgG1. To ascertain whether altering this putative TLR5 ligating motif impaired the GC response to sFliC in these mice, spleens were sectioned for immunohistology

A

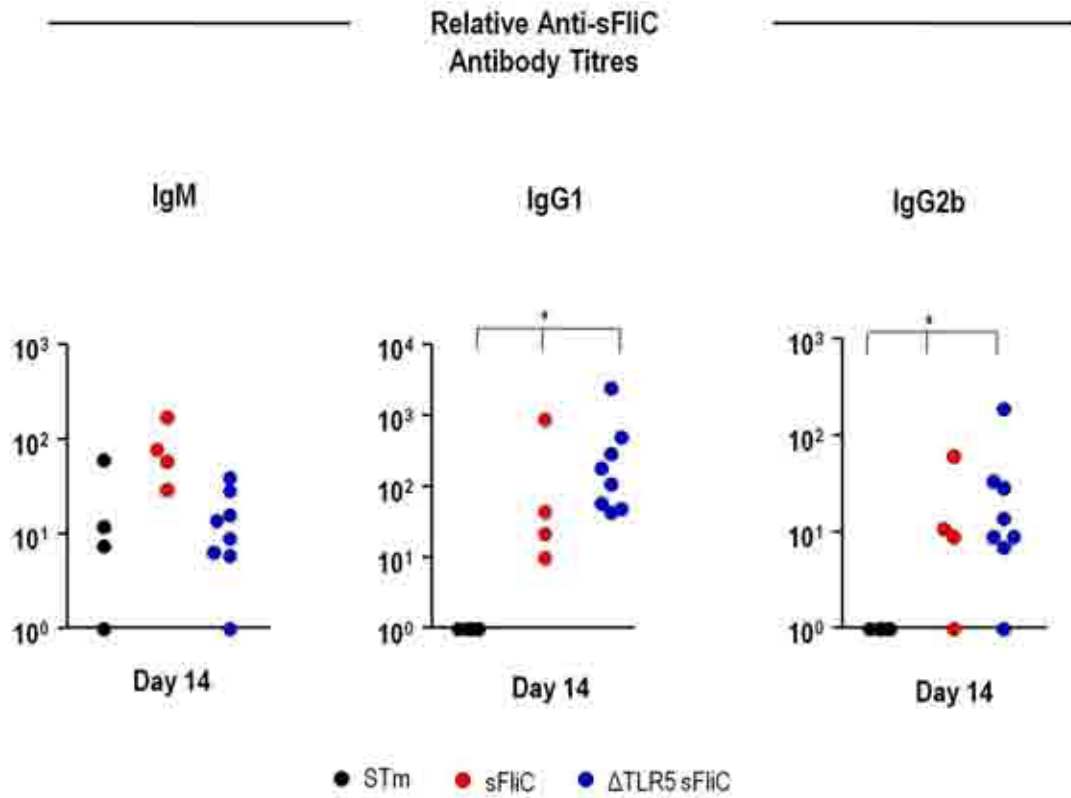


Figure 3. 33

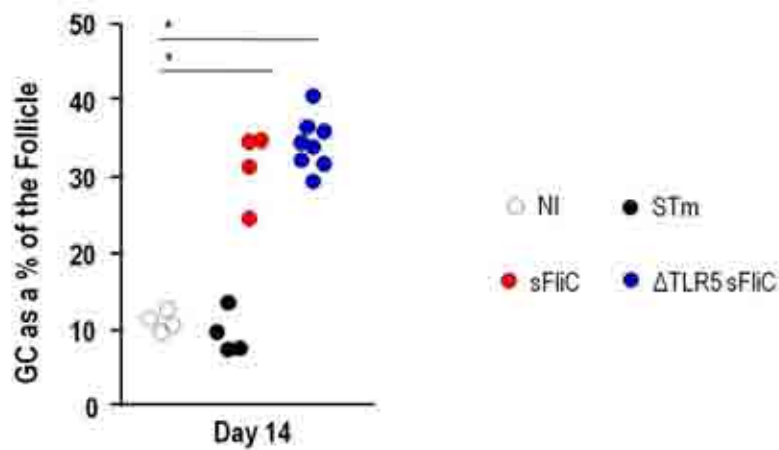
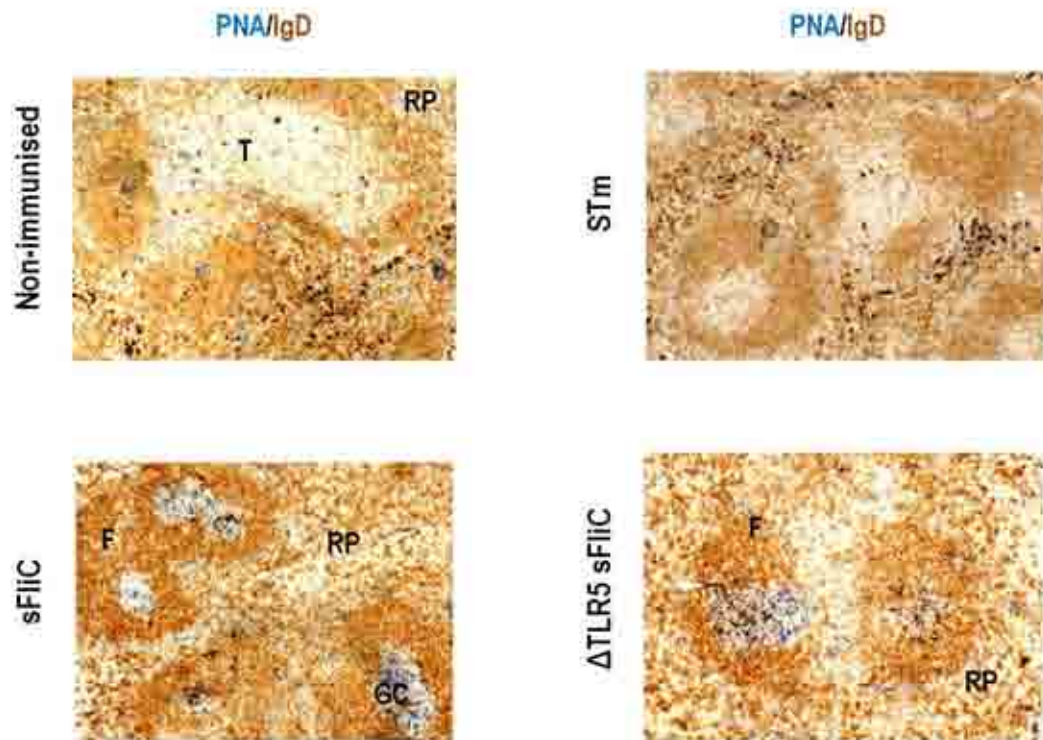
Deletion of part of the putative TLR5 binding site on flagellin does not influence antibody responses to the protein: WT mice were immunised intraperitoneally with 5×10^5 STm or $20 \mu\text{g}$ sFliC or $20 \mu\text{g}$ ΔTLR5 sFliC and antibody responses were assessed 14 days post-immunisation. (A) Anti-sFliC antibody titres for IgM, IgG1 and IgG2b were assessed. Data is representative of 5 mice per group and two independent experiments. * $P < 0.05$.

and double-stained for PNA (to identify GC) and IgD (to identify follicular B cells) (Fig 3.33B). GCs were then quantified as a percentage of the follicle which demonstrated that both sFliC and Δ TLR5 sFliC induced a comparable GC response relative to NI mice and mice infected with STm. This study suggests that some aspects of the sFliC response are not dependent on the amino acids removed in the mutated sFliC protein. Indeed, both sFliC and Δ TLR5 sFliC failed to induce IFN γ and selectively induced expression of IL-4 and GATA-3 mRNA (data not shown).

3.10 The protection seen in sFliC-immunised mice upon STm challenge can be TLR5 independent.

To ascertain whether the benefit afforded by sFliC immunisation upon subsequent challenge with STm at day 18 was observed in the absence of TLR5 signalling, WT and TLR5-deficient mice were immunised with either PBS or 20 μ g sFliC and on day 35 mice were challenged with 5×10^5 STm. Quantification of the splenic bacterial burden 18 days post-STm challenge, consistently revealed both sFliC-immunised WT and TLR5-deficient mice had a significantly lower bacterial burden relative to non-primed counterparts (Fig 3.34A). Interestingly, TLR5-deficient mice demonstrated a trend for greater bacterial numbers relative to sFliC-immunised WT mice. Quantification of CD4 T cell responses in the spleen revealed the proportion and total number of CD4 T cells was comparable between WT and TLR5-deficient mice, irrespective of sFliC immunisation (Fig 3.34B). In terms of the effector CD4 T cell response, TLR5-deficient mice had a significantly greater proportion of effector T cells relative to WT mice, regardless of sFliC immunisation, but total number of effector cells were comparable between all four groups of mice.

Antibody responses in sFliC-primed (35 days) WT and TLR5-deficient mice 18 days post-STm challenge were examined. Assessment of the anti-STm IgM response revealed TLR5-deficient mice demonstrated a trend for lower IgM titres relative to WT mice (Fig 3.35A). This was further apparent on

B**Figure 3.33**

Deletion of part of the putative TLR5 binding site on flagellin does not influence GC responses to the protein: WT mice were immunised intraperitoneally with 5×10^5 STm or 20 μ g sFliC or 20 μ g Δ TLR5 sFliC and responses were assessed 14 days post-immunisation. (B) Spleen sections were generated and double-stained with IgD (brown) and PNA (blue) to identify germinal centres (GC), F = follicle, T = T zone and RP = red pulp. The graph shows the quantification of the GC as a percentage of the follicle. NI= Non-immunised. Data is representative of 5 mice per group and two independent experiments. * $P < 0.05$.

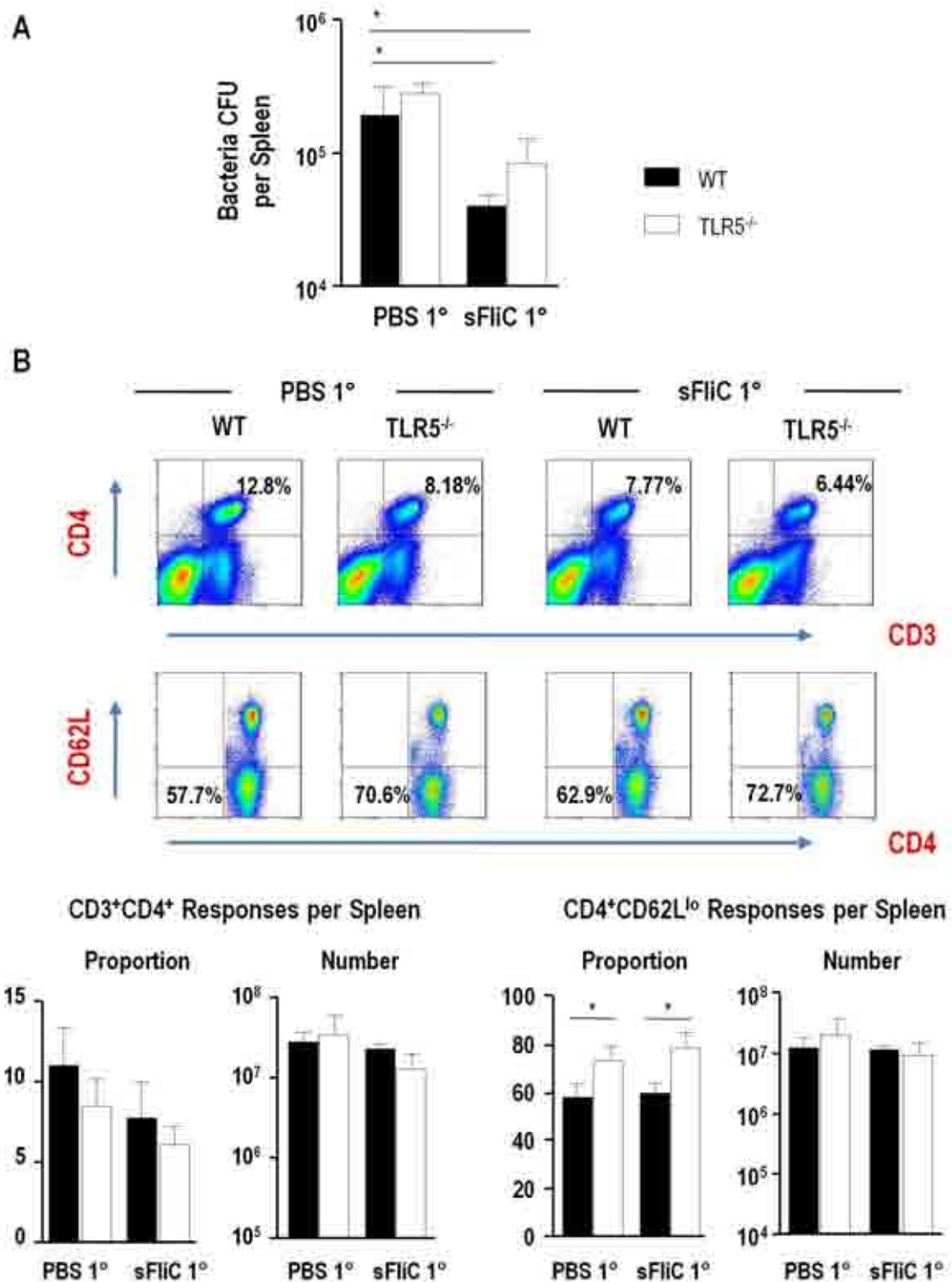
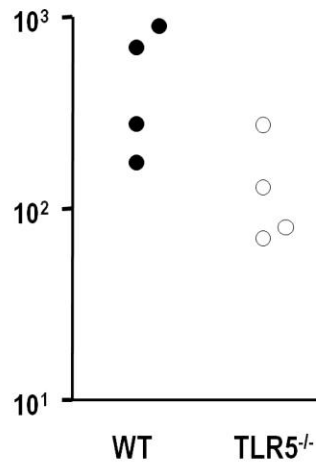


Figure 3. 34

The benefit afforded by sFlitC vaccination upon STm challenge at day 18 can be independent of TLR5: WT and TLR5 deficient mice were immunised with 20 µg sFlitC or PBS and were subsequently challenged on day 35 with 5 × 10⁵ STm and responses were assessed 18 days post-infection: (A) Splenic bacterial numbers were quantified and (B) CD4⁺ (row 1) and CD4⁺CD62L^{lo} (row 2) T cell responses were assessed by FACS. All immunisations were intraperitoneal. Data is representative of 5 mice per group and two independent experiments. *P < 0.05.

A

Relative anti-STm IgM Titres



B

Relative Anti-sFliC Antibody Titres

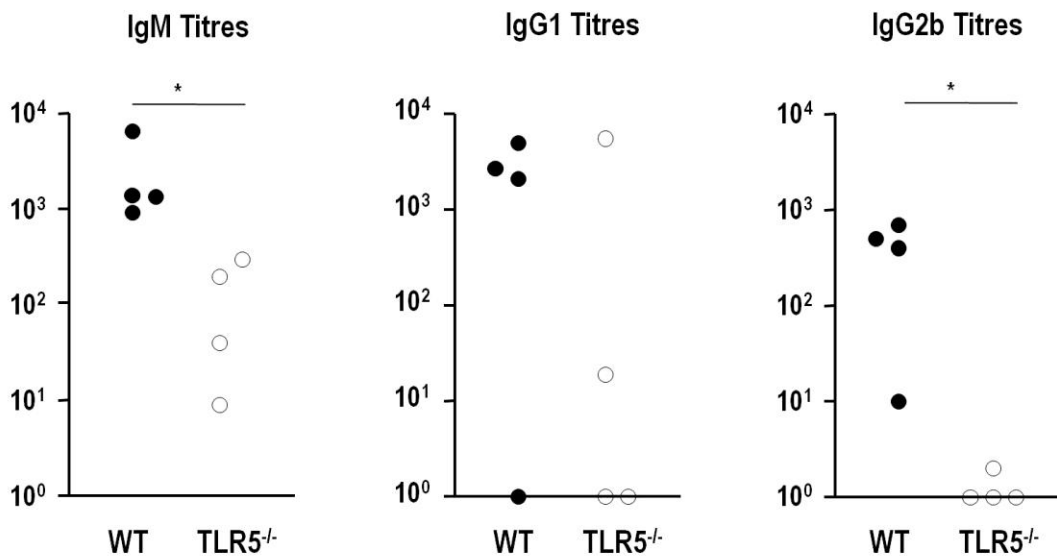


Figure 3. 35

Anti-sFliC antibody titres are reduced in sFliC-primed TLR5 deficient mice after STm challenge: WT and TLR5^{-/-} mice were immunised intraperitoneally with 20 µg sFliC before subsequent challenge with 5x10⁵ STm at day 35 post-priming. Antibody responses were assessed 18 days post-challenge: (A) Serum anti-STm and (B) anti-sFliC antibody titres were then quantified by ELISA. Data is representative of 4 mice per group. *P<0.05.

assessment of the anti-sFliC IgM response where TLR5-deficient mice had significantly lower IgM titres compared to WT mice (Fig 3.35B). Furthermore, TLR5-deficient mice displayed a trend for lower anti-sFliC IgG1 titres, and had significantly lower IgG2b titres relative to WT mice. These data demonstrate that the benefit afforded by sFliC-immunisation upon subsequent STm challenge at day 18 can bypass TLR5 ligation. Although some of the properties of sFliC may be induced independently of TLR ligation, TLR5 ligation may be involved in antibody responses to sFliC.

3.11 Discussion

This study examined the immune response to flagellin which can stimulate innate and adaptive immune responses. Immunisation with sFliC induced a predominantly Th2 response, irrespective of whether mice were immunised with monomeric or polymeric flagellin. This supports earlier studies demonstrating flagellin is capable of provoking T and B cell responses with a strong Th2 bias to itself and co-administered antigens (Cunningham et al., 2004a; Didierlaurent et al., 2004; Huleatt et al., 2007). This work addressed this further by examining responses in flagellin-specific T cells. The Th2 response induced to sFliC in vivo developed in the absence of exogenous adjuvants. Only a select few antigens have been identified with auto-adjuvant activity, tetanus toxoid and pertussis toxin, and in mice and humans responses to these antigens are predominantly Th2 (Barnes et al., 2007; Cunningham et al., 2004a; Didierlaurent et al., 2004; Divekar et al., 2006; Sugai et al., 2005).

It is surprising that flagellin can drive Th2 features, since flagellin signals through TLR5 and initiates signalling through MyD88 (Hayashi et al., 2001; Smith et al., 2003a). MyD88-dependent TLR signalling is classically associated with Th1 responses (Schnare et al., 2001) and the development of Th2 responses has been proposed to be independent of this pathway. Nevertheless, Didierlaurent and colleagues demonstrated flagellin promoted Th2-type immunity through MyD88 signalling (Didierlaurent et al., 2004). Additionally, we found that despite deletion of part of the putative TLR5 binding site

(Andersen-Nissen et al., 2005) immunogenic properties affiliated with sFliC immunisation, such as the induction of GC and antibody switching to IgG1 were maintained. Therefore, it is probable that this region of flagellin is not fully responsible for TLR5 ligation or TLR5 ligation may not be fully responsible for the properties of sFliC which we assessed. Indeed, recently a recombinant flagellin from *C. jejuni* was constructed in which TLR5 activity was reconstituted (de Zoete et al., 2010). Activation of TLR5 in this construct required the presence of a β -hairpin domain in addition to the putative TLR5 motif (Smith et al., 2003a).

Antibody responses (IgM and IgG subclasses) to flagellin were reduced in TLR5 deficient mice relative to WT mice. This was surprising as TLR5-deficient mice have been shown to maintain comparable antibody responses to flagellin as WT mice, whilst the dual loss of TLR5 and Ipaf has been shown to markedly reduce humoral responses to flagellin (Vijay-Kumar et al., 2010). In the main, the mechanism by which flagellin functions remains poorly understood. Although it is not clear why sFliC drives a Th2 response, our ongoing work has now revealed differences in the DC populations in response to STm or sFliC, which can influence the direction of T cell polarisation. This work by Dr. A. Flores-Langarica shows that STm needs to recruit monocyte-derived DC to drive Th1 responses. Thus, the reason sFliC may induce a Th2 response may be because it fails to recruit this additional DC subset.

We found co-immunisation with STm and sFliC suppressed the Th1 response to STm. This was not observed when alum-precipitated ovalbumin was co-administered with STm, nor was it apparent in our dual infection model, with the Th2 inducing parasite *N. brasiliensis* and STm (discussed in Chapter Five). This suggests that Th2 suppression of Th1 responses does not always occur when divergently polarising antigens are given, such as whole pertussis vaccine with alum-proteins. Indeed Th1 and Th2 responses to co-administered antigen can develop in parallel in the same lymph node (Toellner et al., 1998). This implies that sFliC has a specific capacity to mediate this effect, perhaps through the need to signal through TLR5, whilst alum may be able to function through a TLR-independent mechanism

(Gavin et al., 2006). It would be interesting to assess if this suppression is apparent in TLR5 deficient mice and to elucidate the mechanism through which it occurs as IL-10 did not appear to be a likely candidate. Nevertheless, it does suggest that the combination in which component antigens are administered may affect the properties of the primary response initiated, and should be considered in vaccine design.

Directing the immune response to an antigen has been a significant focus of research, particularly for adjuvants, because the type of T cell help initially induced to an antigen can indicate whether it will confer protection. The absolute need for this is challenged by our findings. Indeed, despite the Th2 polarisation of sFliC and its remarkable capacity to suppress IFN γ upon co-administration with STm, sFliC immunised mice showed enhanced protection upon STm challenge. Surprisingly, this benefit afforded by sFliC immunisation was driven by augmentation of the Th1 response to STm upon subsequent infection.

Th2 differentiation to sFliC was the dominant CD4 subset in the primary response, and only low levels of IL-17 producing CD4 T cells, FoxP3 $^{+}$ regulatory T cells and BCL6 $^{+}$ follicular T-helper cells were detected post-sFliC immunisation (data not shown). Nevertheless, it is possible that other primed CD4 T cell subsets were recruited into the response upon STm challenge. Indeed, it is also possible in this model that T cells specific to antigens other than flagellin account for this effect and that flagellin simply mediates the effect. This could be easily tested by priming mice with flagellin and subsequently infecting with aflagellated STm.

Historical studies have postulated that Th1 and Th2 subsets are mutually suppressive as they reinforce their own development whilst reciprocally inhibiting other differentiation programs (Szabo et al., 1997; Usui et al., 2006). For example, several reports have found Th2 cells are stably committed and resistant to re-programming towards Th1 in-vitro (Murphy et al., 1996; Szabo et al., 1995). Nevertheless, it is possible that the polarisation of T cells to Th2 may not be stable throughout the course of a STm

infection in vivo. Thus, the increased numbers of Th1 cells seen after STm infection of sFliC-primed mice could result from the redirection of Th2 cells to Th1. Indeed, it was recently demonstrated that the Th1 cell promoting lymphocytic choriomeningitis virus (LCMV) re-programmed otherwise stably committed Th2 cells to adopt a GATA-3+T-bet+ phenotype, with a remarkable capacity to co-produce Th1 and Th2 cytokines (a feature also displayed by NK T cells) which was stably maintained in vivo for months (Hegazy et al., 2010). Our study supports this and we illustrate the likelihood of Th2 plasticity in vivo in a different infection model. This is of particular importance in allergy and asthma where the persistence of Th2 cytokines is associated with exacerbation of disease and such re-programming may offer an avenue for potential treatment.

As primary T and B cell responses to sFliC were T-bet independent it was possible that the Th1 augmentation after sFliC would also be T-bet independent. Indeed, although the expression of T-bet is often required for the induction of IFN γ , T-bet can be dispensable in other infections (Way and Wilson, 2004; Ylikoski et al., 2005). In contrast, LCMV-specific in vitro-primed Th2 cells can redirect to Th1 in vivo and produce IFN γ in a T-bet-dependent manner (Hegazy et al., 2010). Similarly, our study supports this as we found the enhanced Th1 response in sFliC-immunised mice also required T-bet. Interestingly, the LCMV study demonstrated the acquisition of additional Th1 attributes by stable committed Th2 cells was marked by a mixed response, as cells co-expressed GATA-3 and T-bet as well as Th2 and Th1 cytokines (Hegazy et al., 2010). However, we failed to detect any IL-4 by ELISPOT in sFliC-immunised mice 18 days post STm-challenge suggesting sFliC-primed Th2 cells may not have retained their initial differentiated state (or their frequency may be lower than the detection level for ELISPOT).

Additionally, it has been demonstrated that antigen stimulation of naïve CD4 T cells in vivo and in vitro can lead to the production of a unique subset of primed, but uncommitted (precursor) CD4 T cells which fail to induce the characteristic cytokine profile of Th1 and Th2 responses (Wang and Mosmann, 2001; Yang and Mosmann, 2004). These studies were expanded to show that human CD4 T cells primed by

protein subunit vaccines largely remain uncommitted, and this population of uncommitted CD4 T cells induced by protein vaccines could differentiate into Th1 or Th2 phenotypes under appropriate polarising conditions. These primed, uncommitted precursor cells may constitute an expanded pool of antigen-specific T cells which have the potential to differentiate into Th1 or Th2 phenotypes during subsequent responses (Divekar et al., 2006; Wang and Mosmann, 2001; Yang and Mosmann, 2004). The data from our study may also mean that primed, yet non-committed CD4 T cells, act as a reservoir from which the enhanced Th1 responses after STm infection of sFliC-immunised mice are derived.

The direction of the T-helper response may be of secondary importance if protective antibody responses are induced, as most vaccines work through antibody. Potent antibody responses were induced to flagellin which had the capacity to inhibit the motility of STm. Despite these functional attributes, antibody to flagellin did not have a significant role against systemic STm infection in these studies. Indeed, we found antibody to surface isolated flagella was able to impair subsequent infection with STm at day 5. However, this was attributed to contaminants within the preparation (data not shown) which emphasises the purity of our recombinant flagellin preparation and demonstrates that antibody to highly pure flagellin would not be protective.

Interestingly, although limited, antibody to flagellin was able to moderate infection with STm when the ability of STm to phase-switch its flagellin genes was compromised. There is some evidence that FliB-locked STm is attenuated in vivo (Bergman et al., 2005). Furthermore, O'Brien and colleagues demonstrated FliC-locked STm had a selective advantage over FliB-locked STm once in the tissues (Ikeda et al., 2001), and although we used a different route of infection (intraperitoneal rather than intravenous or oral) we found differences between the two strains to be modest. Indeed, even after infection of sFliC-immunised mice with FliC-locked STm the benefit afforded by antibody was small and was not sustained at day 18. The limited benefit afforded by antibody to flagellin is surprising as we have previously shown antibody can reduce bacterial numbers by several orders of magnitude (Gil-Cruz

et al., 2009), and antibody to STm is clearly important at preventing infection in humans and mice (Gil-Cruz et al., 2009; MacLennan et al., 2008; McSorley and Jenkins, 2000).

The benefit afforded by sFliC immunisation was antibody-independent and provided entirely through CD4 T cells, but only operated after the end of the first week of a subsequent STm infection. The inability of CD4 T cells to protect early in infection was surprising, as more than 50% of CD4 T cells were activated by day 14 in primary responses to sFliC (data not shown) indicating the potency of flagellin for driving adaptive responses, as in agreement with other studies (Alaniz et al., 2006; McSorley et al., 2002b). As the benefit afforded by sFliC immunisation was late in the response to STm this has two implications: firstly, it suggests that T cells in recall responses do not promote accelerated bacterial clearance after STm infection. Thus, sFliC-immunisation may not confer significant protection against virulent strains of STm as its contribution to immunity is made when infection is already well established. Secondly, it suggests that there is some bioavailability of flagellin during infection. There is some evidence that flagellin synthesis, expression and availability is down-regulated during intracellular infection in macrophage-like cells (Alaniz et al., 2006; Cummings et al., 2005; Cummings et al., 2006; Hautefort et al., 2008). However, other studies suggest suppression of flagellin synthesis and expression may not be absolute during infection (Srinivasan et al., 2004; Subramanian and Qadri, 2006). Indeed, it was demonstrated that bacterial recognition and contact with host cell lysophospholipids can induce the active synthesis and secretion of monomeric flagellin, which through activation of TLR5 and subsequent recruitment of inflammatory cells to the site of infection, could promote systemic dissemination (Subramanian and Qadri, 2006).

Nevertheless, it is unclear whether the modest T cell-mediated protection after flagellin immunisation is because CD4 T cells induced after immunisation are not efficient at promoting bacterial killing, or whether only a proportion of infected cells contain flagellin-expressing bacteria. Flagellin phase-switching is unlikely to promote immune evasion since CD4 T cell responses to FliC are directed against

multiple epitopes which co-localise in the conserved regions of the protein. Furthermore, CD4 T cells are able to recognise both FliC and FliB, although with a small bias towards FliC (Bergman et al., 2005; Ikeda et al., 2001). Interestingly, this conserved region of the STM FliC protein also contains the T cell epitope recognised by SM1 CD4 T cells (McSorley et al., 2002b) and this site is conserved across several Gram-negative enteric bacterial species. Therefore, this offers the possibility that sFliC vaccination could provide broad acting T cell, but not B cell, mediated immunity.

The adjuvant properties of flagellin (Bergman et al., 2005; Cunningham et al., 2004a; Didierlaurent et al., 2004) underlie the rationale of employing it in vaccine preparations. Indeed, its intrinsic adjuvant activity is currently being employed in experimental recombinant vaccines against human influenza, West Nile fever, malaria and tuberculosis (Bargieri et al., 2008; Honko et al., 2006; Le Moigne et al., 2008; McDonald et al., 2007; Song et al., 2008). In closing, although sFliC induced potent humoral and cellular immune responses, only cellular immunity was able to modestly contribute to controlling infection, and then only from the second week. Therefore, the potential of sFliC as a subunit vaccine has major drawbacks, largely due its failure to promote antibody-mediated immunity means it may not be sufficient to induce rapid control, despite providing T cell mediated benefits at later time-points.

4.0 The porin OmpD is a key target for a protective B1b cell antibody response against NTS

4.1 Introduction

The identification of antigens that can induce protective antibody is fundamental in vaccine design. Whilst antibody does not impact upon clearance of primary STm infection or the intracellular phase of bacterial growth (Cunningham et al., 2007; Mastroeni et al., 2000; McSorley et al., 2000; Mittrucker et al., 2000), the extracellular growth of bacteria in the blood requires humoral immunity. Antibody present at the time of infection has been shown to be effective in moderating bacteraemia (Cunningham et al., 2007; Mastroeni and Menager, 2003) which may be pivotal in the control of NTS infections.

NTS infections in SSA cause a progressive bacteraemia in HIV⁺ adults and children, and is a strong correlate of disease severity, killing around 50% of infected individuals. Additionally, NTS infections also cause fatal disease in children under the age of two who are HIV negative. The period of initial susceptibility in these infants coincides with when maternally acquired antibody levels wane and occurs before the development of STm-specific antibody (MacLennan et al., 2008). Indeed, this risk inversely correlates with the presence of specific antibody which provides compelling evidence that antibody is important in NTS infections, and restricting bacteraemia is likely to be central to controlling disease.

Previously we reported that STm induces atypical antibody responses characterised by an early, accelerated expansion of extra follicular (EF) plasma cells without the parallel induction of Germinal Centres (GCs). The early switched antibody response was T-dependent (TD), and OMPs were major targets of this response (Cunningham et al., 2007). The outer membrane of STm contains many OMPs including the related porins OmpC, OmpD and OmpF which can associate to form trimers. In the absence of exogenous adjuvants purified porins from ST can induce an atypically sustained antibody response that confers bacteriocidal benefit against ST (Secundino et al., 2006). These properties in

combination with our earlier work showing OMPs were an early antibody target (Cunningham et al., 2007), prompted this assessment of the properties of the antibody response to purified porins isolated from STm. Particular emphasis was placed on whether immunisation with STm porins can provide protection against a systemic STm infection.

4.1.1 Experimental Design

The characteristics of the antibody response to purified porins from STm were examined by assessing the following:

1. Early B cell switching to IgG2a in response to OMPs was TD. Using TCR-deficient mice, whether induction of the early EF plasma cell response to OMPs also required T cells was assessed.
2. To ascertain whether porin immunisation could provide protection against a subsequent systemic STm infection, and whether this could offer cross-protection against other *Salmonella* serovars a prime-challenge model of infection was used. All challenges post-porin immunisation were with the attenuated SL3261 strain unless stated otherwise. Highly purified porins isolated from STm were kindly provided by our collaborator Prof. C. Lopez-Macias.
3. The STm porins in this study are trimeric complexes, by utilising different STm strains bearing selective deletions of specific OMPs the relative contribution of the individual porins OmpC, OmpF and OmpD was assessed.

4.2. STm induces a TI plasma cell response against porins in the outer membrane

Previous studies have reported STm induces a rapid and extensive antibody response, and that a major target of the early switched response were the OMPs, and not LPS or sFliC (Cunningham et al., 2007). As this EF plasma cell response was apparent by day 4 its induction suggested a T-independent (TI) element. To further dissect this, the response in $\beta\delta$ TCR-deficient mice (TCR-deficient) was assessed. TCR-deficient mice were infected intraperitoneally with 5×10^5 STm for 4 days. Spleens were isolated and sections were generated for immunohistology which were double-stained for CD138 (a plasma cell and plasmablast marker) and IgD (to identify follicular B2 cells) (Fig 4.1A). Quantification of the number of CD138⁺ cells demonstrated that infection with STm resulted in an increase in these cells by day 4 relative to NI TCR-deficient mice.

The specificity of this antibody response against a preparation of homogenised total STm by ELISA was then assessed. This revealed that the observed increase in CD138⁺ plasma cells upon STm infection was paralleled by a rapid induction of STm-specific serum IgM, which was significantly enhanced relative to NI mice (Fig 4.1B). Subsequently, this anti-STm IgM response against a range of surface components associated with the outer membrane of STm was further examined. This revealed serum IgM in TCR-deficient mice infected with STm was also strongly induced to LPS and a preparation containing a mixture of total OMPs, but was less marked in response to sFliC (Fig. 4.1B). Therefore, this early EF plasma cell response developed rapidly in the absence of T cells and a major target of this antibody response were OMPs. Since IgG levels did not increase above background levels (data not shown) it suggests that T cell help was required to induce switching as demonstrated by our previous work (Cunningham et al., 2007).

It was surprising that proteins could induce a TI antibody response, so we focussed on this by assessing the response to a highly purified OMP preparation from STm. This preparation consisted primarily of the

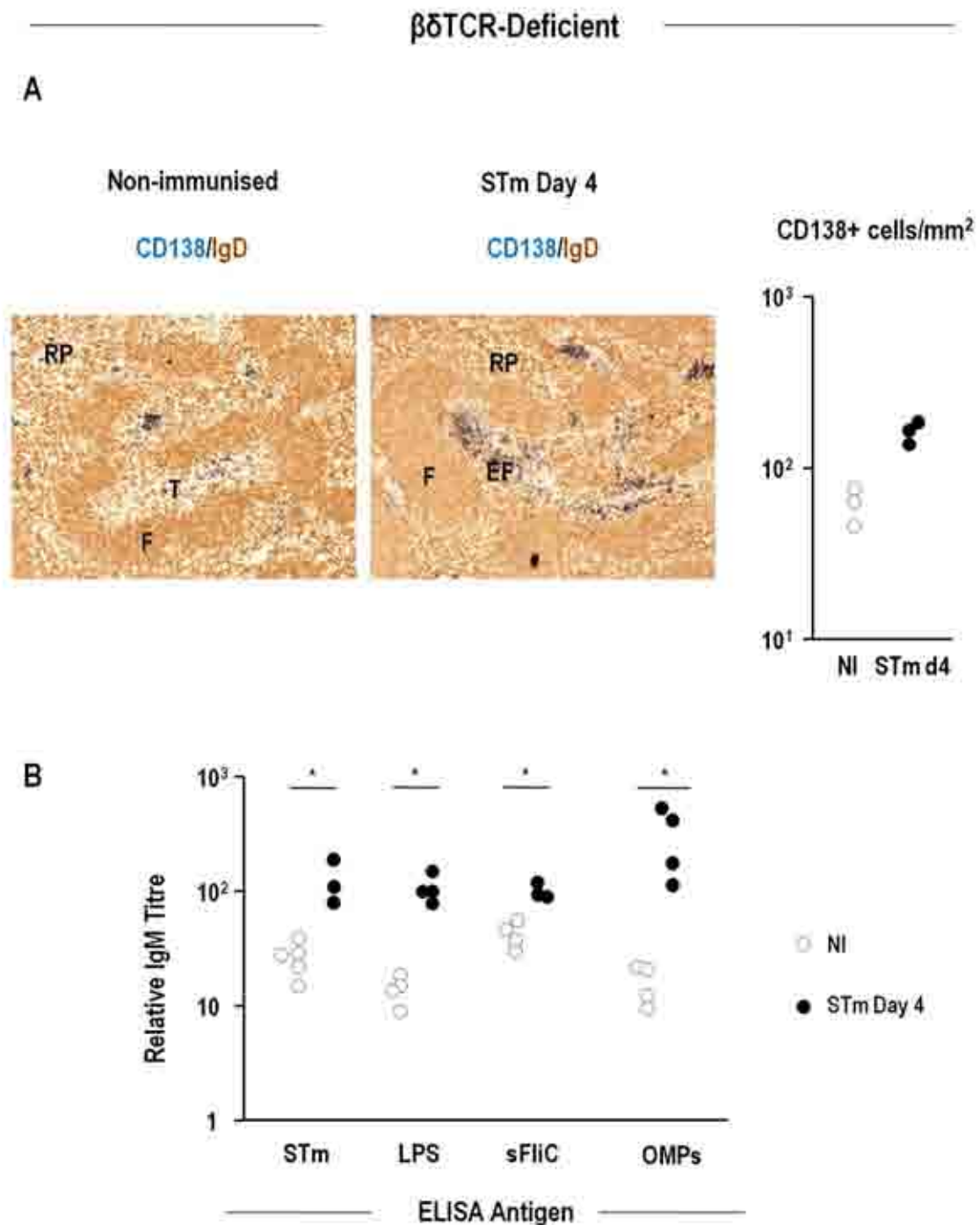


Figure 4.1

STm induces a rapid TI plasma cell response: (A) Spleens sections were generated for histology from βδTCR^{-/-} (TCR-deficient) mice infected intraperitoneally with 5x10⁵ STm for 4 days and NI controls: Representative photomicrographs of spleen sections stained for CD138 (blue) and IgD (brown) by immunohistology (left panel) were used to quantify splenic CD138⁺ plasmacytoid cells per square millimeter (right panel) from these mice. F = follicle, T = T zone, RP = red pulp, EF= extra follicular plasmablasts. (B) Serum IgM titres were assessed from these mice by ELISA against STm, LPS, soluble recombinant flagellin (sFlIC) and a total OMP preparation (OMPs). Data is representative of two independent experiments and 4 mice per group. *p<0.05

co-purifying porins OmpC, OmpD, and OmpF, which are referred to as porins hereafter. The porins were prepared by Dr Cristina-Gil Cruz in collaboration with Prof. C. Lopez-Macias as described in Appendix C. Before assessing the *in vivo* response to the porins their identity was confirmed by trypsin digest and QTOF mass spectrometry (data not shown). Subsequent to this, their purity was examined by SDS PAGE which revealed a highly purified protein at the expected molecular weight ($M_r = \approx 38$ kDa). Upon more extensive separation using a higher percentage acrylamide gel it was apparent that the purified porins contained OmpC (36 kDa) and OmpD (34 kDa), with trace amounts of OmpF (35 kDa) (Fig. 4.2A).

Once the purity and identity was confirmed the structural composition of the porins was examined. Quantitative structural analysis using circular dichroism (CD) revealed a characteristic CD spectra of a folded protein with an absorbance spectrum consistent with that of β -sheets (minimum at 218 nm), suggesting that the protein was folded in a native conformation (Fig. 4.2B). Furthermore, the porins were separated by SDS-PAGE under heat-denaturing conditions at 100°C or under semi-native conditions at 25°C. This showed when heated to 100°C in SDS PAGE sample buffer, purified porins migrated at their expected molecular mass of 38 kDa, but under semi-native conditions porins migrated mostly as a 70-kDa protein (Fig 4.2B). This suggested that a large portion of the porins adopted a β -barrel conformation, which is stable in SDS unless heated (Sugawara et al., 1996). Interestingly, this heat-modifiable mobility displayed by porins to SDS PAGE is a feature of folded β -barrels such as porins (Nikaido, 1996). Finally, the structural conformation of porins in solution was examined using analytical ultracentrifugation. The mass profile from the sedimentation coefficient (rate of movement) of the porins had a broad distribution with a peak coefficient of 8.1, indicative of oligomeric structures with a molecular weight of 120-240 kDa, suggesting they adopted a trimeric or hexameric configuration (Fig 4.2B). The structural and biophysical assessments demonstrated the porins isolated from STm were folded in a native conformation and formed oligomeric structures, which was consistent with their normal conformation in the outer membrane.

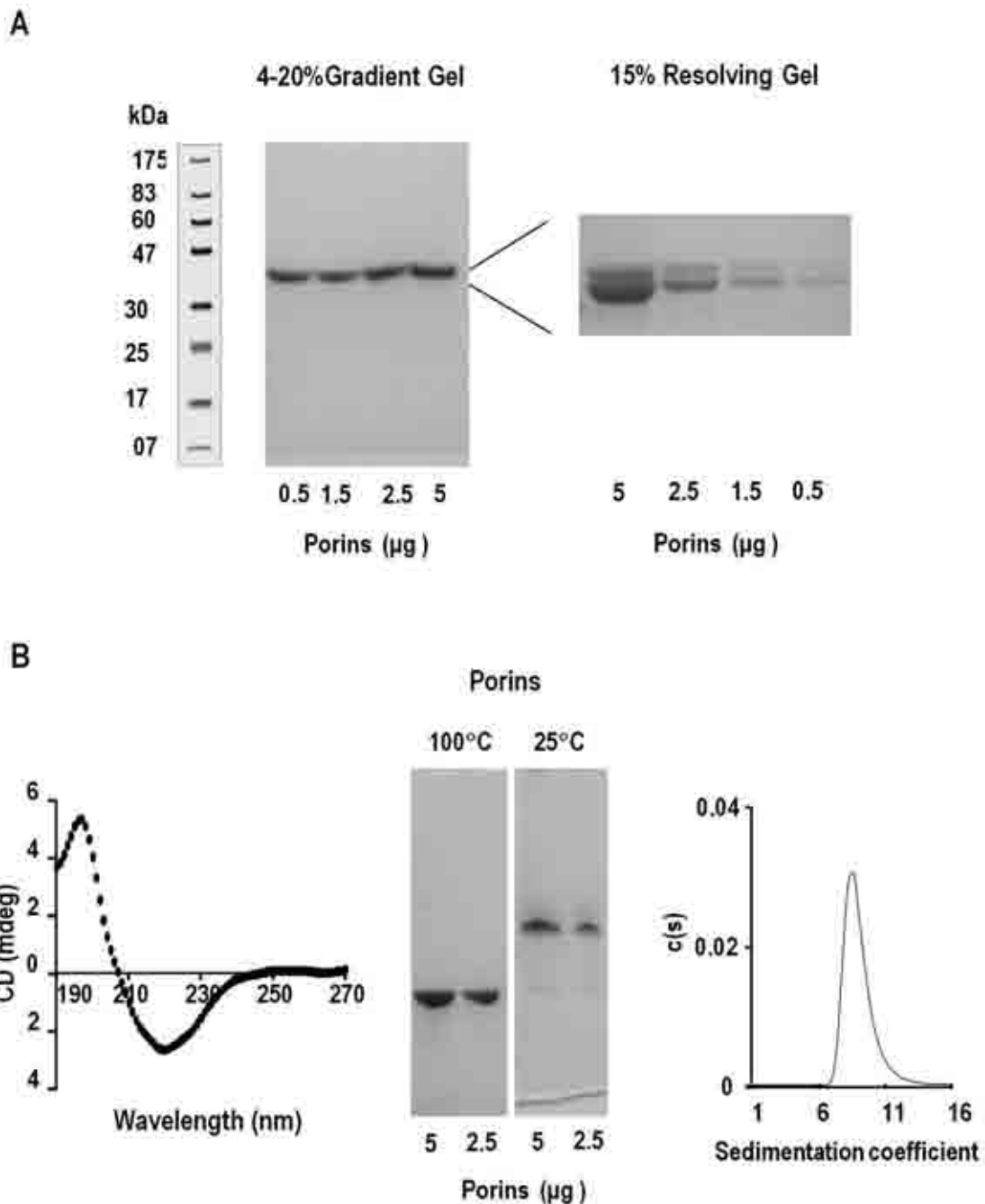


Figure 4.2:

STm porins are highly pure and adopt a native conformation in solution: (A) Analysis of porin purity and composition: Porins were electrophoretically separated using a 4-20% gradient gel showing their purity (left panel) or a 15% polyacrylamide gel to reveal two major species OmpC and OmpD with trace amounts of OmpF (right panel). (B) Analysis of porin structure: Circular dichroism (CD) trace of purified porins following far UV CD measurements on a JASCO J-715 spectropolarimeter (values in millidegrees (mdeg)) (left panel). Porins were electrophoretically separated using a 4-20% gradient gel under denaturing (100°C) or semi-native (25°C) conditions (middle panel). Sedimentation coefficient distributions for porins after analytical ultracentrifugation (AUC) (right panel). CD and AUC data was generated in conjunction with Dr. Karina Tveen-Jensen.

Following porin characterisation, it was assessed whether they could induce an antibody response in the absence of T cells by immunising TCR-deficient mice with 20 µg porins intraperitoneally. Spleen sections were generated 7 days post-immunisation for immunohistology and double-stained for IgM and IgD (Fig 4.3A). Qualitatively, this revealed mice immunised with porins induced a substantial IgM response relative to NI mice. We next examined antigen-specificity by ELISA and western blot analysis. Both methods confirmed that immunisation with porins induced a large increase in anti-porin IgM relative to NI mice (Fig 4.3B). Western blot analysis demonstrated that IgM in sera from NI and porin-immunised TCR-deficient mice were both able to detect non-porin targets induced independently of porin immunisation (Fig 4.3C the band at 27 kDa). Thus, both groups of mice had natural IgM that bound some STm proteins. However, only porin-immunised mice specifically reacted with the porin target, which was rarely bound by natural antibody (only one out of seven sera showed some reactivity). These data demonstrate that a porin preparation can induce an early and prominent plasma cell response in the absence of T cells. Furthermore, since natural antibody can recognise some components in STm, the binding of natural antibody is likely to be insufficient in immunity to STm. This point has been verified by challenge of WT and B cell deficient mice (data not shown).

4.3 The anti-porin IgM response is sufficient to impair STm infection

The antibody titres induced by vaccines can be a good marker for the degree of protection (Engels et al., 1998; Xu et al., 1993). Since immunisation with porins induced a robust antibody response, whether antibody induced after immunisation with a single dose of porins could reduce the bacterial burden of a subsequent STm infection was examined. WT mice were immunised intraperitoneally with 20 µg porins or 5×10^6 heat-killed STm and on day 35 they were infected with 5×10^6 STm alongside NI WT mice (Fig 4.4A). Heat-killed organisms are used in current vaccines against ST and were therefore, an ideal comparative. Assessment of the splenic bacterial burden at day 5 post-STm infection showed mice immunised with porins or heat-killed STm were both able to reduce bacterial numbers, and to a similar

$\beta\delta$ TCR-Deficient

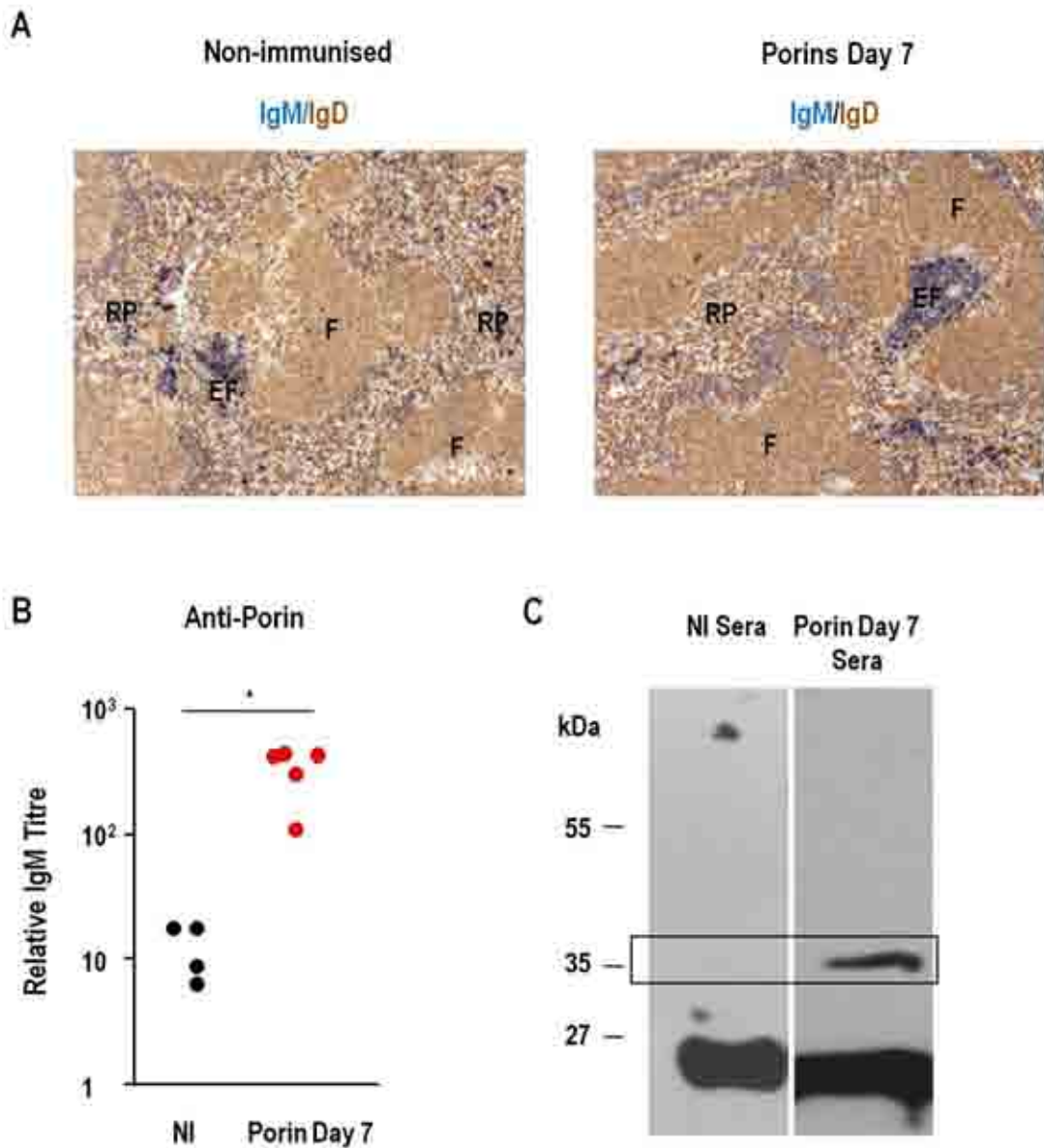


Figure 4.3:

Porins from STm induce a TI plasma cell response: (A) Representative photomicrographs of spleen sections stained for IgM (blue) and IgD (brown) by immunohistology from TCR-deficient mice immunised with 20 μ g porins for 7 days and NI controls. F = follicle, RP = red pulp, EF= extra follicular plasmablasts. (B) Serum anti-porin IgM titres were assessed by ELISA in these mice. (C) Immunoblot analysis of binding of IgM from sera isolated from NI or day 7 porin-immunised TCR-deficient mice against a total OMP preparation isolated from STm. Boxed area highlights approximate MW of the porins. The binding of sera to other proteins (such as at approximately 27 kDa) reflects the presence of natural antibody which is independent of porin immunisation. Non-immune and immune sera were added at 1:50 and 1:150, respectively. ELISA data is representative of three experiments; immunoblots representative of 5 non-immune sera and 5 immune sera. The Mann-Whitney test was used to compare the statistical significance between groups. * $p < 0.05$.

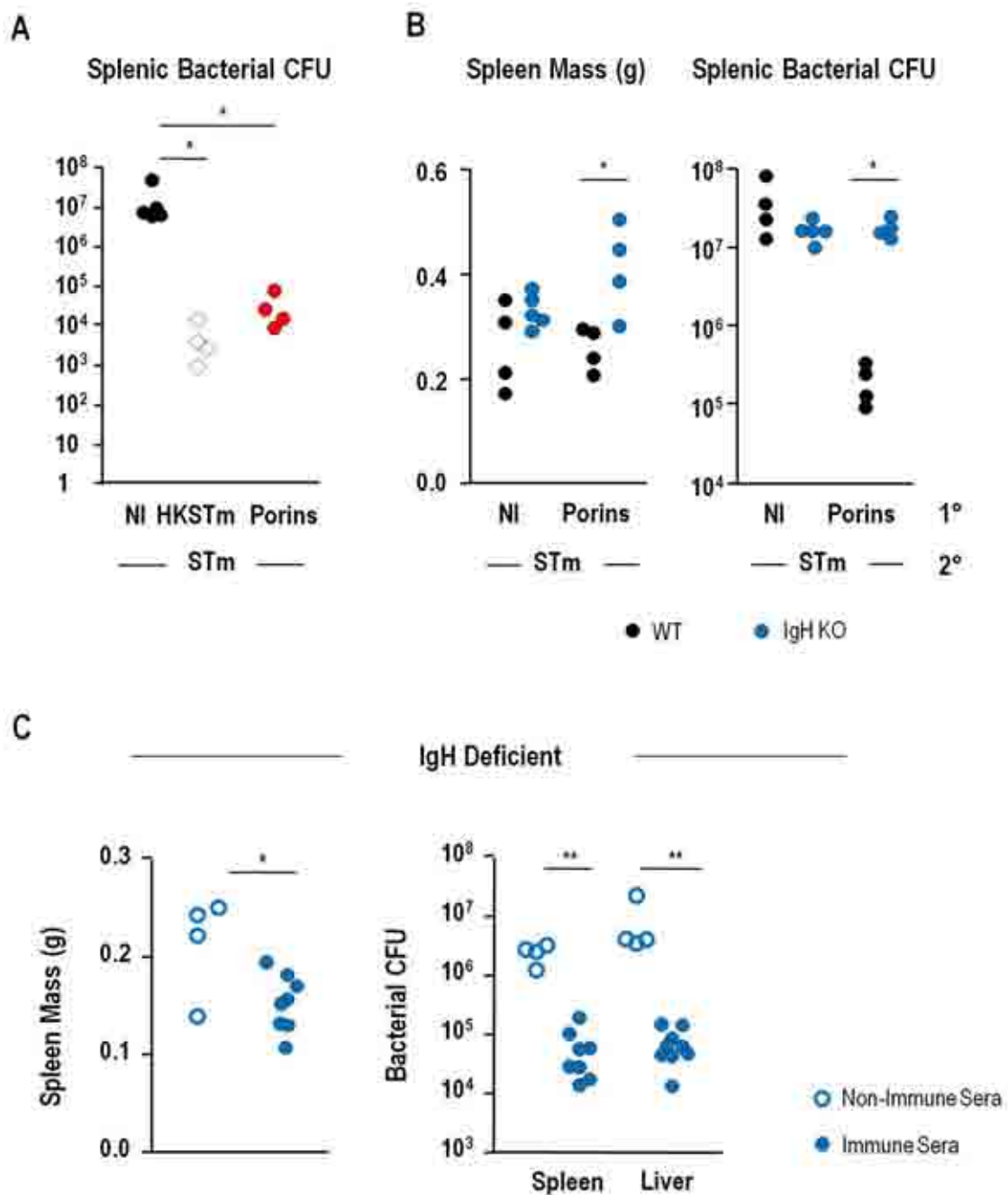


Figure 4.4:

Immunisation with porins impairs STm infection in a B cell-dependent manner: (A) WT mice immunised with 20 μ g porins or 5×10^6 heat-killed STm were infected on day 35 with 5×10^6 STm alongside NI controls. Splenic bacterial numbers were enumerated at day 5 post-challenge. This data was generated in conjunction with Dr. C. Gil-Cruz. (B) NI or porin-immunised WT and IgH^{-/-} (B-cell deficient) mice were infected as in (A). Spleen mass (left) and splenic bacterial CFU (right) were enumerated. This data was generated in conjunction with Dr. C. Gil-Cruz. (C) B cell-deficient mice were infected with STm opsonised with complement-inactivated sera from NI or day-4 porin-immunised TCR-deficient mice. Spleen mass (left) and splenic and liver bacterial CFU (right) were enumerated 5 days post-infection. Data is representative of two separate experiments and 4 mice per group. The Mann-Whitney test was used to compare statistical significance between groups * $p < 0.05$.

capacity, as both immunogens reduced splenic bacterial burdens by a median 2-3 logs compared to NI WT mice. Therefore, a single dose of porins was sufficient to impair subsequent STm infection.

4.4 The protection conferred by porin immunisation requires B cells

To identify the cellular components necessary for the protection observed upon immunisation with porins, the role of B cells was examined. To do this, WT mice and B cell-deficient mice (IgH-deficient) were immunised intraperitoneally with 20 µg porins and on day 35 they were infected intraperitoneally with 5×10^6 STm for 5 days alongside NI counterparts (Fig 4.4B). The spleen mass was broadly equivalent between the groups, except porin-immunised IgH-deficient mice had significantly larger spleens relative to porin-immunised WT mice. Assessment of the splenic bacterial load revealed bacterial numbers were equivalent between immunised and NI B-deficient mice, whereas porin immunised WT mice had a markedly reduced bacterial load relative to NI mice and porin-immunised B-cell deficient mice. This suggested, B cells were required for the protection provided by porin-immunisation.

As the protection was B cell mediated and early it suggested that antibodies may be involved through their ability to opsonise STm and facilitate phagocytosis. STm was opsonised with complement-inactivated sera from NI TCR-deficient mice or from TCR-deficient mice immunised with 20 µg porins for 4 days, and IgH-deficient mice were immediately infected (Fig 4.4C). The spleen mass 5 days post-infection was significantly lower in mice infected with STm opsonised with immune sera relative to those infected with STm opsonised with non-immune sera. Quantification of the splenic and liver bacterial burden showed that mice infected with STm opsonised with immune serum, had significantly lower bacterial numbers relative to mice infected with STm opsonised with non-immune serum. Therefore, the benefit of porin immunisation was mediated through B cells as reductions in bacterial titres were ablated if B cells were not present during immunisation, but were restored upon opsonisation of STm.

It was apparent that the protection provided by porin immunisation to STm infection was B cell-mediated and TI. To assess whether switched antibody conferred additional benefit over and above the IgM response, WT and TCR-deficient mice were immunised with 20 µg porins intraperitoneally and on day 35 they were infected intraperitoneally with 5×10^5 STm alongside NI counterparts. Assessment of the spleen mass 5 days post-infection revealed no differences between WT or TCR-deficient mice, irrespective of porin-immunisation, but the spleen masses in porin-immunised mice were significantly lower than non-immunised mice (Fig 4.5A). Quantification of the splenic and liver bacterial burden demonstrated porin-immunised TCR-deficient mice had a lower bacterial burden than NI TCR-deficient mice. However, porin-immunised TCR-deficient mice had a 15-fold and 10-fold higher bacterial load in the spleen and liver, respectively relative to porin-immunised WT mice. Subsequently, the anti-porin antibody response was assessed by ELISA (Fig 4.5B). This revealed that although the induction of IgM was unimpaired in porin-immunised TCR-deficient mice relative to porin-immunised WT mice after infection, porin-specific IgG was largely undetectable. Since >90% of the benefit after porin immunisation was mediated through switched antibody it means promoting T cell recruitment is important because in the absence of T cells, IgG switching is diminished.

4.5 Porin immunisation is effective in eliminating bacteraemia and protecting against virulent STm

Bacteraemia is a marker of severity during NTS infections so the influence of porin immunisation on the blood bacterial burden was assessed. WT mice were immunised intraperitoneally with 20 µg porins and infected on day 35 with 5×10^6 STm alongside NI mice. Quantification of the bacterial burden in the blood on day 5 post-infection (which we have historically shown is the peak of the bacteraemia (unpublished findings)) revealed the median bacteraemia in NI mice was 6000 bacteria per ml, but no bacteria were cultured from the blood post porin-immunisation (Fig. 4.6A). Thus, antibody induced against porins is effective at eliminating bacteraemia.

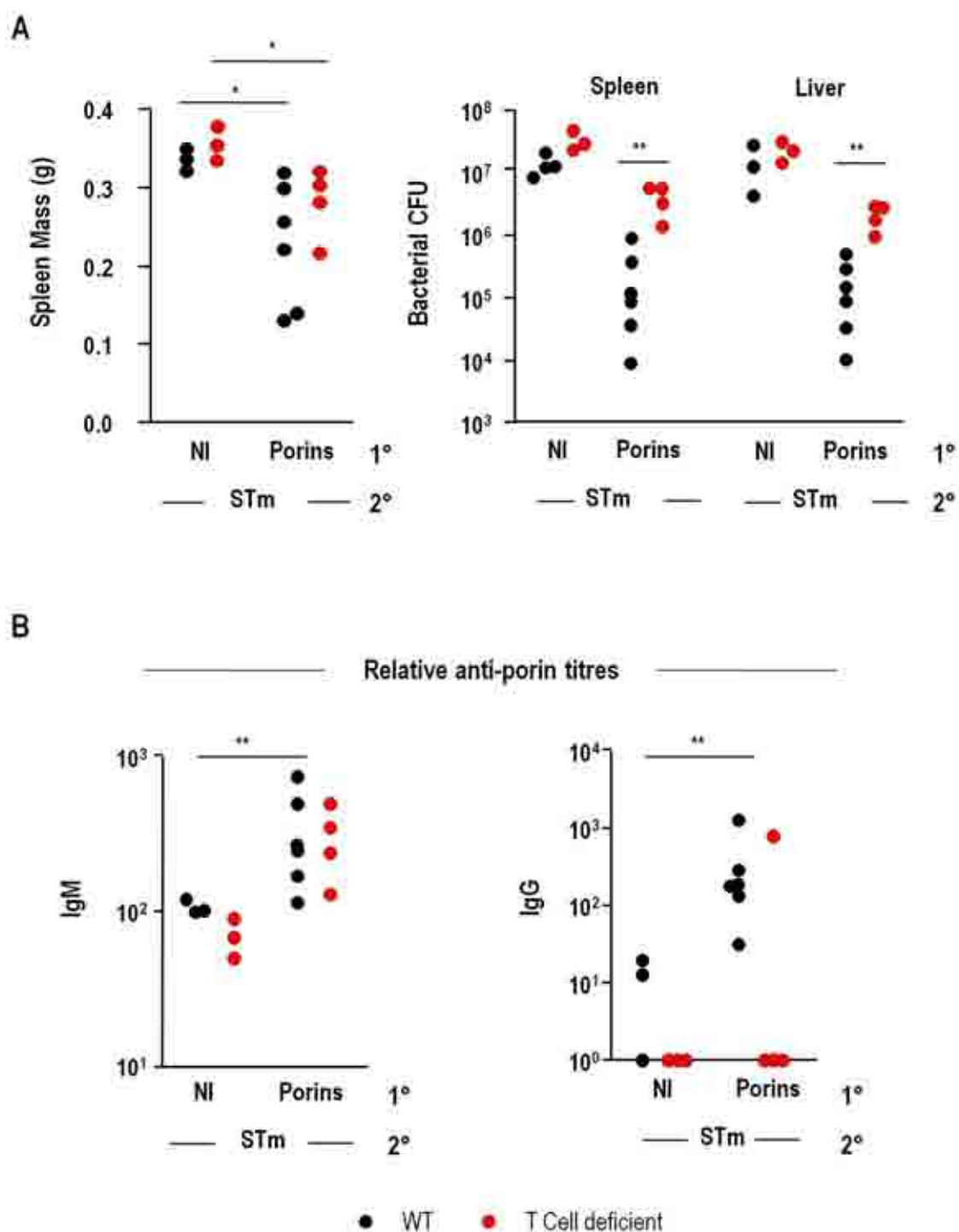


Figure 4.5:

Benefit afforded by porin immunisation is enhanced by T cells: (A) NI or porin-immunised WT and TCR-deficient mice were infected with 5×10^8 STm on day 35. Spleen mass (left panel) and splenic and liver bacterial CFU (right panel) were enumerated 5 days post-infection. (B) Serum IgM (left panel) and IgG (right panel) anti-porin titres were analysed by ELISA in these mice. Data is representative of two independent experiments and 4 mice per group. The Mann-Whitney test was used to compare the statistical significance between groups. * $p < 0.05$, ** $p < 0.01$

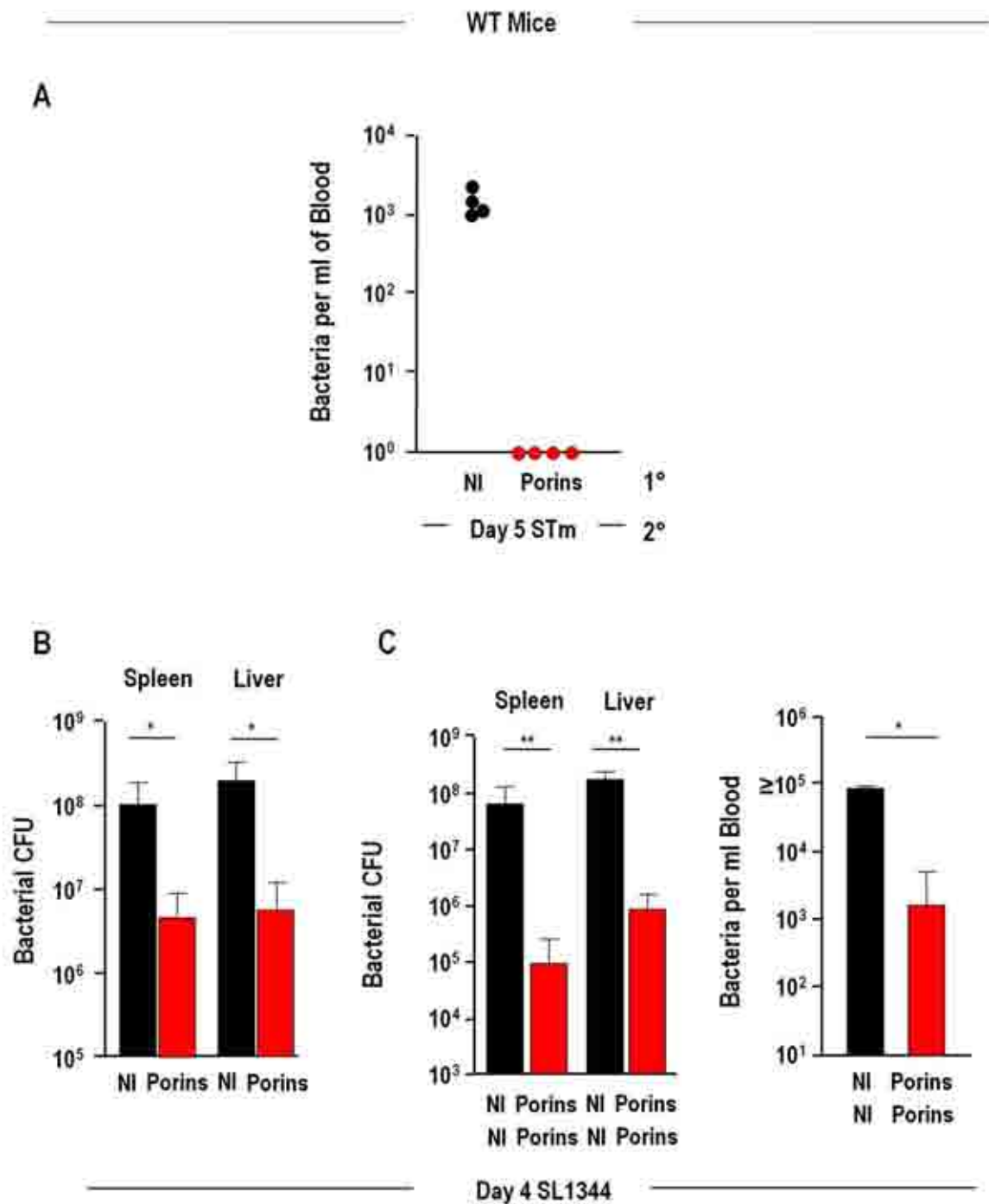


Figure 4.6:

Immunisation with porins is sufficient to impair infection with virulent STm and bacteraemia: (A) Blood CFU counts from NI and porin-immunised WT mice which were infected with 5×10^5 STm on day 35 for 5 days. (B) Spleen and liver bacterial CFU counts from NI WT mice or WT mice immunised with 20 μ g porins either once or (C) twice (left panel) for 14 days before intraperitoneal infection with 3×10^3 virulent STm (SL1344) for 4 days. Blood CFU counts were also enumerated from NI mice or mice immunised twice with porins before infection with SL1344 (right panel). Data with virulent and attenuated STm is representative of two and three individual experiments, respectively. The Mann-Whitney test was used to compare the significance between groups. $p^* = < 0.05$; $p^{**} = < 0.01$.

As virulent strains of NTS induce infections in clinical settings it was assessed whether anti-porin antibody could moderate infection with the virulent STm strain, SL1344. WT mice were immunised with 20 µg porins and infected intraperitoneally on day 35 with 3000 SL1344 organisms alongside NI mice (Fig. 4.6B). A single porin immunisation reduced splenic and liver bacterial numbers 4 days after infection by 20 and 40 fold respectively relative to NI mice. Whether a second porin immunisation could enhance this effect was then examined. WT mice were immunised with 20 µg porins twice for 14 days and subsequently infected with SL1344 as before (Fig 4.6C). Quantification of the bacterial burden demonstrated that when porin-immunised mice were boosted with a second porin immunisation, splenic and liver bacterial numbers were reduced by 1700 and 400 fold respectively at day 4 post infection relative to NI mice. Additionally, assessment of the blood bacterial load revealed mice boosted with porins had a significantly lower blood bacterial load (median = 30) relative to NI mice (median = 10⁵).

To eliminate the possibility that residual LPS contamination could account for these findings, and to elucidate whether porins from ST could provide cross-protection against STm, WT mice were immunised intraperitoneally with either 20 µg of TLR grade LPS from STm or 20 µg of highly purified porins from ST or STm. Mice were then infected on day 35 with 5x10⁵ STm (Fig 4.7). Quantification of the splenic bacterial burden 5 days post-infection showed that TLR grade LPS did not confer protection against STm. Surprisingly, the same porin preparation from ST did not confer protection to STm relative to porins isolated from STm, despite the high degree of homology between OmpC and OmpF from these two serovars. These data show that a single immunisation with porins can moderate subsequent infection with virulent STm through reduction in bacterial numbers, which although could prolong survival, would not prevent death. Interestingly, the protection conferred by porins showed species-specificity, since immunisation with porins from ST did not protect against infection with STm.

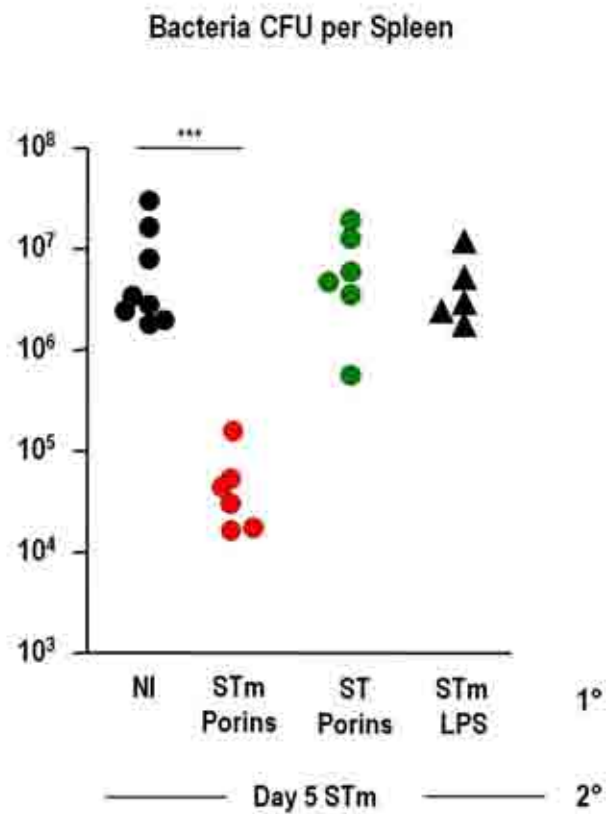


Figure 4.7:

Immunisation with ST porins does not offer cross-protection against STm: Splenic bacterial CFU in NI WT mice or WT mice immunised with 20 µg of either STm porins, ST porins or LPS for 35 days prior to infection with 5×10^6 STm for 5 days (Data was generated in conjunction with Dr. C. Gil-Cruz). Data is representative of three individual experiments. The Mann-Whitney test was used to compare the significance between groups. $p^{***} = <0.001$.

4.6 Antibody to the porin OmpD is sufficient to impair STm infection

A major difference between the outer membranes of STm and ST is the absence of the porin OmpD in ST (Santiviago et al., 2003). This suggested that responses observed against STm porins may be directed against this third STm porin. To test whether OmpD specific antibody could account for the differing results observed after immunisation with STm or ST porins, WT mice were immunised intraperitoneally with 20 µg porins and infected on day 35 alongside NI mice with either STm or STm lacking OmpD, and responses assessed 5 days post-infection (Fig. 4.8A). Assessment of the spleen mass showed no differences between mice infected with STm or STm lacking OmpD, irrespective of porin immunisation. As expected, splenic and liver bacterial numbers were markedly reduced in porin-immunised mice upon subsequent challenge with STm relative to NI mice. Interestingly, splenic and liver bacterial burdens were equivalent between NI and porin-immunised mice after subsequent challenge with OmpD-deficient STm. This suggests deletion of OmpD is not further attenuating for STm, at least at this stage of the infection. Subsequently, bacterial numbers in the blood were assessed and despite the failure to confer protection in the tissues, porin-immunisation offered some protection against bacteraemia upon challenge with OmpD-deficient STm (Fig. 4.8B).

The failure to confer protection upon infection with OmpD-deficient STm in porin-immunised mice could be correlated with the failure of anti-porin antibody to bind to the porin target in OmpD-deficient bacteria, as identified earlier. IgM-specificity of sera isolated from TCR-deficient mice immunised with porins for 7 days and NI controls was examined by western blot analysis, against a total OMP preparation isolated from STm and OmpD-deficient STm (Fig. 4.8C). This showed that non-immune sera recognised the same targets in the OMP preparation from STm and STm-lacking OmpD. However, anti-porin IgM bound the porin fraction in STm that were OmpD sufficient, but failed to bind to the porin fraction in the OMP preparation from STm that were OmpD-deficient. This suggested that OmpD was possibly a target of the anti-porin antibody response.

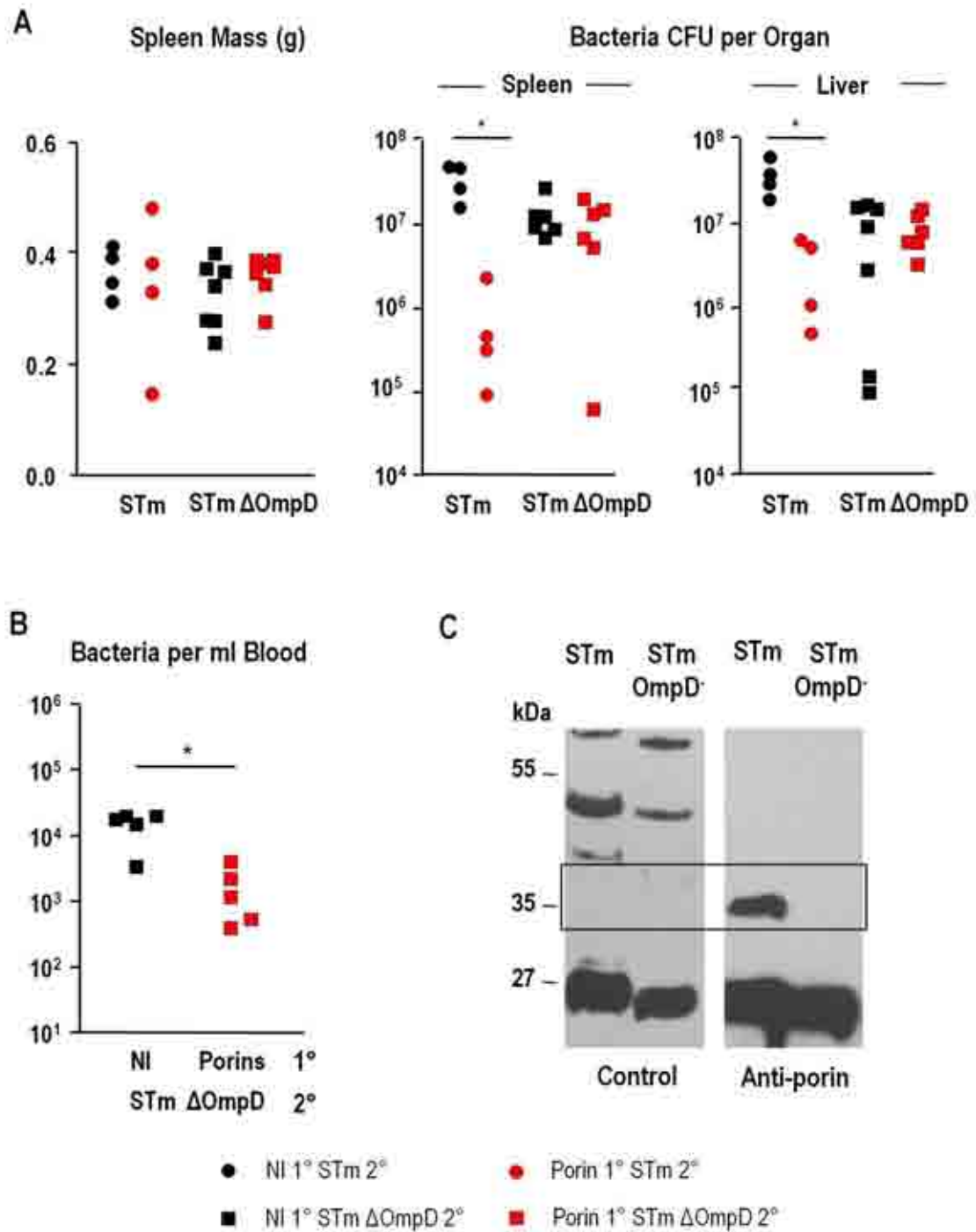


Figure 4.8:

Antibody to OmpD is sufficient to impair STm infection: (A) NI WT mice or WT mice immunised with porins were infected on day 35 with 5×10^6 STm (circles) or 5×10^6 STm lacking OmpD (strain RAK126) (squares). Spleen mass (left), splenic (centre) and liver (right) bacterial numbers were enumerated at day 5 post-infection. (B) Bacterial blood counts were quantified from these mice at sacrifice. (C) Immunoblot analysis of binding of IgM from sera isolated from NI or day 7 porin-immunised TCR-deficient mice against total OMPs from STm or STm lacking OmpD. The boxed region shows the approximate MW of the porins OmpC and F. Non-immune and immune sera were added at 1:50 and 1:150, respectively. Data is representative of 3 separate experiments and at least 4 mice per group. The Mann-Whitney test was used to compare statistical significance between groups, $p^* = < 0.05$

As an additional control, the relative contribution of the individual porins to offer protection against STm infection was investigated by repeating this study using STm lacking the transcription factor OmpR, which fail to express OmpC and OmpF, but continue to express OmpD. WT mice were immunised with 20 µg porins and infected on day 35 alongside NI mice with either STm or STm lacking OmpR and responses were assessed 5 days post-infection (Fig. 4.9A). Assessment of the spleen mass showed porin-immunised mice had significantly smaller spleens relative to NI mice, irrespective of infection with STm or OmpR-deficient STm.

Quantification of the splenic and liver bacterial burden demonstrated that OmpR-deficient STm were intrinsically more attenuated than STm, as bacterial numbers were significantly lower than mice infected with STm, regardless of porin-immunisation, in agreement with earlier studies (Dorman et al., 1989; Meyer et al., 1998). Nevertheless, porin-immunisation markedly reduced bacterial numbers further in the tissues of mice subsequently infected with OmpR deficient STm (Fig. 4.9A). Furthermore, assessment of the blood bacterial load revealed bacteraemia was not detectable in porin-immunised mice upon subsequent infection with OmpR deficient STm, relative to NI mice (Fig. 4.9B). This indicates that OmpF and OmpC are dispensable for the protective function of porins.

Finally the IgM-specificity of sera was assessed by western blot analysis as before, against a total OMP preparation isolated from STm and OmpR-deficient STm (Fig. 4.9C). Immunoblot analysis revealed that anti-porin IgM antibody bound the porin fraction in STm that were OmpR-sufficient and OmpR-deficient. These data demonstrate that whereas porin-immunisation induced protection against STm lacking both OmpF and OmpC, porin-immunisation did not moderate infection when mice were subsequently challenged with STm lacking OmpD. This indicates that OmpD is an important immunogenic target of antibody to STm, and antibody to OmpD alone is sufficient to provide protection against subsequent STm infection.

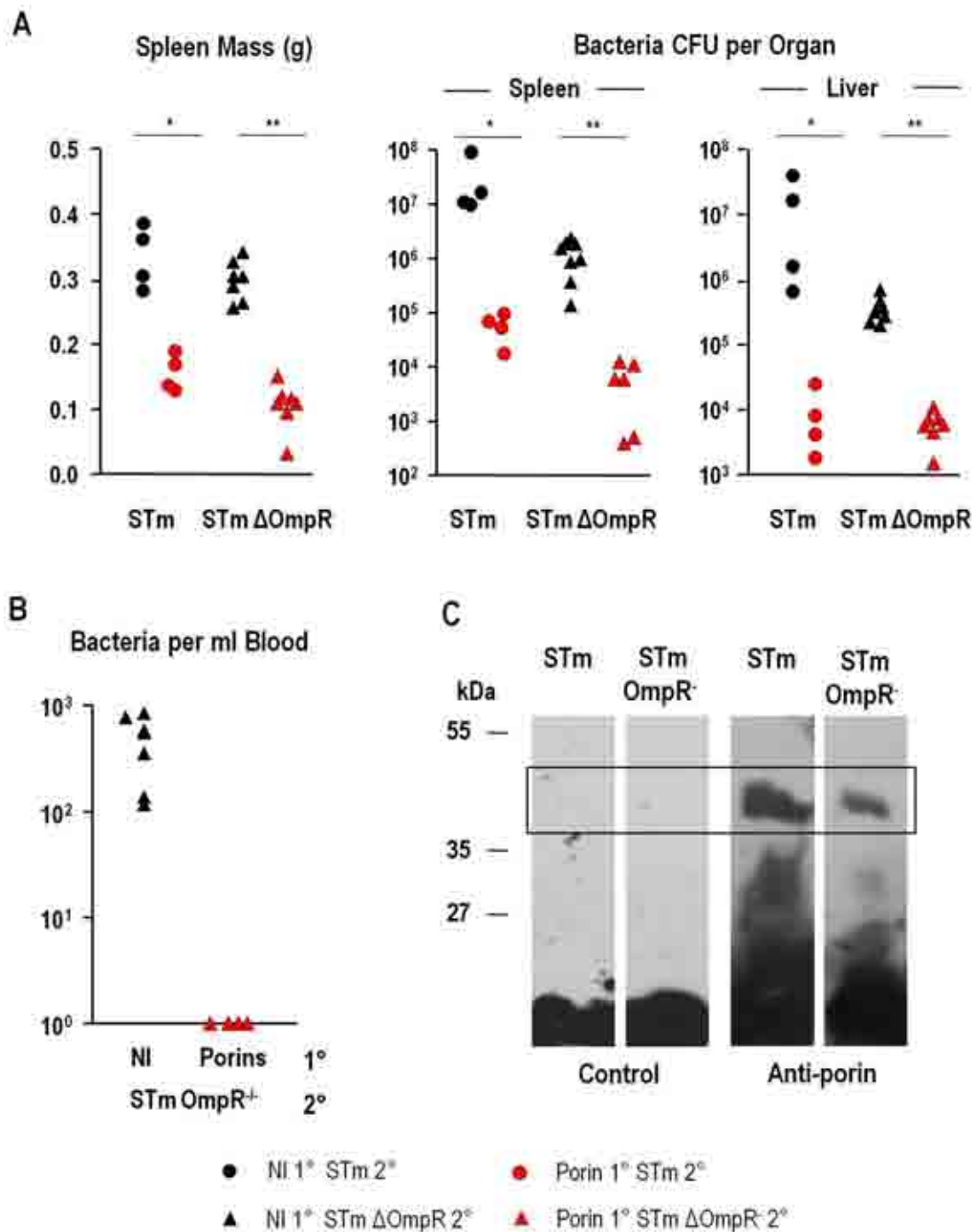


Figure 4.9:

Loss of OmpR expression does not lead to loss of protection in porin-immunised mice: (A) NI WT mice or WT mice immunised with porins were infected on day 35 with 5×10^6 STm (circles) or 5×10^6 STm lacking OmpR (strain RAK83) (triangles). Spleen mass (left), splenic (centre) and liver (right) bacterial CFU were enumerated at day 5 post-infection. (B) Bacterial blood counts were quantified from these mice at sacrifice. (C) Immunoblot analysis of binding of IgM from sera isolated from NI or day 7 porin-immunised TCR-deficient mice against total OMPs from STm or STm lacking OmpR. The boxed region shows the approximate MW of the porin OmpD. Non-immune and immune sera were added at 1:50 and 1:150, respectively. Data is representative of 3 separate experiments and at least 4 mice per group. The Mann-Whitney test was used to compare statistical significance between groups. $p^* = < 0.05$ $p^{**} = < 0.01$

4.7 Porin-Immunisation induces an increase in the peritoneal B1b cell population

The data thus far showed that the effectiveness of porin immunisation was wholly dependent upon B cells, and the induction of antibody to these proteins could occur in the absence of T cells. In conjunction with Drs. JL Marshall and C. Gil-Cruz WT mice were immunised intraperitoneally with either 20 µg of the following: porins, TLR Grade LPS or sFliC or 5×10^6 heat killed STm or 5×10^5 STm and peritoneal responses were assessed 4 days later using 7 colour FACS, alongside NI mice (Fig. 4.10). B cells were gated from the lymphocyte population as CD19⁺ and IgM⁺ cells. In the peritoneum B1 cells were gated to remove B2 cells as a CD21 and CD23 low/negative population. This population of B cells was examined for CD5 and B220 expression, and the B1b B cell population was gated as CD5⁻B220^{int}. Phenotypically, the B1b population was characterised as: CD19⁺IgM⁺B220^{int}CD5⁻ with low CD21 and CD23 expression.

Relative to NI mice immunisation with porins showed a significant induction of B cells with characteristics of B1b B cells (Fig 4.10). This population of B1b B cells was also significantly induced in response to viable STm and surprisingly by highly purified LPS, but it was not induced by heat-killed STm or sFliC. We then characterised CD11b expression by B1b B cells, as this has historically been used to define B1 B cells (Wells et al., 1994). This revealed that CD11b expression was significantly lower in mice infected with viable STm relative to NI mice, and generally, CD11b expression was variable between the groups. However, this was not surprising since CD11b⁻ B1b B cells have the potential to become CD11b⁺ after transfer into lymphopenic recipients, and its expression instead may distinguish distinct stages in the development of peritoneal B1 B cells (Ghosn et al., 2008).

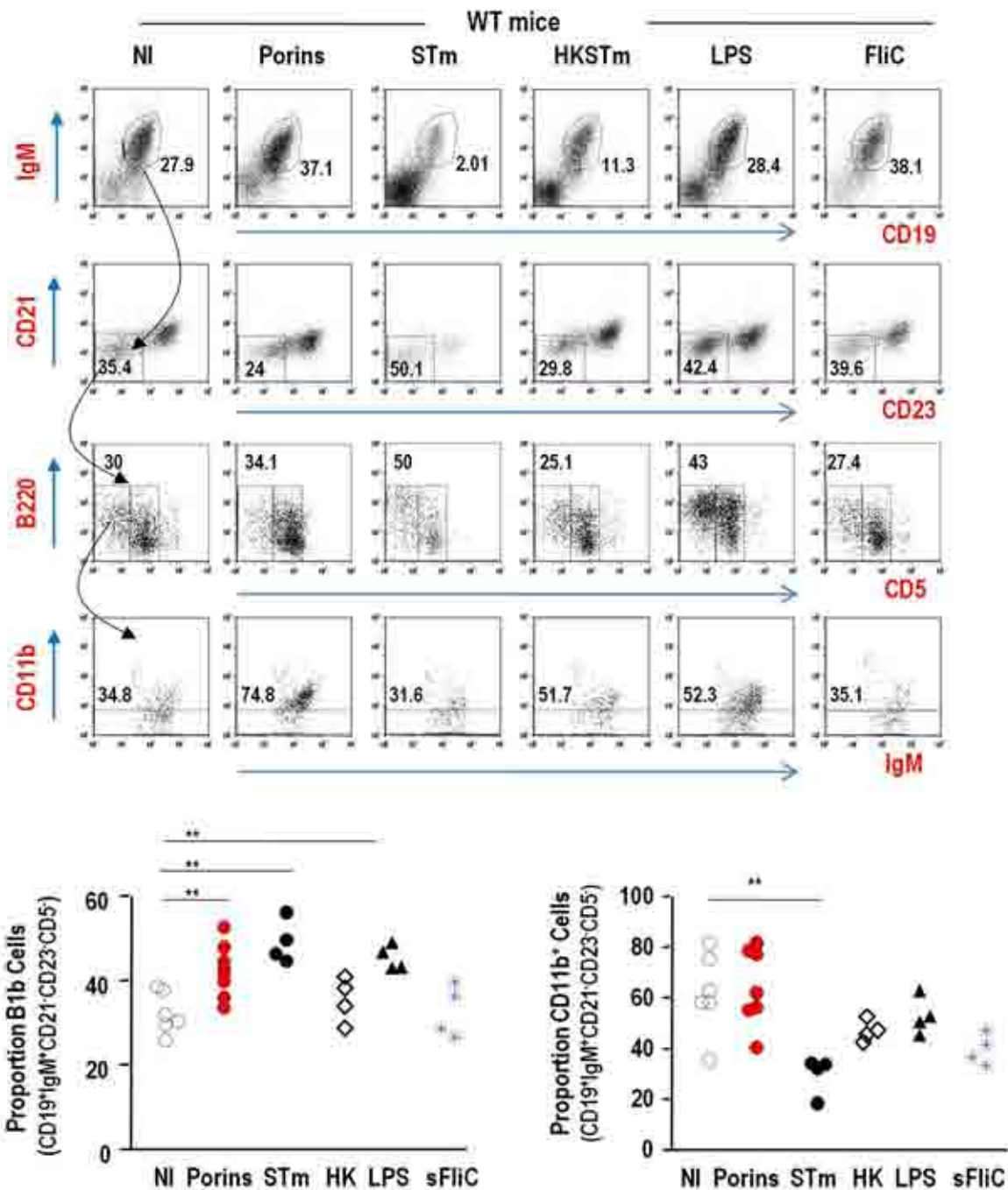


Figure 4.10:

STm porins induce recruitment of a population of peritoneal B cells with a B1b phenotype: Peritoneal exudate cells (PEC) were isolated from NI WT mice and WT mice immunised intraperitoneally with the described antigens for 4 days. Cells were stained and the IgM⁺CD19⁺ cells were gated from the lymphocyte population (Row 1). Cells that were low for CD21 and CD23 were gated from this population (Row 2), the B1b cells were IgM⁺CD19⁺CD21^{lo}CD23^{lo} cells that were CD5⁺B220⁺ (Row 3). The final FACS gate (Row 4) shows the proportion of B1b cells that express CD11b. Graph show the proportion of B1 cells that are B1b (left panel) and the proportion of B1b cells that are CD11b⁺ (right). Data representative of at least 2 repeat experiments. HK = heat-killed STm. Numbers refer to the % of cells in the gated boxes. This data was generated in conjunction with Drs. JL Marshall and C. Gil-Cruz. The Mann-Whitney test was used to compare the statistical significance between groups. p** = <0.01

Subsequent to this, we assessed if this B1b population detected in response to porins was induced in the absence of T cells. TCR-deficient mice were immunised with 20 µg porins or infected with 5×10^5 STm intraperitoneally, and peritoneal B cell responses were assessed 4 days later alongside NI mice which were gated as before (Fig 4.11). As can be seen mice immunised with porins or infected with STm showed a significant induction of B1b B cells relative to NI mice. Furthermore, as observed with WT mice the expression of CD11b by B1b B cells was variable. These data presented here demonstrate that viable STm and porins induce a peritoneal population of T1 B cells that have a B1b B cell phenotype.

4.8 Anti-Porin antibody from B1b B cells is sufficient to impair subsequent STm infection

Finally we sought to assess whether antibody produced by B1b B cells in response to porin immunisation could impair STm infection. To do this, peritoneal B220⁺CD5⁻ B cells were sorted from NI WT mice. 2×10^5 of these sorted were adoptively transferred into IgH-deficient mice to generate B1b B cell chimeras that lack B1a cells and have relatively few B2 cells (Fig. 4.12A) (Hsu et al., 2006). Although this population of transferred cells would include some B2 follicular cells, these cells do not expand after transfer into lymphopenic mice, and consequently will only contribute a small proportion of the final B cell population after expansion of B1b cells (Sprent et al., 1991). To establish that the transferred B cells re-populated the IgH deficient mice the serum IgM levels of the B1b B cell chimeras was assessed by ELISA 20 days after adoptive transfer. This revealed the chimeras were able to produce natural IgM antibody in the absence of porin immunisation, and as expected, this did not react with porins.

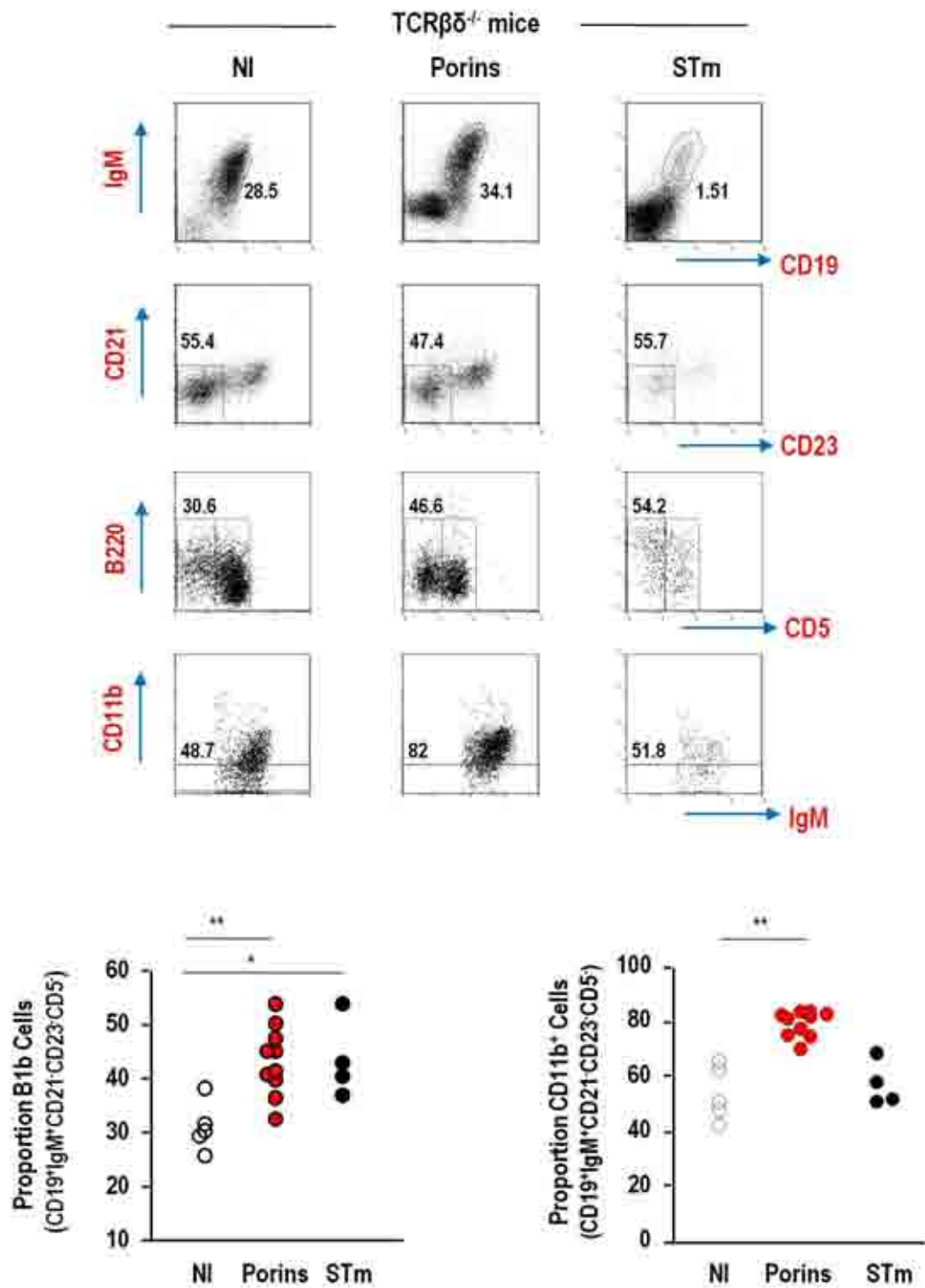


Figure 4.11:

B1b cells recruited in response to STm are induced independently of T cells: B1b cells from either NI, porin-immunised or STm-infected TCR-deficient mice were identified as in Figure 4.10. Graphs show the proportion of B1 cells that are B1b (right) and the proportion of B1b cells that are CD11b⁺ (left). Numbers refer to the % of cells in the gated boxes and are representative of at least 2 experiments. This data was generated in conjunction with Drs. JL Marshall and C. Gil-Cruz. The Mann-Whitney test was used to compare the statistical significance between groups. $p^* = <0.05$; $p^{**} = <0.01$

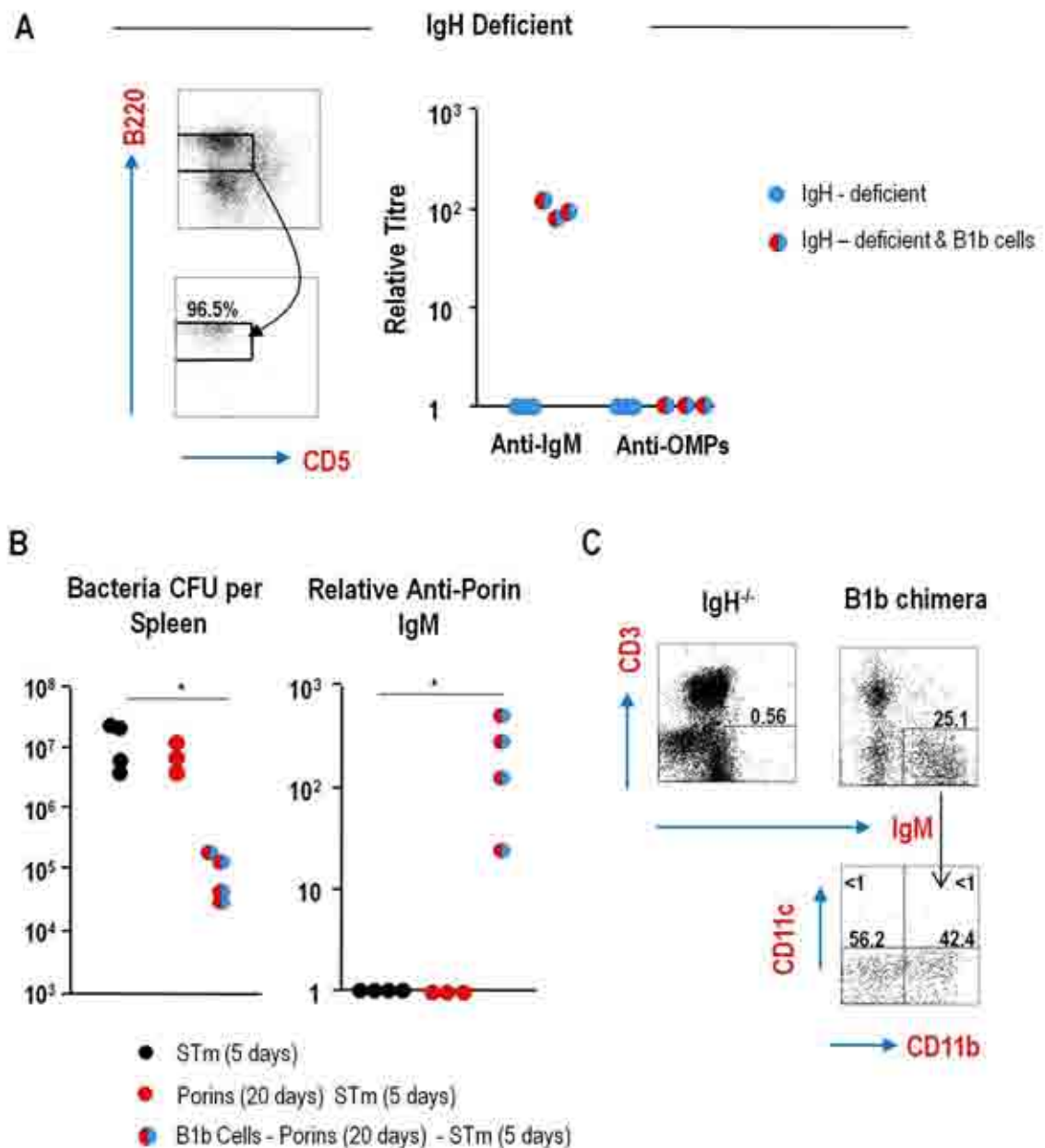


Figure 4.12:

Anti-porin antibody from B1b cells is sufficient to impair STm infection: (A) FACS plot showing the population of B220⁺CD5⁺ cells (B1b cells) (boxed) which were sorted from PEC cells isolated from NI WT mice before 2×10^5 cells were transferred into IgH-deficient mice (upper plot). Plot showing the purity of the sorted B1b population was >95% and they were CD5⁺ (lower plot). Graph shows relative anti-IgM antibody titres assessed by ELISA from B1b chimeras 20 days after transfer. (B) Splenic bacterial CFU (left graph) and serum IgM titres (right graph) 5 days post-STm infection in IgH-deficient mice that have either: only been infected with STm, or infected with STm 20 days post-porin immunisation, or infected with STm post-porin immunisation and transfer of B1b cells. (C) FACS plots show the absence of B cells in the PEC of porin-immunised IgH-deficient mice (left) and their presence in porin-immunised B1b chimeras (right). The bottom plot shows the proportion of these transferred B cells that express CD11b. This data was generated in conjunction with Drs. JL Marshall and C. Gil-Cruz. The Mann-Whitney test was used to compare the statistical significance between groups. $p^* = <0.05$.

Having established that the transferred B cells re-populated in IgH deficient mice we generated B1b B cell chimeras as before. After transfer (48 hours), B1b B cell chimeras and IgH-deficient mice were immunised with 20 µg porins. After 20 days the success of immunisation was confirmed by measuring anti-porin IgM titres from these mice. This showed that only immunised-chimeric mice induced an anti-porin IgM response relative to IgH-deficient mice (data not shown). The next day, the porin-immunised chimeras, porin-immunised IgH-deficient mice and NI IgH-deficient were infected with 5×10^5 STm. Quantification of the splenic bacterial burden at day 5 post STm-infection revealed that only the chimeric-mice that received both peritoneal B1b B cells and were immunised with porins, were able to impair STm infection. IgH-deficient mice that were immunised with porins in the absence of B1b cells had bacterial numbers equivalent to NI IgH-deficient mice (Fig 4.12B). This demonstrates that only those mice that had anti-porin antibody at the time of infection were able to impair STm infection. As a further control we also examined the B cell content of the peritoneal cavity in the generated chimeras (Fig. 4.12C). This revealed an absence of B cells in porin-immunised IgH-deficient mice that were present in porin-immunised chimeras. The peritoneal B cell population in the chimeras was IgM⁺ and approximately half of these isolated cells were also CD11b⁺. This suggested that TI antibody isolated from B1b B cells induced in response to porins is sufficient to impair infection with STm.

Importantly, this study shows that a single immunisation with porins from STm, but not ST can reduce bacterial colonisation by both attenuated and virulent strains of STm. Protection was shown to be due to antibody directed against OmpD, a porin present in STm but absent in ST, since ST porins fail to protect against STm infection and STm porins fail to protect against OmpD-deficient STm. This protection was mediated through the induction of TI B1b B cells and was enhanced by T cells.

4.9 Discussion

This study examined responses to a highly purified preparation of porins isolated from STm containing the OMPs, OmpC, OmpD and OmpF. OmpD is a trimeric porin that shares a degree of homology with OmpF and OmpC, primarily in the barrel structure (Benz et al., 1980; Nikaido, 2003). Despite representing the most abundant OMP in STm its role in infection has been understudied relative to OmpF and OmpC, presumably due to its absence in ST. The loss of OmpD on virulence has not been fully investigated whilst the loss of OmpR, which regulates expression of OmpC and OmpF, profoundly affects virulence (Dorman et al., 1989; Meyer et al., 1998). Although, this is likely to be because of other effects of OmpR, including its proposed regulation of SPI-2 genes through interactions with the SpiR/SsrB two-component system (Feng et al., 2003). Interestingly, the virulence of Salmoporc (a licensed live STm vaccine for pigs) and a mutant lacking OmpD (Salmoporc Δ OmpD) are indistinguishable in terms of the LD₅₀ in BALB/c mice. This suggests that loss of OmpD is not further attenuating in STm Salmoporc (Selke et al., 2007) which is in agreement with our studies using OmpD-deficient SL3261.

Purified porins are immunogenic in mice and rabbits (Hofstra and Dankert, 1981; Overbeeke et al., 1980) and were identified over 20 years ago as potentially protective antigens. However, there has been debate about the role of contaminating LPS in purified porin preparations, and whether the response was actually directed against LPS (Barber and Eylan, 1976; Bhatnagar et al., 1982). Indeed, some porins including OmpF are known to exist as a protein-LPS complex, with at least one LPS molecule tightly associated with each trimer. Consequently, the immunogenicity of porins has been disputed (Kuusi et al., 1981; Ried et al., 1990). Furthermore, a recent study identified that classically prepared LPS preparations are often contaminated with compounds such as peptidoglycan or proteins such as OmpA (Hirschfeld et al., 2000; Ried et al., 1990). In this study LPS was unlikely to be a potential confounder since purified porins had undetectable levels of LPS contamination (< 0.01 EU/100 μ g of

porins). Furthermore, immunisation with highly purified STm LPS did not moderate infection with STm. Additionally, infecting porin-immunised mice with OmpD-deficient STm that were LPS-sufficient, resulted in the loss of binding of anti-porin antibody to OmpD-deficient bacterial cell walls, and the associated loss of benefit from porin immunisation. Together these findings strongly implicate that antibody to porins, and in particular OmpD, not LPS can moderate infection with STm.

Multiple studies have assessed the potential of porins as vaccines to STm, often with conflicting results. Some studies have demonstrated that immunisation with porins can induce long-lasting immunity against STm (Isibasi et al., 1994; Matsui and Arai, 1990; Singh et al., 1999). Other studies have shown no benefit from porin immunisation (Singh et al., 1996). This latter study demonstrated that the passive transfer of anti-porin monoclonal and polyclonal antibodies could not prevent death, but significantly extended survival. However, it was not clear if the antibodies used by (Singh et al., 1996) sufficiently covered the full breadth of the potential porin epitope repertoire. Indeed, epitope mapping studies have shown a lack of cross-reactivity between surface binding monoclonal antibodies to these three porins. Moreover, even within OmpD many antibodies only recognise the protein when in trimeric form but not in monomeric form (Pai et al., 1992). This suggests that these exposed regions are under selective pressure by the immune system and may mean that a broad range of epitopes need to be recognised to obtain the maximum benefit from immunisation.

Isolated porins from STm containing the OMPs OmpC, OmpD and OmpF could induce a rapid and strong antibody response which had the capacity to moderate infection with STm. Moreover, this porin-induced protection could reduce STm infection as effectively as immunisation with heat-killed STm. Heat-killed bacteria have been established as an effective vaccine used to protect against typhoid in humans, and can confer comparable protection as current subunit vaccines (Guzman et al., 2006), possibly even more so than live vaccines (Engels et al., 1998). In addition to reducing bacterial loads in the tissues we found anti-porin antibody was effective at restricting bacteraemia. The greatest proxy for

disease severity in NTS is bacteraemia which can be fatal (Graham et al., 2000). The peak incidence of this complication is in children and coincides with the loss of passively acquired maternal IgG, and before acquisition of adaptive immunity. Indeed, nearly all children have antibody to NTS by the age of three which correlates with decreased susceptibility (MacLennan et al., 2008). It is tempting to hypothesise that since children in this age-group have an impaired capacity to respond to T1-2 antigens that one of the predisposing risks to NTS, is this inability to make antibody responses to STm porins, which enhances the likelihood of bacteraemia and complications resulting in death.

The sterilising immunity necessary to protect against infection with virulent STm SL1344 in susceptible strains of mice may overstate the degree of immunity required to protect against NTS or typhoid in humans. This is because whilst protection to SL1344 identifies absolute protection anything less will mean potentially effective vaccine candidates will be missed. Furthermore, NTS bacteraemia is generally of a low grade (less than a hundred organisms per ml of blood) and therefore, the same kinetics of protection may not apply for NTS and typhoid. We consistently saw reductions in bacterial numbers of 50-1000 fold in the tissues, irrespective of whether the infecting organism was virulent or attenuated - levels of protection comparable to killed organisms that are used as current vaccines against typhoid. Whilst it is unlikely that antibody to STm-porins can offer sterilising immunity, it may be sufficient to diminish bacterial numbers at the time of infection and the extracellular dissemination that can lead to fatal bacteraemia. Thus, even modest reductions in bacterial numbers may result in substantial clinical benefits for NTS. In this study we have not attempted to optimise the potential dose or route of porin administration but to identify OmpD as a vaccine candidate that may work primarily through a T1 route. Indeed, unsurprisingly in WT animals an additional dose of porins enhanced protection and we are currently assessing whether this is sufficient to augment the response.

Both viable STm and STm porins induced antibody through recruitment of a population of B1b B cells in the absence of T cells. Antibody from B1b cells induced in response to porin immunisation was sufficient

on its own to moderate infection. For typhoidal strains of *Salmonella*, responses induced upon vaccination of adults with capsular polysaccharide (Vi antigen) may involve B1b cells, as well as other B cell subsets, and so immunisation with STm porins or Vi antigen may act similarly in their respective infections. However, antibody to purified Vi antigen in humans is wholly TI (Guzman et al., 2006) whilst immunisation with porins has the added attraction of inducing antibody responses with bactericidal activity, as well as cell-mediated immunity (Salazar-Gonzalez et al., 2004). Indeed, we found that T-dependent induction of IgG upon porin-immunisation enhanced protection and additionally, T cell help is essential for the induction of life-long B cell memory, a desirable feature of vaccine design.

TI antibody to protein antigens have been described previously to the glycoprotein of vesicular stomatitis virus (Freer et al., 1994) and complement factor H-binding protein from the bacterial pathogen *Borrelia hermsii* (Colombo and Alugupalli, 2008). For the latter, self-renewing B1b cells have been shown to confer long-lasting immunity to re-infection (Alugupalli et al., 2004). Furthermore, B1b cells have been shown to be important in the early defence to pneumococcal infections (Haas et al., 2005). These studies, together with the work presented here suggests B1b cells may play significant roles in a range of infections and reflects our ever increasing understanding of the diversity of B cell responses to pathogens (Racine et al., 2008). Furthermore, this work broadens the repertoire of TI protein antigens, and specifically suggests that the B1b cell repertoire may be broader than originally thought.

Infants and the elderly have impaired responses to TI-2 antigens such as capsular polysaccharides, despite the presence of B1b cells. Whilst the reason for this is complicated, and it is tempting to speculate, as others have, that it is lack of B1b memory in children that is responsible for their susceptibility to encapsulated bacteria (Alugupalli, 2008), in mice it is likely to involve two sets of B cell responses to TI-2 antigens, B1b cells and MZ B cells. Indeed, mice deficient for the tyrosine kinase *pyk2* lack MZ B cells, but not B1b B cells, and remain able to induce potent IgM and IgG responses to the TI-2 antigen TNP-Ficoll (Guinamard et al., 2000). Furthermore, the transfer of B1b cells alone into RAG1-

deficient mice is sufficient to support antibody responses to NP-Ficoll (Hsu et al., 2006). TI responses in humans and mice share some similarities (Gonzalez-Fernandez et al., 2008). For instance serum from both species during *B. hermsii* infection can react to FhbA, an antigenic target of B1b cells (Colombo and Alugupalli, 2008; Hovis et al., 2006), suggesting humans may have equivalent of B1b cells.

Despite earlier reports appreciation of the importance of antibody in STm infection is now increasing (Mastroeni et al., 2000; McSorley and Jenkins, 2000; Mittrucker et al., 2000). There are at least two roles antibody could fulfil in restricting infection: firstly, to prevent the dissemination and subsequent bacteraemia during primary infections and secondly, prevent macrophage colonisation during secondary infections. Earlier studies have shown that antibody can perform both these roles (Cunningham et al., 2007) and we now extend these findings to show that the natural response of antibody to OmpD is sufficient to reduce colonisation in the tissues and bacteraemia in a mouse model of NTS infection caused by STm. The protective benefit of antibody to porins has also been documented in our recent work on human responses to NTS (MacLennan et al., 2010). Interestingly, porcine responses to *Salmoporc* Δ OmpD revealed that OmpD may not be a dominant protective antibody target as some of the protective efficacy of *Salmoporc* was largely retained upon its loss (Selke et al., 2007). Despite this, in the absence of OmpD the capacity of *Salmoporc* to reduce colonisation in the caecum, colon and ileum was reduced relative to mice immunised with the parent vaccine supporting a role for protective antibody to OmpD in the early colonisation of STm as in agreement with this study. The importance of antibody to OmpD does not exclude roles for antibody to OmpF and OmpC because other reasons may account for their minor role here, such as the amount of OmpF and OmpC in the preparations, or their expression in STm at different growth stages.

In closing, we propose OmpD could be a possible candidate for a subunit vaccine against NTS. Indeed, porins from ST which are essentially prepared in the same manner as the STm porins described here, have already been shown to be safe and efficacious in humans (Salazar-Gonzalez et al., 2004).

Additionally, the importance of antibody to OmpD, a porin absent from ST may in part explain why porins from ST did not cross-protect despite homology between the shared porins OmpF and OmpC, supporting earlier studies showing anti-porin sera (ST) did not cross-protect against STm (Secundino et al., 2006). It is important to emphasise that the absence of OmpD from ST indicates that any vaccine based on OmpD would not be effective against ST strains, but may be of use against paratyphoid strains.

5.0 Disrupted protective immunity in *N. brasiliensis* & *Salmonella* co-infection models

5.1 Introduction

Classically, models of experimental immunity to infection have addressed immune regulation to a single pathogen. Such studies have shaped our understanding of how infections develop and how they are controlled. Naturally we are exposed to multiple pathogens throughout our lives, and often by more than one pathogen at any given time. The relationship between co-infection and host immune responses is likely to be important, but has received limited detailed attention. Furthermore, how infectious history impacts on immunity to novel pathogens is relatively unknown. One model to assess this is how helminth infections, which are widespread and induce Th2 responses, can affect immune regulation to the Th1 inducing pathogen, STm.

Previous studies have shown helminths can modulate host responses and this can influence the outcome to an unrelated pathogen (Correale and Farez, 2009; Zaccone et al., 2003). For instance, helminth infection is associated with increased susceptibility to infections such as malaria and TB (Su et al., 2005; Tetsutani et al., 2009). Interestingly, malaria-helminth co-infection can either exacerbate the severity of malarial disease (Helmbj et al., 1998; Legesse et al., 2004; Tetsutani et al., 2009) or benefit host responses through counteracting strong inflammation (Hoeve et al., 2009; Lyke et al., 2005; Nacher et al., 2000). Moreover, emerging evidence from animal and human studies suggests the impact of chronic helminth infections can significantly impair responses to standard childhood immunisations (Cooper et al., 2001; Sabin et al., 1996).

Generating a detailed understanding of how immunity induced to bacterial pathogens such as STm influences responses to a second pathogen such as a helminth that requires a different, distinct form of immune response for protection represents an important and poorly understood field. STm infections

are the leading cause of childhood bacteraemia in SSA (Graham et al., 2000) and parasitic nematode infections are extremely common throughout this region (Hotez, 2009). Moreover, the highest incident rates of NTS overlap with areas where helminth infections are common (Hotez, 2009). Due to this demographic overlap the likelihood of concomitant infections with STm and hookworm early in infancy in endemic areas is highly plausible, and lends an important relevance to this study. Three approaches were used in this study as detailed below. The findings from this work demonstrate clear correlates of immunity against both pathogens which are disrupted by exposure to an unrelated pathogen.

Effect of primary *N. brasiliensis* infection on subsequent STm infection

How helminth induced immunity influences the host's ability to launch a protective response to STm was one aspect of this study. Mice were infected with *N. brasiliensis* and following worm expulsion (in an immune competent animal *N. brasiliensis* is expelled by day 9-11) mice were challenged with STm and the effects of *N. brasiliensis* on the kinetics of immunity to STm were examined. Furthermore, how pre-exposure to *N. brasiliensis* can influence the efficacy of potential STm vaccine candidates, where protection requires the presence of porin specific antibody (Gil-Cruz et al., 2009) was addressed.

Effect of primary STm infection on subsequent *N. brasiliensis* infection

The influence of primary infection with STm on subsequent expulsion of *N. brasiliensis* was investigated. Mice were infected with STm and then challenged with *N. brasiliensis* when the STm infection was either still active or had resolved. The influence of STm on worm expulsion and the correlates of protection against *N. brasiliensis*: IL-13 production and goblet cell hyperplasia were then examined.

Effect of dual STm and *N. brasiliensis* infection

Particular importance was placed on how synchronous challenge with these potent Th1 and Th2 pathogens impacted on the development of protective immunity against both pathogens relative to single infection. Throughout this study all infections with *N. brasiliensis* and STm were subcutaneous and intraperitoneal, respectively.

5.2 Primary infection with *N. brasiliensis* impairs the resolution of subsequent STm infection.

Whether primary infection with *N. brasiliensis* (Nb) disrupted protective host responses to subsequent STm infection was investigated. BALB/c mice were infected with 500 L3 Nb larvae and at day 16 post infection (6-7 days post worm expulsion) mice were challenged with 5×10^5 STm (Fig 5.1A). Bacterial burdens in the spleen and liver at day 6 post-STm challenge were comparable between non-Nb primed and Nb-primed mice (Fig 5.1B). By day 25 post-STm challenge although both groups had significantly reduced bacterial numbers relative to day 6, Nb-primed mice had significantly elevated bacterial numbers when compared to non-Nb primed mice. Equivalent spleen cellularity and weight was found in both groups at days 6 and 25 post-STm infection (Fig 5.1C). This suggested that primary infection with NB negatively impacted on subsequent control of STm.

Splenic CD4⁺ T cell populations showed increased cell numbers and proportions in both non-Nb primed and Nb-primed mice at day 25 post-STm challenge relative to day 6 (Fig 5.2A). Comparison of non-Nb primed and Nb-primed CD4⁺ T cell numbers and proportions were broadly equivalent, although the proportion of CD4⁺ T cells in Nb-primed mice when compared to non-Nb primed mice at day 25 post-STm challenge was marginally, but significantly lower. Analysis of T cell activation revealed largely comparable proportions of CD62L^{lo} T cells at both time points. Although consistently, there was a significantly higher proportion of CD62L^{lo} T cells in Nb-primed mice when compared to non-Nb primed mice at day 25 post STm challenge (Fig 5.2B). Nevertheless, there were no differences in activated CD4 T cell numbers at both time-points between the two groups.

Splenocytes were stimulated ex-vivo with pathogen-specific antigens or anti-CD3 for 72 hours and cytokine secretion in the supernatant was measured by ELISA. Anti-CD3 re-stimulation revealed equivalent levels of IFN γ in non-Nb and Nb-primed mice at both time points (Fig 5.3A). Anti-CD3 induced IL-13 was higher in both non-Nb and Nb-primed mice at day 25 compared to day 6 post-STm

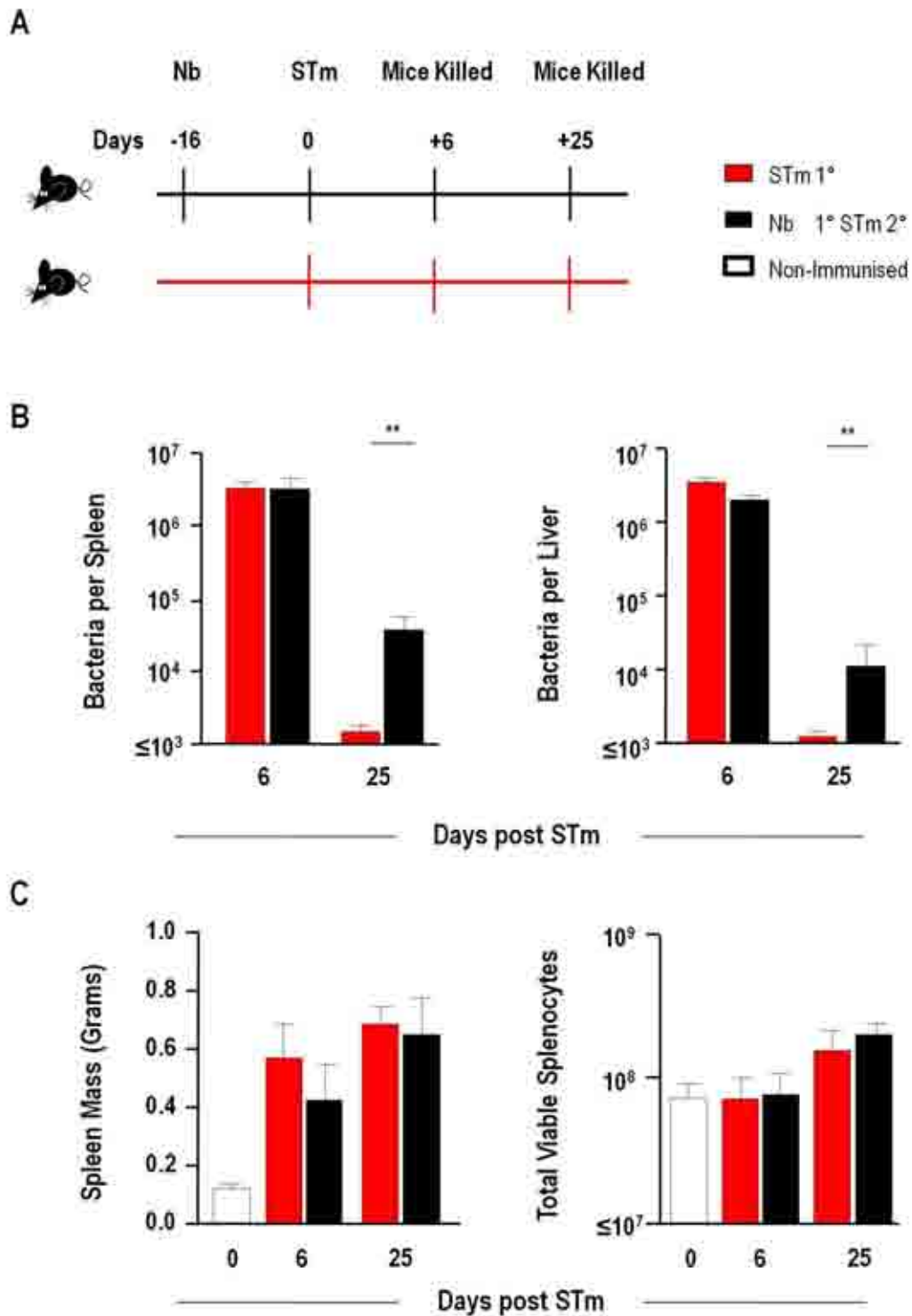


Figure 5.1

Primary *N. brasiliensis* infection impairs the hosts ability to control subsequent STm infection: (A) BALB/c mice were infected with 500 L3 Nb and challenged on day 16 alongside naïve control mice with 5×10^5 STm and responses were assessed at day 6 and 25 post-STm challenge. (B) Splenic and liver bacterial numbers were enumerated and (C) spleen mass and total spleen cellularity was assessed. Infections with STm and *N. brasiliensis* were intraperitoneal and subcutaneous, respectively. The Mann-Whitney Test was used to compare the significance between groups. Data is representative of 6 mice per group. ** $p=0.0043$

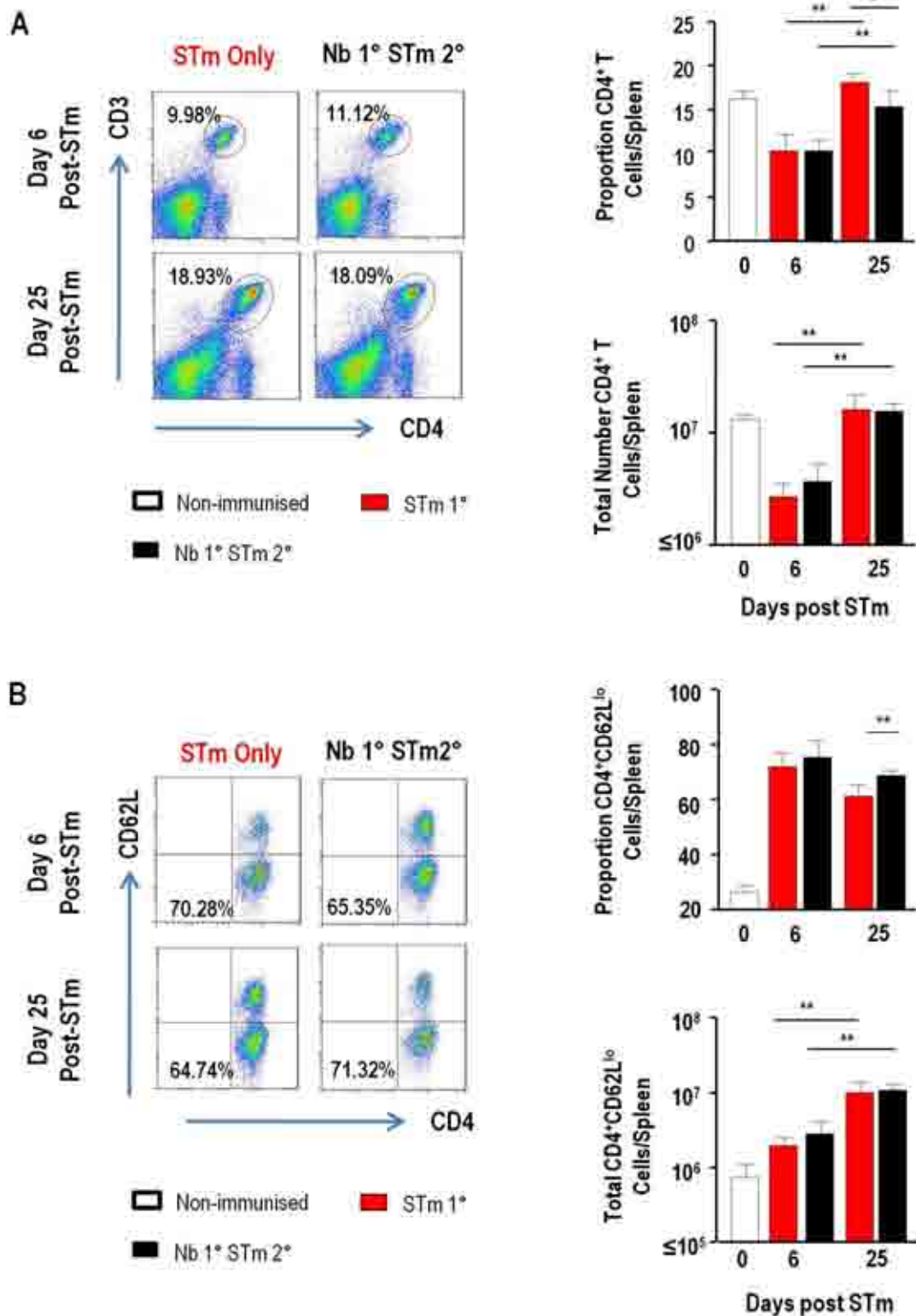


Figure 5.2

Effect of *N. brasiliensis* on STm associated CD4 T cell populations is marginal: BALB/c mice were infected with 500 L3 Nb and challenged on day 16 alongside naïve control mice with 5×10^5 STm and responses assessed at day 6 and 25 post-STm challenge. (A) Proportion and number of: splenic CD4⁺ T cells quantified from the lymphocyte population and (B) those that have down-regulated expression of CD62L. Infections with STm and *N. brasiliensis* were intraperitoneal and subcutaneous, respectively. Data is representative of 6 mice per group. * $p < 0.05$ and ** $p < 0.01$.

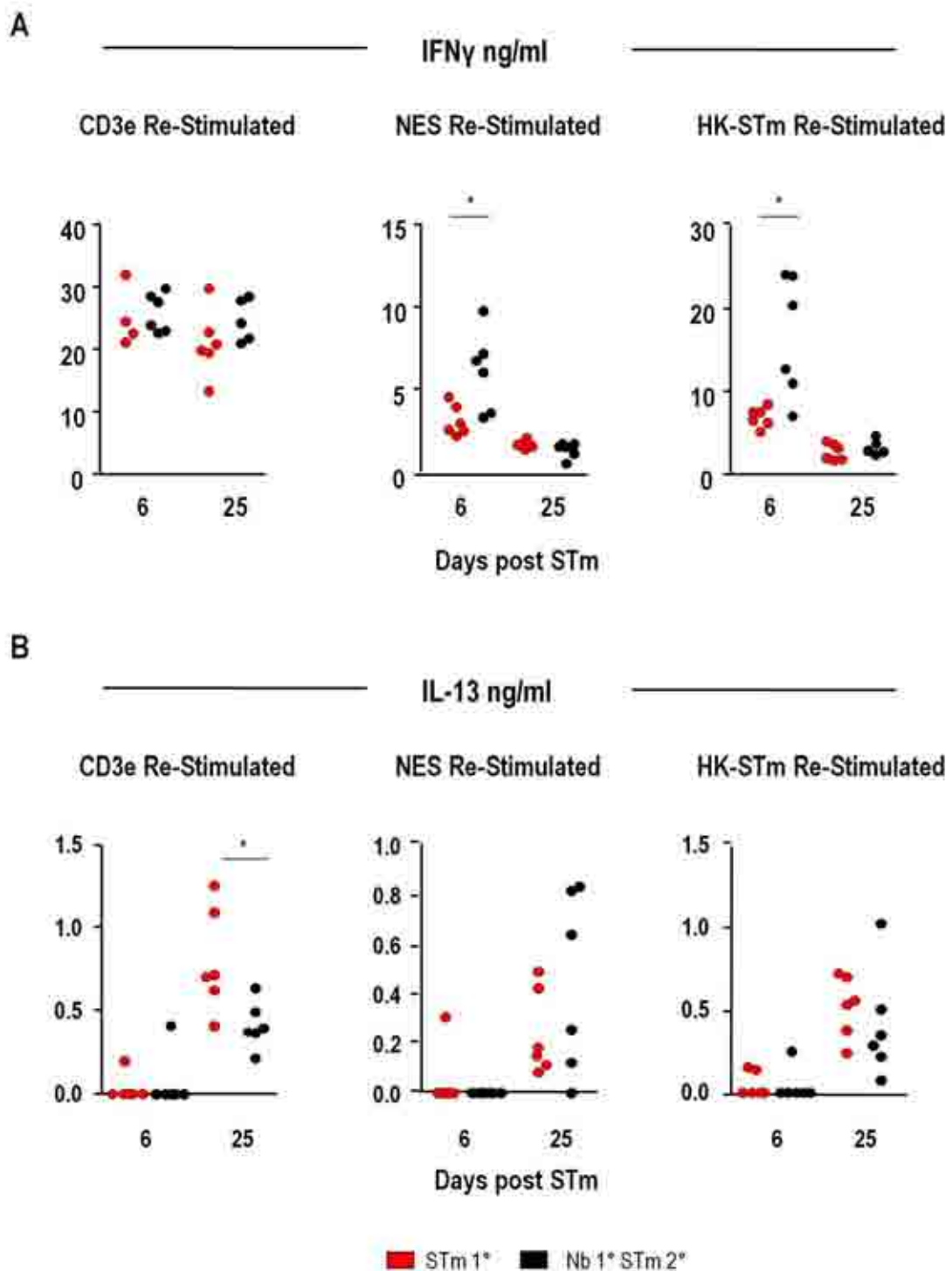


Figure 5.3

Primary *N. brasiliensis* infection increases early induction of Nb and STm specific IFN γ : BALB/c mice were infected with 500 L3 Nb and challenged on day 16 alongside naïve control mice with 5×10^5 STm. Splenocytes were isolated at days 6 and 25 post-STm infection and 2×10^5 cells were re-stimulated in-vitro for 72 hours with either anti-CD3, somatic NES or Heat-Killed (HK-STm). (A) IFN γ and (B) IL-13 were then quantified by ELISA from the supernatants post-stimulation. Infections with STm and *N. brasiliensis* were intraperitoneal and subcutaneous, respectively. The Mann-Whitney Test was used to compare the significance between groups. Data is representative of 6 mice per group. * $p < 0.05$

challenge, and surprisingly, highest in the non-Nb primed group (Fig 5.3B). Cytokine secretion post-antigen specific re-stimulation of splenocytes showed increased levels of IFN γ in Nb-primed mice compared to non-Nb primed mice at day 6 post-STm infection, following re-stimulation with somatic Nb (NES) or Heat-killed STm. Levels were equivalent between both groups at day 25. As was observed in CD3 re-stimulated splenocytes, IL-13 secretion increased in both groups at day 25 post-STm infection following antigen-specific stimulation, and to a similar level.

Assessment of serum anti-STm IgM showed titres increased similarly in both Nb and non-Nb primed mice at day 25 compared to day 6 post-STm challenge, whilst anti-NES IgM titres did not increase above background levels for non-Nb primed mice (Fig 5.4A). Anti-STm IgG titres increased in both groups at day 25 relative to day 6 post-STm challenge and interestingly, titres were significantly lower in Nb-primed mice compared to non-Nb primed mice. Anti-NES IgG titres were only detected in non-Nb primed mice and titres were comparable at each time-point (Fig 5.4B). Subsequently, IgG2a responses were assessed which revealed IgG2a was absent from either group at day 6 post STm-challenge, irrespective of antigen-specificity (Fig 5.4C). Anti-STm IgG2a was detected at day 25 in both groups and titres were equivalent, and as expected, anti-NES IgG2a titres were only induced in Nb-primed mice. Anti-STm IgG1 responses did not increase above background in both groups (data not shown).

These data demonstrate that a prior infection with Nb disrupts the host's ability to control a subsequent STm infection. Initial control of STm, when historically we and others have shown innate immunity is important (Fig 3.1) appears to be unaffected by a prior Nb infection. The impaired resolution of STm in Nb infected hosts correlated with the time when the adaptive immune response is required to control the later stages of infection. Initial analysis of the underlying immune responses associated with this heightened susceptibility did not reveal any obvious correlates of reduced T cell mediated protection. Nevertheless, these data do not exclude factors such as impaired communication and/or migration of immune effectors.

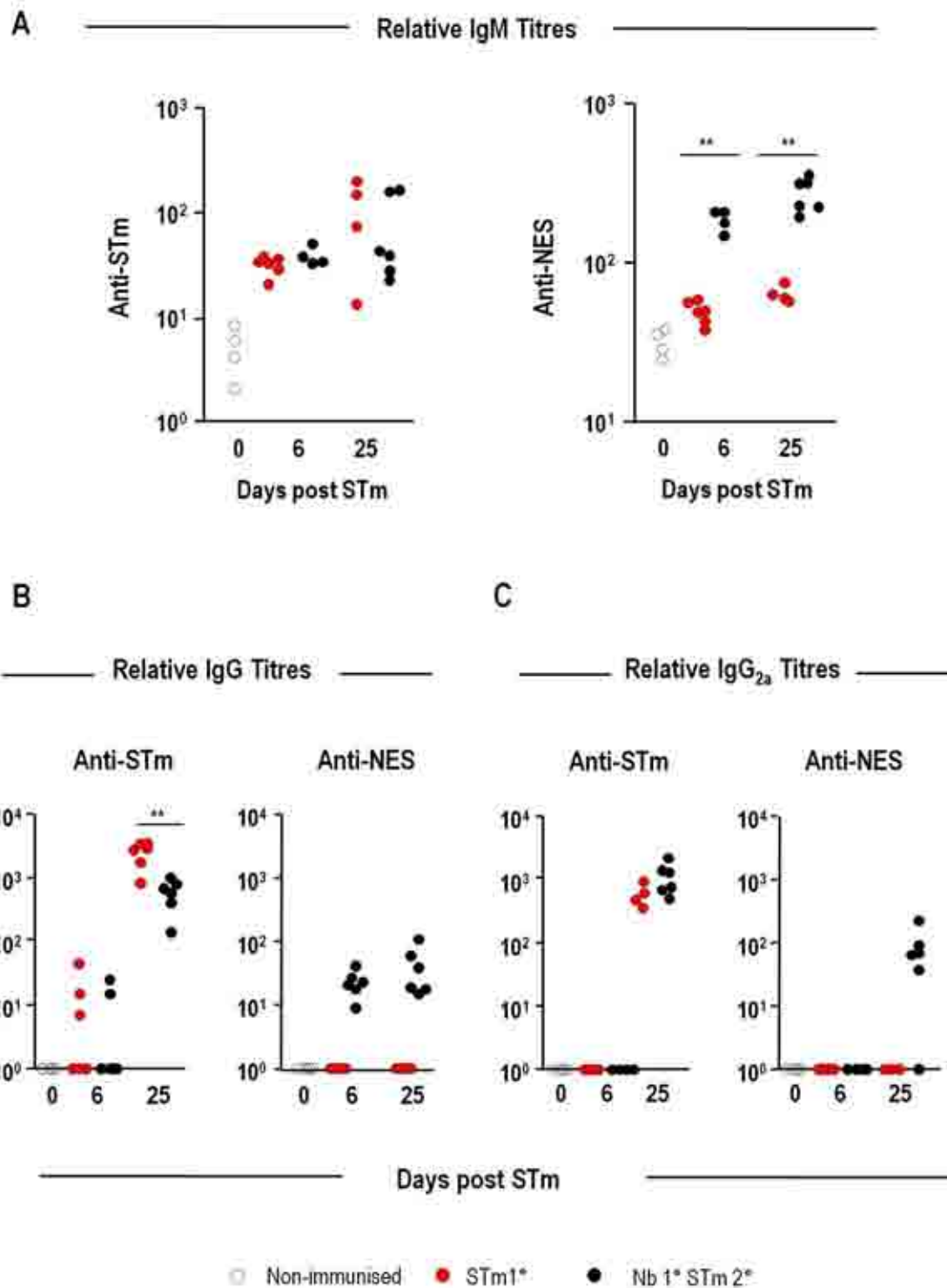


Figure 5.4

Antibody switching in response to STm remains faithful to IgG_{2a} despite priming with *N. brasiliensis*: BALB/c mice were infected with 500 L3 Nb and challenged on day 16 alongside naïve control mice with 5×10^5 STm. Mice were sacrificed at day 6 and 25 post-STm challenge. Serum anti-STm and anti-NES (A) IgM, (B) IgG, and (C) IgG_{2a} antibody titres were quantified by ELISA. Infections with STm and *N. brasiliensis* were intraperitoneal and subcutaneous, respectively. The Mann-Whitney Test was used to compare the significance between groups. Data is representative of 6 mice per group. ** $p < 0.01$

5.3 Primary STm infection causes delayed expulsion of *N. brasiliensis*

Whether primary infection with STm influenced the response to subsequent challenge with Nb was then investigated. BALB/c mice were infected with 5×10^5 STm and at day 17 post-STm infection mice were challenged with 500 L3 Nb larvae (Fig 5.5A). Quantification of the worm burden at day 5 post Nb infection (22 days post-STm) revealed no differences between non-STm primed and STm-primed mice. In contrast, at day 11 post Nb infection (28 days post-STm) STm-primed mice showed a significant delay in worm expulsion compared to non STm-primed mice.

A caveat in this first experiment was that the initial STm-infection had not resolved so discrete stages of a STm infection were examined. BALB/c mice were infected with STm for 32 or 42 days prior to infection with 500 L3 Nb larvae for 11 days (Fig 5.5B). At the end of this experiment assessment of the splenic bacterial burden showed that mice infected with STm for 32 days prior to Nb challenge (43 days post-STm) had some residual bacteria, compared to mice infected with STm for 42 days before Nb infection (53 days post STm-challenge). This latter group had undetectable levels of infection, bar one mouse (which had 10 bacteria). Quantification of the worm burden revealed mice infected with STm for 32 days prior to Nb infection (43 days post-STm) had a significant delay in worm expulsion relative to non-STm primed mice (Fig 5.5B). Interestingly, mice primed with STm for 42 days before Nb challenge (53 days post-STm) revealed Nb infection in a single animal, the same animal that had a residual STm infection. This suggests impaired worm clearance requires the presence of detectable numbers of STm.

As expected, spleen weights for STm-primed mice were significantly greater compared to non-STm primed mice, irrespective of the number of days previously infected with STm, whilst differences in spleen cellularity were generally small (Fig 5.6). As the worm burdens were comparable in STm-primed and non-STm primed mice 5 days post-Nb infection, responses in these mice were not examined further.

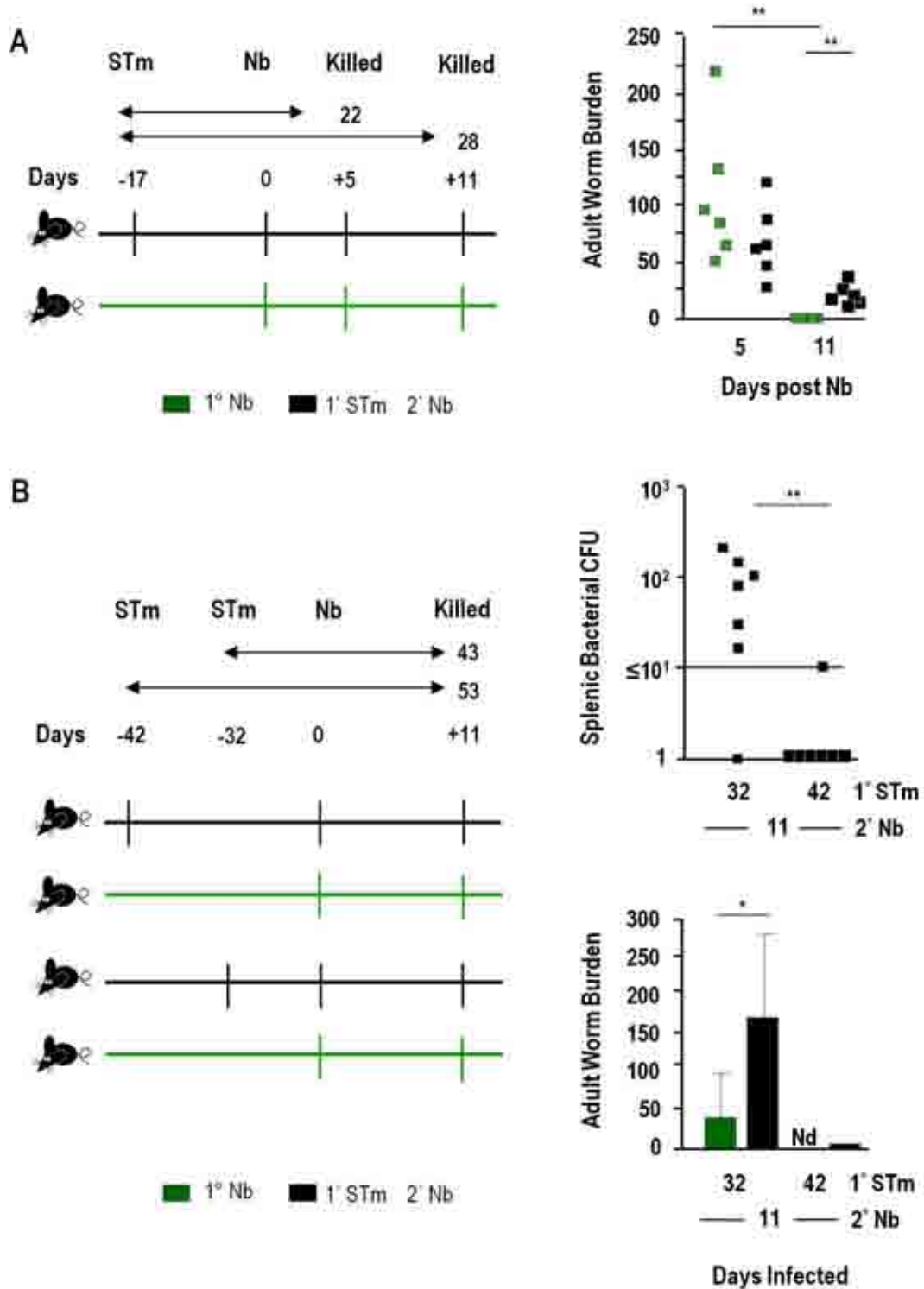


Figure 5.5

Primary STm infection impairs the host's ability to control subsequent *N. brasiliensis* infection in mice still infected with STm: (A) BALB/c mice were infected with 5×10^5 STm and challenged on day 17 with 500 L3 Nb alongside naive control mice. Small Intestines were isolated and worm burdens assessed at days 5 and 11 post Nb-infection. (B) BALB/c mice were infected as outlined and splenic bacterial CFU (top) and total worm burdens were assessed post Nb-infection (bottom). Infections with STm and Nb were intraperitoneal and subcutaneous, respectively. The Mann-Whitney Test was used to compare the significance between groups. Data is representative of 6 mice per group. * $p < 0.05$ ** $p < 0.01$

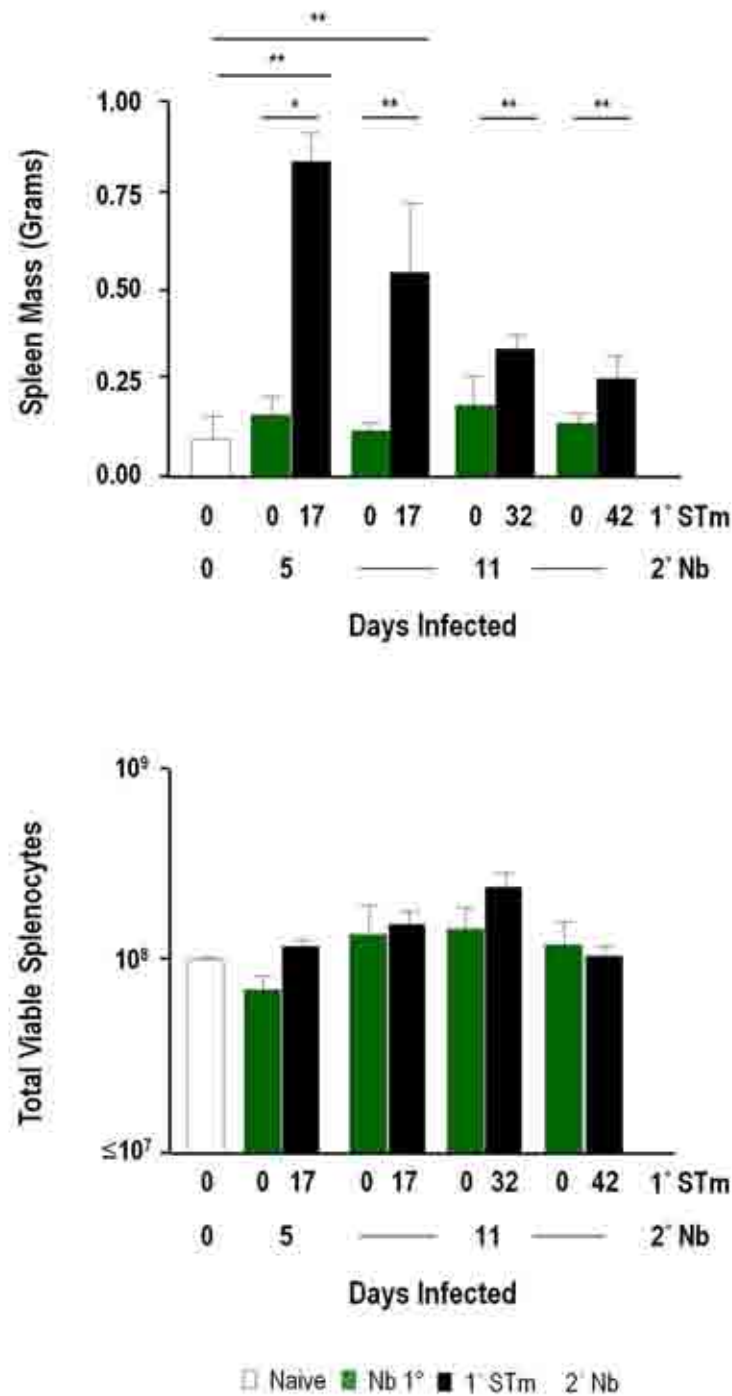


Figure 5.6

Infection with *STm* induces splenomegaly but this is not reflected in the total cellularity: Spleens were harvested and weighed (upper panel) and total cellularity determined (lower panel) from non-primed BALB/c mice or mice primed with 5×10^5 *STm* for either 17, 32 or 42 days before challenge with 500 L3 Nb for either 5 or 11 days. Infections with *STm* and *N. brasiliensis* were intraperitoneal and subcutaneous, respectively. The Mann-Whitney Test was used to compare the significance between groups. Data is representative of 6 mice per group. ** $p < 0.01$.

Quantification of splenic CD4⁺ T cell responses in STm-primed and non-STm primed mice infected with Nb for 11 days revealed that total numbers were broadly similar (Fig 5.7). The proportion and number of CD4 T cells per 0.1 g spleen showed the greatest variability. Additionally, when effector CD4 T cell responses were examined, the proportion and density of effector CD4 T cells showed some variability but total numbers were broadly similar, bar mice-primed with STm for 32 days prior to infection with Nb for 11 days (43 days post-STm), which had significantly more effector CD4 T cells relative to non-STm primed mice (Fig 5.8). In the main, assessment of total and effector CD4 T cells did not demonstrate a clear correlation between the proportion or number of cells, and the failure to control Nb infection in STm-primed mice.

As expulsion of Nb is associated with induction of IL-13 splenocytes were re-stimulated in-vitro with anti-CD3 in the presence of anti-CD28 and cytokine responses were assessed by intracellular FACS. This showed the proportion, total number and density of CD4⁺IFN γ ⁺ T cells in STm-primed Nb-infected mice was consistently greater than in non-STm primed mice (Fig 5.9A). Despite this, there was no clear effect on IL-13 production with the exception of mice primed with STm for 42 days before Nb infection (53 days post-STm), which had a lower proportion and density of CD4⁺IL-13⁺ T cells compared to non-STm primed mice (Fig 5.9B). However, this group had cleared the Nb infection suggesting a highly complex relationship between Th1 and Th2 responses to these pathogens. As an additional control the intestinal mucus response was assessed by PAS staining (Fig 5.10). Indeed, induction of IL-13 typically correlates with goblet cell hyperplasia which is critical for the expulsion of many intestinal helminths, including Nb (Horsnell et al., 2007; Marillier et al., 2008). As expected, the response was comparable on day 5 post Nb-infection in mice primed with STm for 17 days before Nb infection (22 days post-STm) and non-STm primed mice. However, the response was qualitatively reduced on day 11 post Nb-infection in mice primed with STm for 17 days before Nb-infection (28 days post-STm) relative to non-STm primed mice. This suggests that the reduced mucus response upon priming with STm may contribute to the delay in worm expulsion.

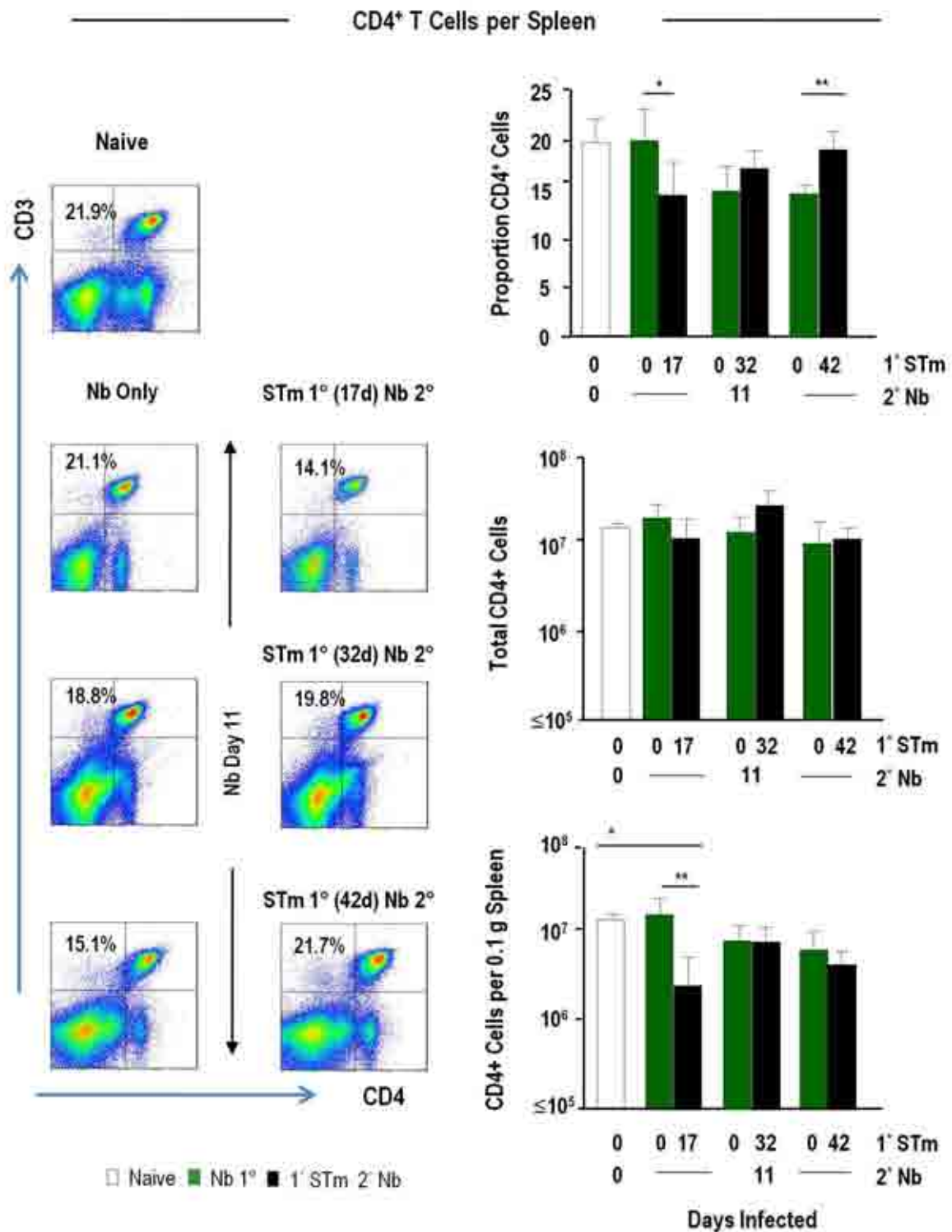


Figure 5.7

CD4⁺ T cell responses are comparable after infection with *N. brasiliensis* in naïve or STm-primed mice: BALB/c mice were infected with 5×10^5 STm for 17, 32 or 42 days before challenge with 500 L3 Nb for 11 days alongside naïve control mice. Splenic CD4⁺ T cells were gated from the lymphocyte population and are represented as a proportion (top), total number per spleen (central) and total number per 0.1 g spleen (bottom). Infections with STm and *N. brasiliensis* were intraperitoneal and subcutaneous, respectively. The Mann-Whitney Test was used to compare the significance between groups. Data is representative of 6 mice per group. * $p < 0.05$, ** $p < 0.01$.

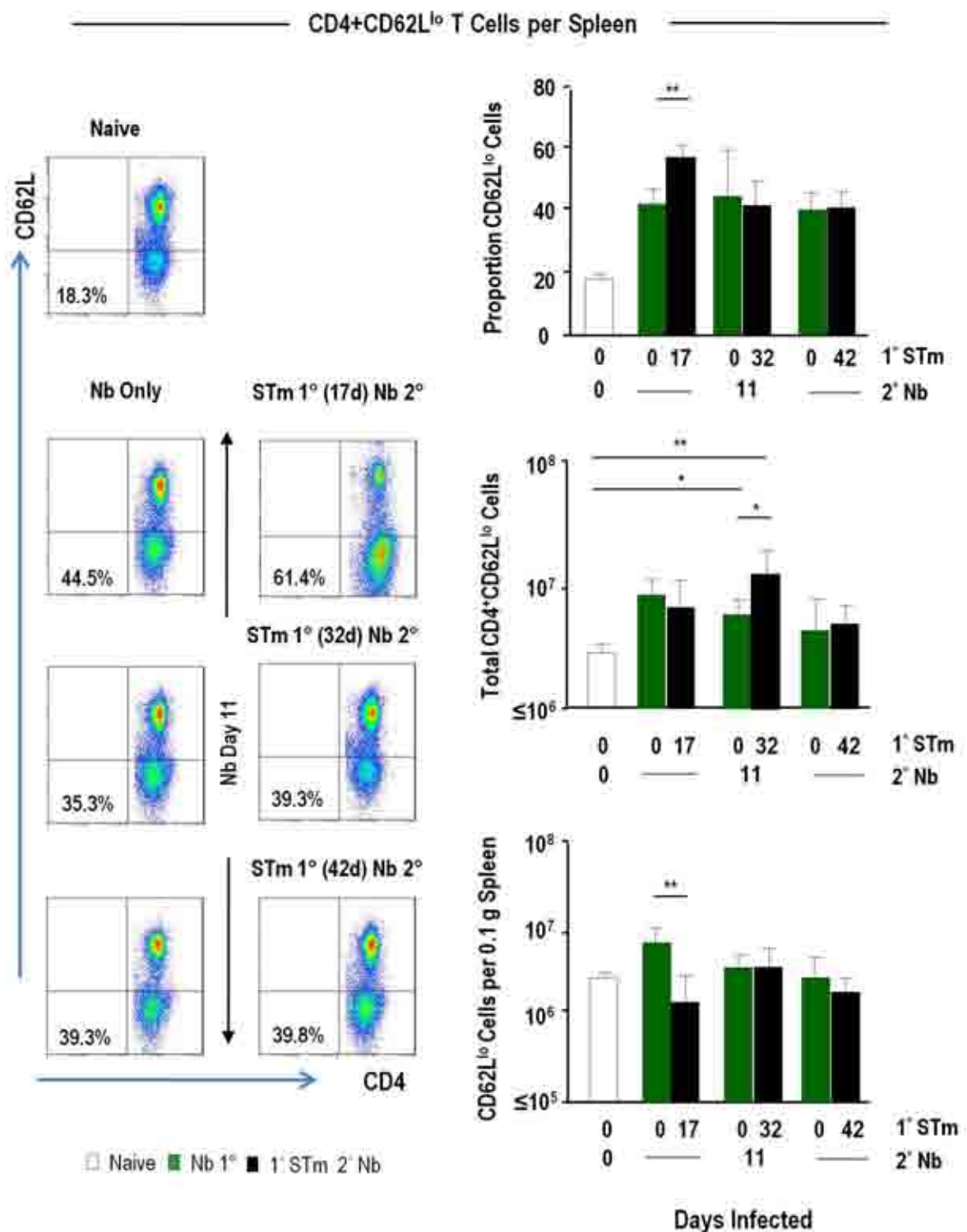


Figure 5.8

Effector CD4 T cell responses are comparable after infection with *N. brasiliensis* in naïve or STm-primed mice: BALB/c mice were infected with 5×10^5 STm for 17, 32 or 42 days before challenge with 500 L3 Nb for 11 days alongside naïve control mice. Splenic CD4⁺CD62L^{lo} T cells were quantified by FACS and are represented as a proportion (top), total number per spleen (central) and total number per 0.1 g spleen (bottom). Infections with STm and *N. brasiliensis* were intraperitoneal and subcutaneous, respectively. The Mann-Whitney Test was used to compare the significance between groups. Data is representative of 6 mice per group. * $p < 0.05$, ** $p < 0.01$.

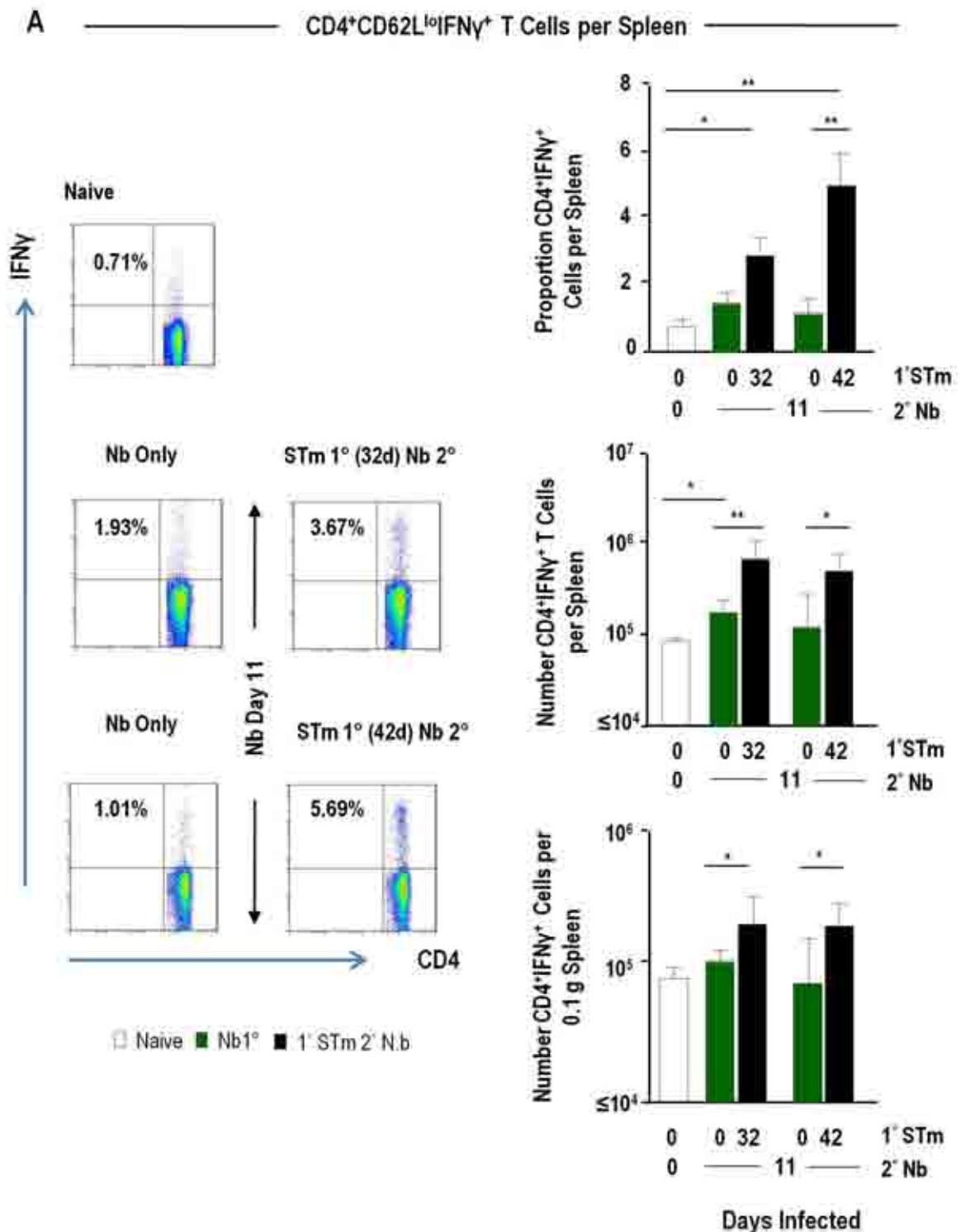


Figure 5.9

Th1 responses are enhanced in STm-primed mice 11 days after subsequent Nb infection compared to non-STm primed mice: (A) BALB/c mice were infected with 5×10^5 STm for 32 or 42 days before challenge with 500 L3 Nb larvae alongside non-primed mice. Spleens were harvested 11 days post-Nb-infection and 5×10^5 splenocytes were re-stimulated in-vitro with anti-CD3 in the presence of anti-CD28 for 6 hours. IFN γ expression was measured by intracellular FACS and is represented as: a proportion (top), total number per spleen (centre), and total number per 0.1 g spleen (bottom) of CD4⁺CD62L^{lo} T cells. Infections with STm and Nb were intraperitoneal and subcutaneous, respectively. The Mann-Whitney Test was used to compare the significance between groups. Data is representative of 6 mice per group. * $p < 0.05$, ** $p < 0.01$.

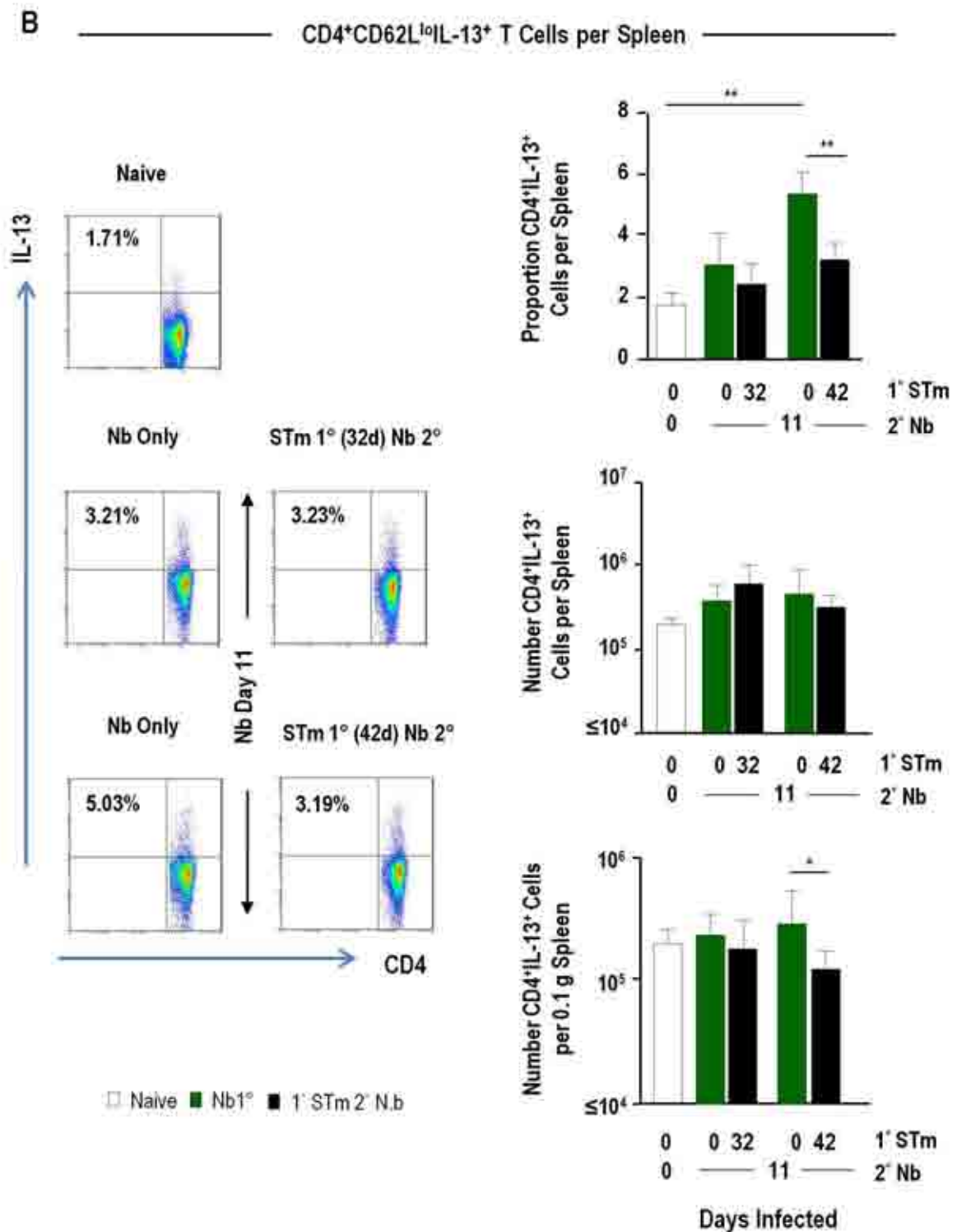


Figure 5.9

Mice primed with STm for 42 days before subsequent Nb challenge have a reduced IL-13 response relative to non-STm primed mice: (B) BALB/c mice were infected and spleens were harvested as described in (A). 5×10^5 splenocytes were re-stimulated in-vitro with anti-CD3 in the presence of anti-CD28 for 6 hours. IL-13 expression was measured by intracellular FACS and is represented as: a proportion (top), total number per spleen (centre), and number per 0.1 g spleen (bottom) of CD4⁺CD62L^{lo} T cells. Infections with STm and Nb were intraperitoneal and subcutaneous, respectively. The Mann-Whitney Test was used to compare the significance between groups. Data is representative of 6 mice per group. * $p < 0.05$, ** $p < 0.01$.

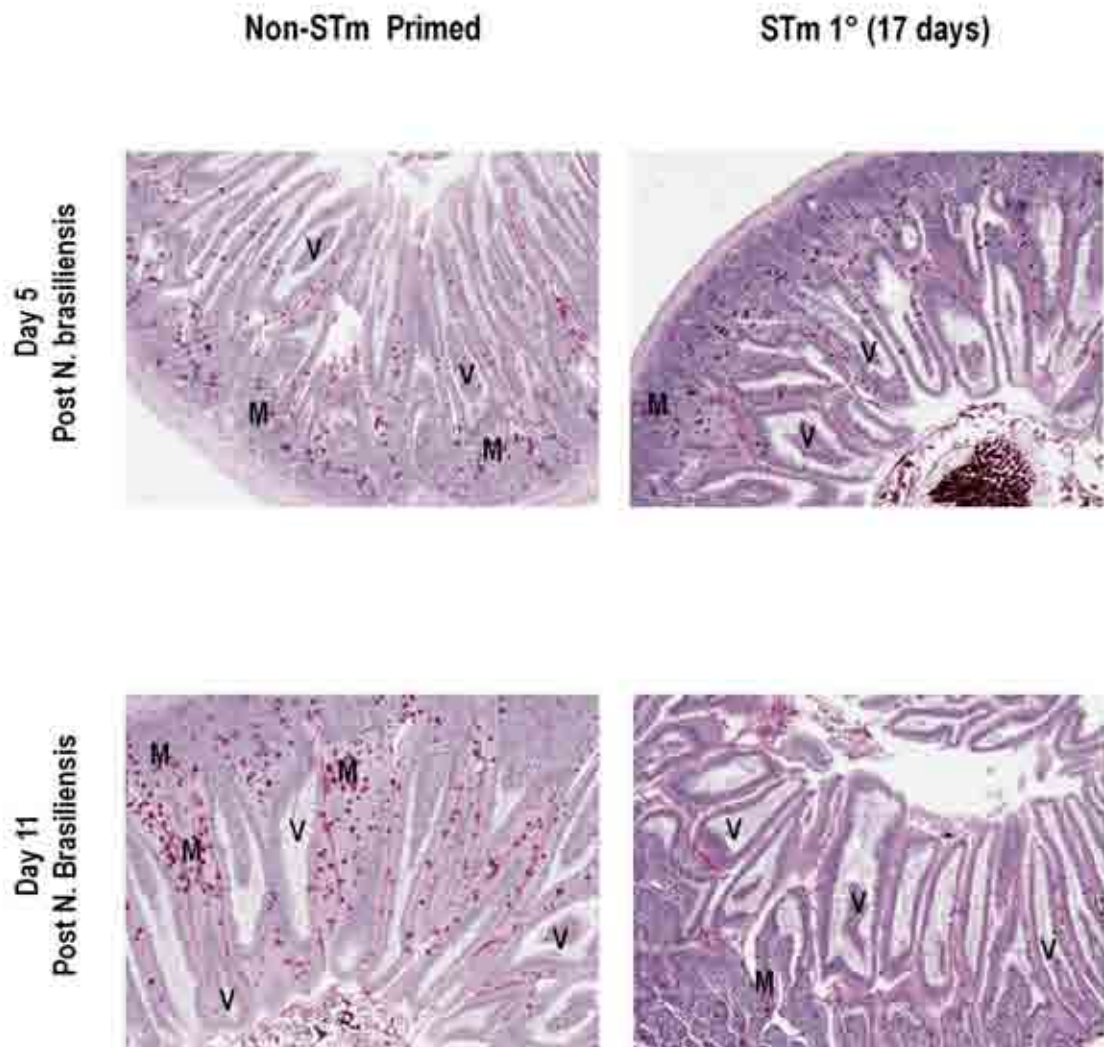


Figure 5.10

Primary infection with STm impairs goblet cell mucus production upon subsequent challenge with *N. brasiliensis*: BALB/c mice were infected with 5×10^5 STm for 17 days before challenge with 500 L3 Nb larvae alongside naïve control mice. Small intestines were removed at days 5 and 11 post Nb-infection and a portion of the jejunum was fixed in formalin, sectioned and stained with PAS to detect mucus production. V = villi and M = mucus-producing cells (stain deep purple). Infections with STm and Nb were intraperitoneal and subcutaneous, respectively. Data is representative of 6 mice per group.

These data demonstrate primary infection with STm negatively influences the host's response to subsequent infection with Nb. This delayed expulsion requires the persistence of STm in the host which strikingly prolongs Nb infection. Of interest, this study identified a reduction in the mucus response by STm-primed mice which could underlie the delayed expulsion of Nb. However, assessment of the CD4 T cell response failed to identify any clear correlates of reduced protection, except for a small trend towards lower IL-13 expression.

5.4 Dual Infection with STm and *N. brasiliensis* act reciprocally to impair the host's response to both pathogens

Since primary infection with either STm or Nb could negatively influence the subsequent host response upon challenge, the impact of simultaneous infection with Nb and STm on the host response to each pathogen at discrete stages of infection was investigated. BALB/c mice were infected with 5×10^5 STm, or 500 L3 Nb larva or both pathogens for 5, 10, 18 or 32 days. Quantification of bacterial burdens in the spleen and liver at day 5 post-infection revealed no differences in bacterial numbers between STm-only and co-infected mice (Fig 5.11A). From day 10 onwards splenic and liver bacterial numbers were consistently higher in dual-infected mice. Quantification of intestinal worm burdens revealed Nb-only or co-infected mice had a comparable worm burden at day 5 post-infection (Fig 5.11B). As expected, at day 10 post-infection Nb-only infected mice had largely cleared the infection and by day 18 no worms were detectable. Numbers were not assessed at day 32. In contrast, co-infected animals had consistently higher worm burdens from day 10, which persisted at day 18, with worms still being detected at day 32.

STm-only and co-infected mice had bigger spleens from day 5 onwards and both the spleen mass and cellularity was significantly enhanced compared to NI or Nb-only infected mice at each time-point assessed (Fig 5.11C). No differences between STm-only and co-infected mice were apparent, except at day 18 where co-infected mice had significantly larger spleens, but comparable cellularity.

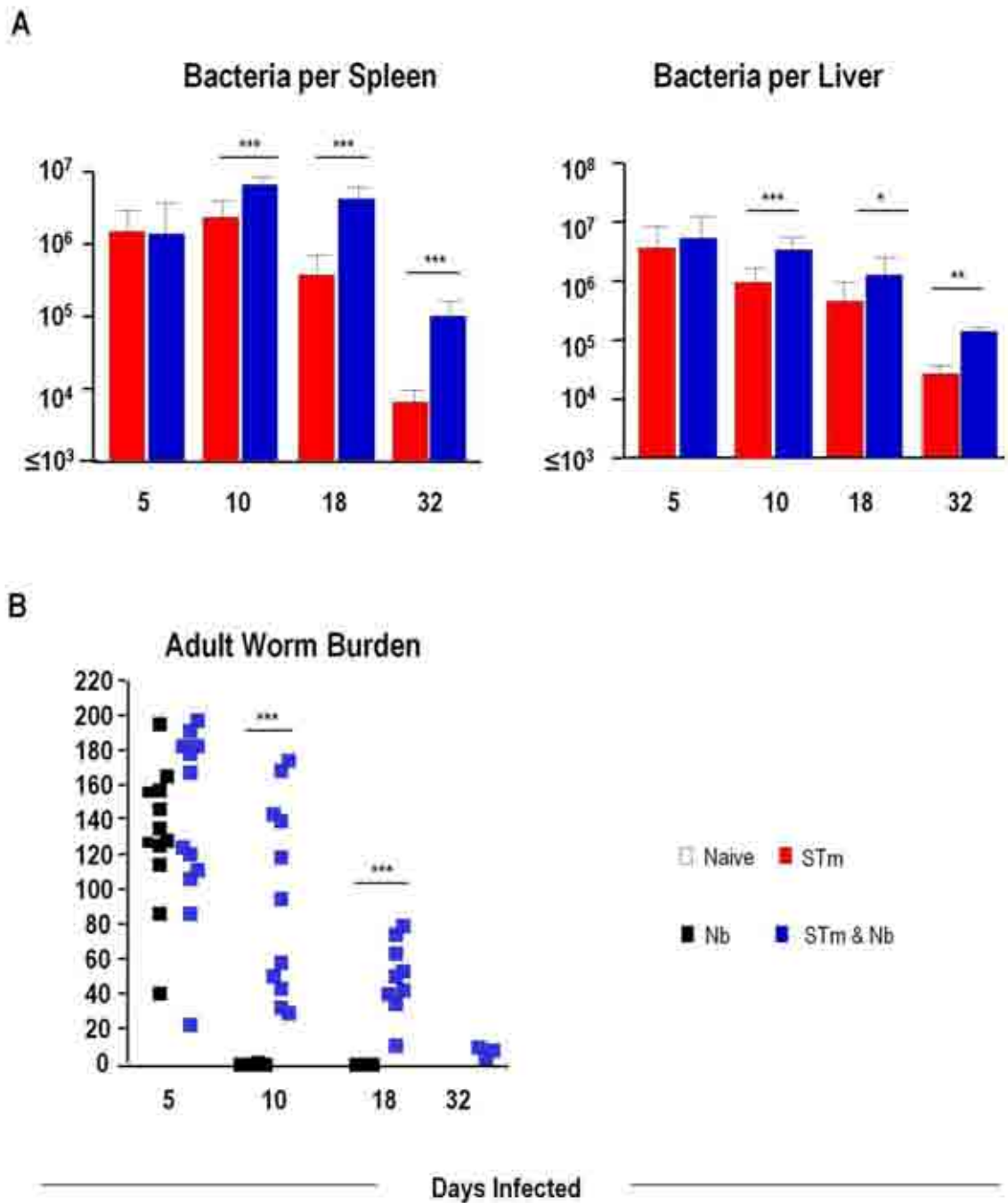


Figure 5.11

Dual Infection with STm and Nb impairs the hosts ability to resolve either infection: BALB/c mice were infected with either 5×10^5 STm, 500 L3 Nb or both for either 5, 10, 18 or 32 days: (A) Spleens and livers were harvested and bacterial CFU enumerated from STm-infected animals. (B) Small intestines were isolated and total worm burdens assessed from Nb infected mice. Infections with STm and Nb were intraperitoneal and subcutaneous, respectively. The Mann-Whitney Test was used to compare the significance between groups. Groups contained 4-6 mice and experiments were performed at least twice for each time point except day 32, which was done once. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$.

C

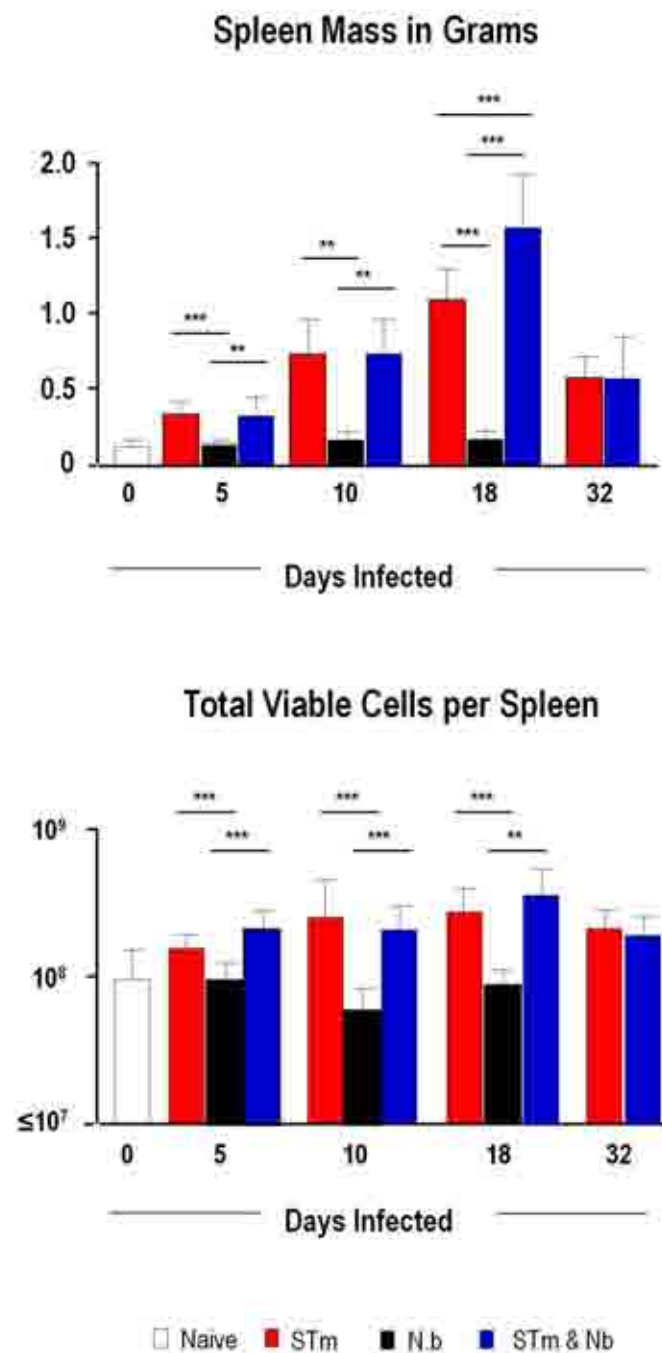


Figure 5.11

Spleen mass of dual infected mice mirrors the profile of STm-only infected mice: BALB/c mice were infected with either 5×10^5 STm, 500 L3 Nb or both for either 5, 10, 18 or 32 days; (C) Spleens were weighed (top panel) and total cellularity quantified (bottom panel). Infections with STm and Nb were intraperitoneal and subcutaneous, respectively. The Mann-Whitney Test was used to compare the significance between groups. Groups contained 4-6 mice and experiments were performed at least twice for each time point except day 32, which was done once. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$.

5.4.1 Th1 and Th2 profiles remain faithful to STm and *N. brasiliensis* upon co-infection

Quantification of splenic CD4⁺ T cell responses revealed broadly equivalent T cell numbers and proportions in NI and Nb-only infected mice. The proportion and density of CD4⁺ T cells was lower in STm-only infected mice compared to both these groups at each stage (Fig 5.12). Primarily, CD4⁺ T cell counts in dual infected mice were equivalent to STm-only infected mice, except at day 5, where co-infected mice had more CD4⁺ T cells than both STm-only and Nb-only infected mice. As expected, analysis of T cell activation in the spleen revealed proportions of CD4⁺CD62L^{lo} T cells increased after infection in all groups compared to NI mice (Fig 5.13). The proportion of effector CD4⁺ T cells was consistent throughout infection in Nb-only infected mice. Once more, responses in co-infected mice largely resembled STm-only infected mice. Differences were marginal, but significant, at day 5 post-infection where the density of effector CD4⁺ T cells was greater in co-infected mice relative to STm-only infected mice, and at day 18, where the proportion was lower compared to STm-only infected mice. This suggested that the CD4 T cell response, in terms of T cell activation, may not be responsible for the impaired clearance of STm and Nb in dual-infected mice.

As distinct CD4 T cell responses are required for the resolution of STm and Nb, Th1 and Th2 responses were examined by intracellular FACS following re-stimulation of splenocytes with anti-CD3 in the presence of anti-CD28. This revealed the proportion, number and density of CD4⁺IFN γ ⁺T cells from Nb-only infected mice was equivalent to NI mice at each time point assessed (Fig 5.14A). Similar levels of IFN γ were apparent between STm-only and co-infected mice throughout, in terms of the proportion, absolute numbers and density, with the exception of a small augmentation in co-infected mice at day 5 post-infection. As expected, the proportion of CD4⁺IL-13⁺T cells was significantly greater in Nb-only and co-infected mice when compared to NI and STm-only infected mice until day 32 post-infection (Fig 5.14B). Nevertheless, IL-13 T cell numbers were broadly equivalent except at day 5, where STm-only

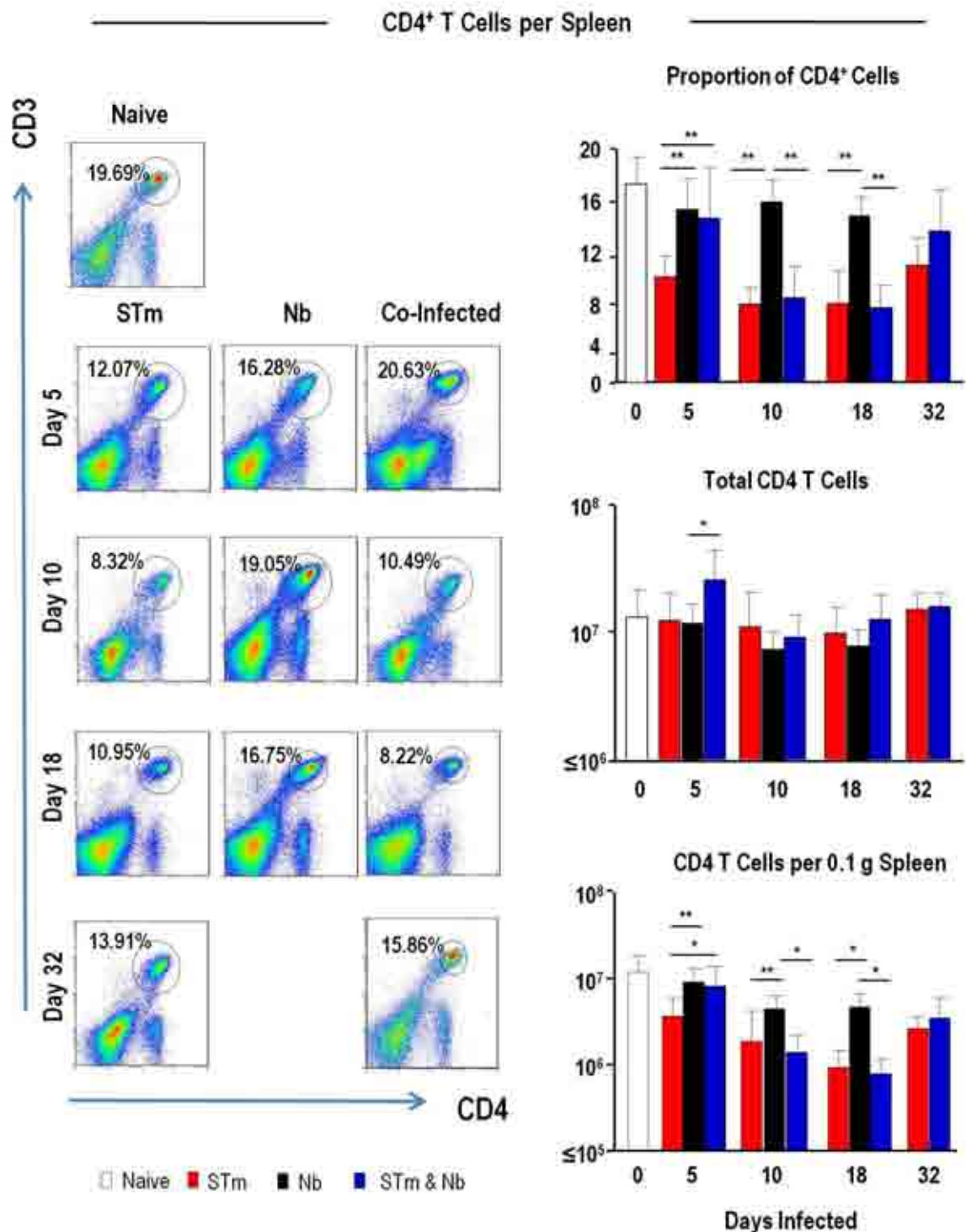


Figure 5.12

Splenic CD4 T cell responses in dual infected mice are comparable to STm-only infected mice: BALB/c mice were infected with either 5×10^5 STm, 500 L3 Nb or were co-infected with both for either 5, 10, 18 or 32 days. Splenic CD3⁺CD4⁺ T cells were gated from the lymphocyte population and are represented as a proportion (top), total number per spleen (centre), and total number per 0.1 g spleen (bottom). Infections with STm and Nb were intraperitoneal and subcutaneous, respectively. The Mann-Whitney Test was used to compare the significance between groups. Groups contained 4-6 mice and experiments were performed at least twice for each time point except day 32, which was done once. * $p < 0.05$, ** $p < 0.01$.

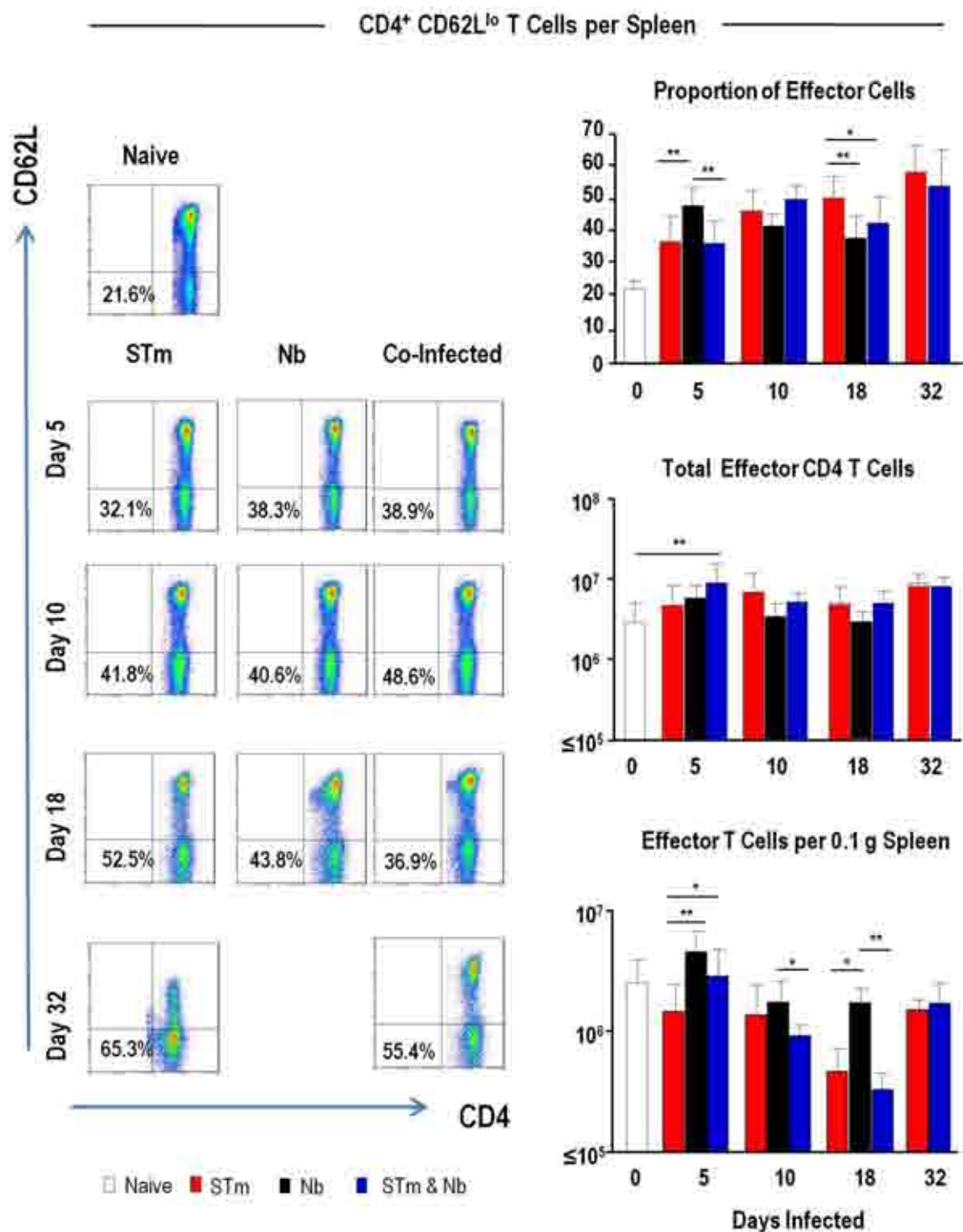


Figure 5.13

CD4⁺CD62L^{lo} T cell responses in dual infected mice are comparable to STm-only infected mice: BALB/c mice were infected with either 5×10^5 STm, 500 L3 Nb or both for 5, 10, 18 or 32 days: Splenic CD4⁺ T cells that had down-regulated expression of CD62L were quantified and are represented as a proportion (top), total cells per spleen (centre), and total cells per 0.1 g spleen (bottom). Infections with STm and Nb were intraperitoneal and subcutaneous, respectively. The Mann-Whitney Test was used to compare the significance between groups. Groups contained 4-6 mice and experiments were performed at least twice for each time point except day 32, which was done once. * $p < 0.05$, ** $p < 0.01$.

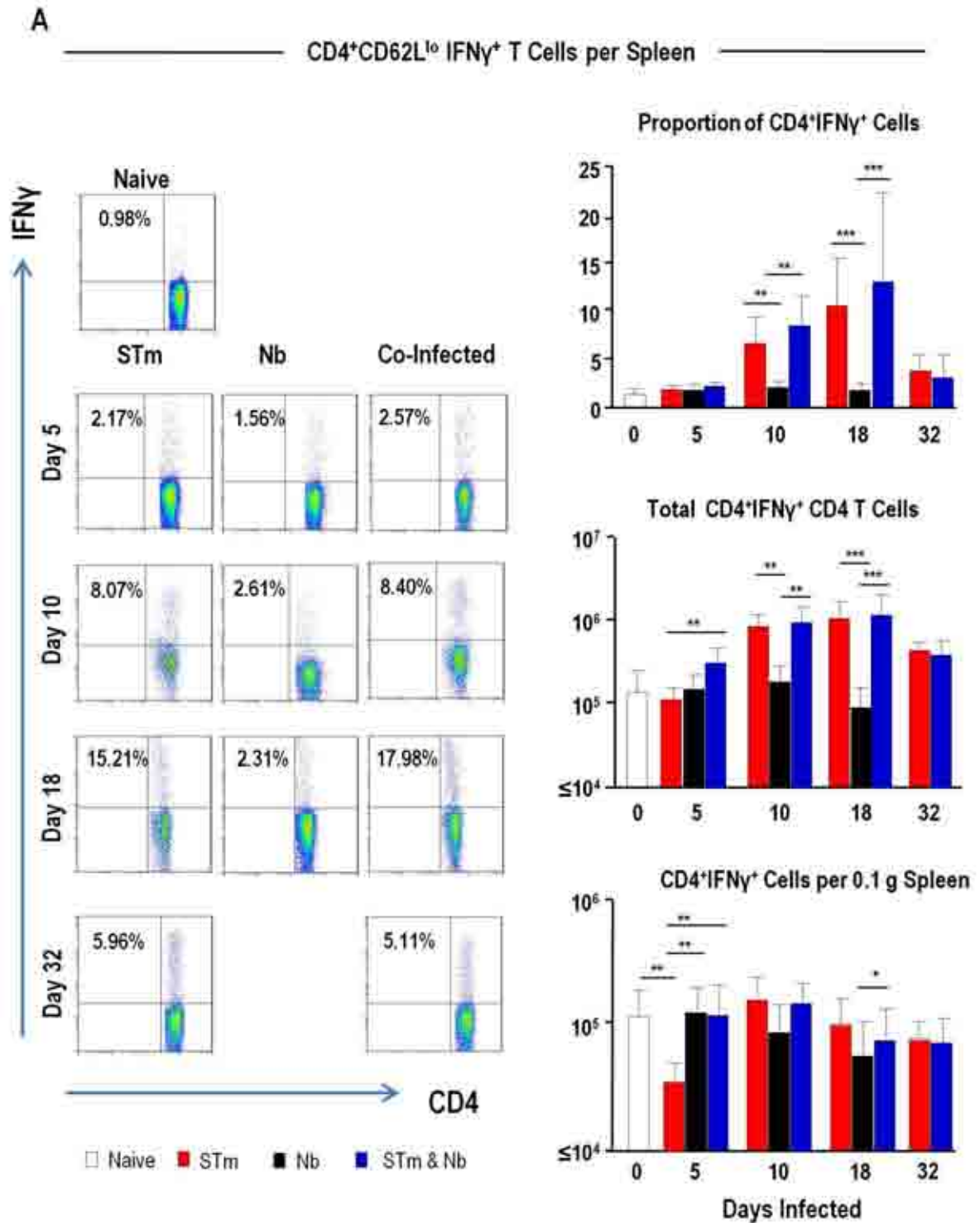


Figure 5.14

The IFN γ response in dual infected mice mirrors the response in STm-only infected mice: (A) BALB/c mice were infected with either 5×10^5 STm, 500 L3 Nb or both for either 5, 10, 18 or 32 days and 5×10^6 splenocytes were re-stimulated in-vitro with anti-CD3 in the presence of anti-CD28 and IFN γ expression was assessed in CD4⁺CD62L^{lo} T cells and is represented as a proportion (top), total numbers per spleen (centre), and total numbers per 0.1 g spleen (bottom) alongside NI mice. Infections with STm and Nb were intraperitoneal and subcutaneous, respectively. The Mann-Whitney Test was used to compare the significance between groups. Groups contained 4-6 mice and experiments were performed at least twice for each time point except day 32, which was done once. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

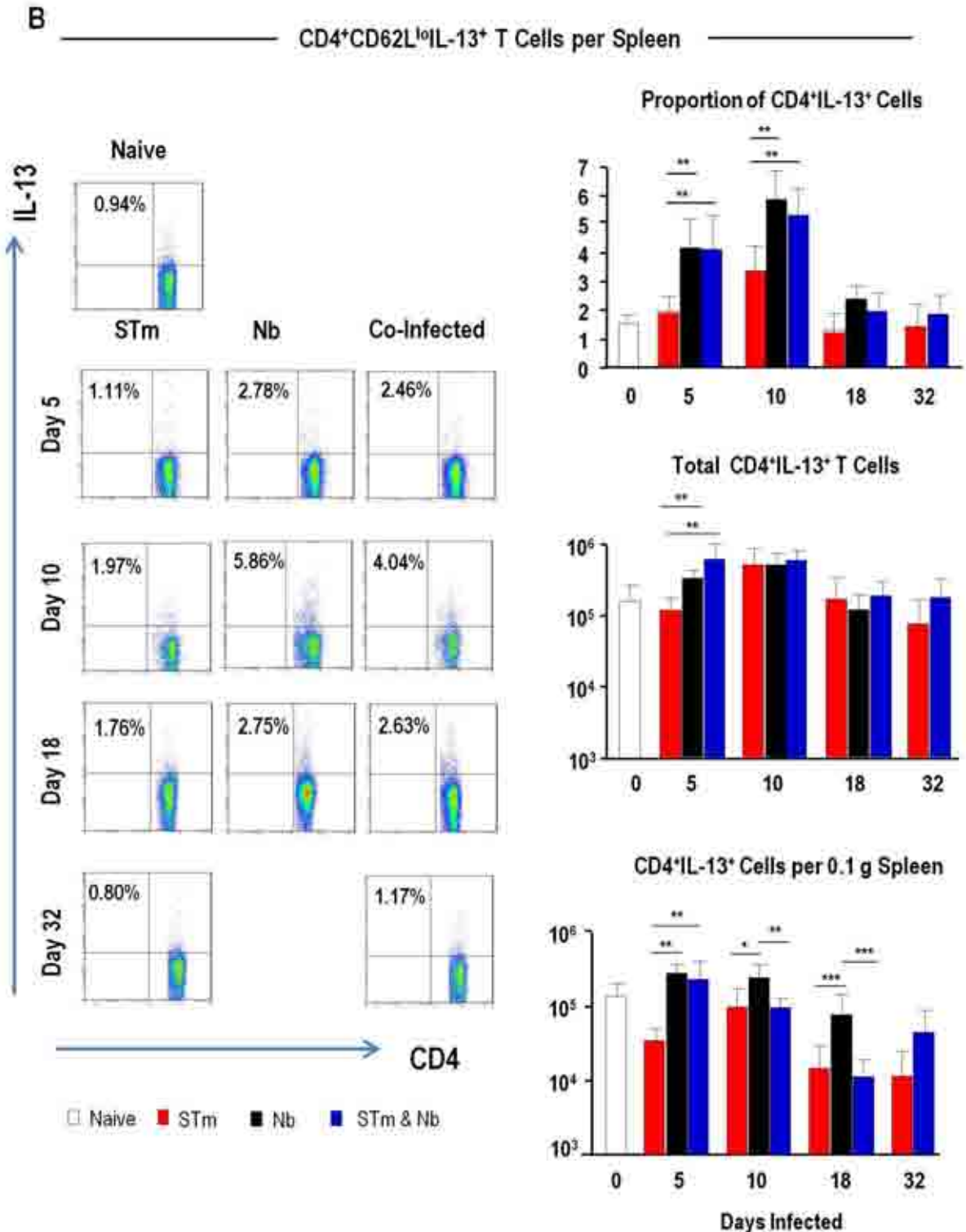


Figure 5.14

Levels of IL-13 fall in dual infected mice from day 10 compared to Nb-only infected mice: (B) BALB/c mice were infected as in (A) and 5×10^6 splenocytes were re-stimulated in-vitro with anti-CD3 in the presence of anti-CD28. IL-13 expression was assessed in CD4⁺CD62L^{lo} T cells and is represented as a proportion (top), total number per spleen (centre), and total number per 0.1 g spleen (bottom). Infections with STm and Nb were intraperitoneal and subcutaneous, respectively. The Mann-Whitney Test was used to compare the significance between groups. Groups contained 4-6 mice and experiments were performed at least twice for each time point except day 32 which was done once. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

infected mice had fewer cells. Interestingly, the number of CD4⁺IL-13⁺ T cells per 0.1 g spleen fell from day 10 onwards in co-infected mice relative to Nb-only infected mice, despite the presence of a pronounced worm burden. Despite this, in the main the intracellular FACS data suggested that CD4 T cell polarisation to Th2 and Th1 in response to Nb and STm respectively, remained largely intact in co-infected mice.

IL-10 can be secreted by regulatory T and B cells which can suppress CD4 T cell responses (Bai et al., 1997). To examine IL-10 responses, splenocytes were re-stimulated with anti-CD3 for 72 hours and total IL-10 responses were analysed in the supernatants by ELISA (Fig 5.15). This revealed Nb-only and co-infected mice had a greater IL-10 response compared to STm-only infected mice at the equivalent time-points. Responses in co-infected mice closely resembled Nb-only infected mice, except at day 10, where responses were lower. This suggested that on the basis of cytokine secretion a highly complex host response was involved and in depth analysis of regulatory T cells may be warranted.

5.4.2 Goblet cell hyperplasia is reduced co-infected mice

Expulsion of Nb is associated with goblet cell hyperplasia and mucus secretion which is critical for expulsion of Nb (Horsnell et al., 2007). The mucus response was visualised in the small intestine by PAS staining which found no differences between mice infected with STm-only and NI mice throughout infection (Fig 5.16). As expected, at day 10 post-infection Nb-only infected mice displayed a pronounced mucus response and by day 18, this returned to levels observed in NI mice. Surprisingly, although the goblet cell response in co-infected mice at day 10 post-infection was enhanced relative to STm-only infected mice, it was reduced relative to Nb only infected mice. At day 18, the mucus response in co-infected animals was enhanced relative to mice infected with a single pathogen, reflecting an active Nb infection. Moreover, despite a residual Nb infection in co-infected mice at day 32 the goblet cell response was comparable to STm-only infected animals. This suggests that the delay in worm expulsion in co-infected mice may be related to a reduced production of mucus.

CD3 Re-stimulated Splenocytes
IL-10 ng/ml

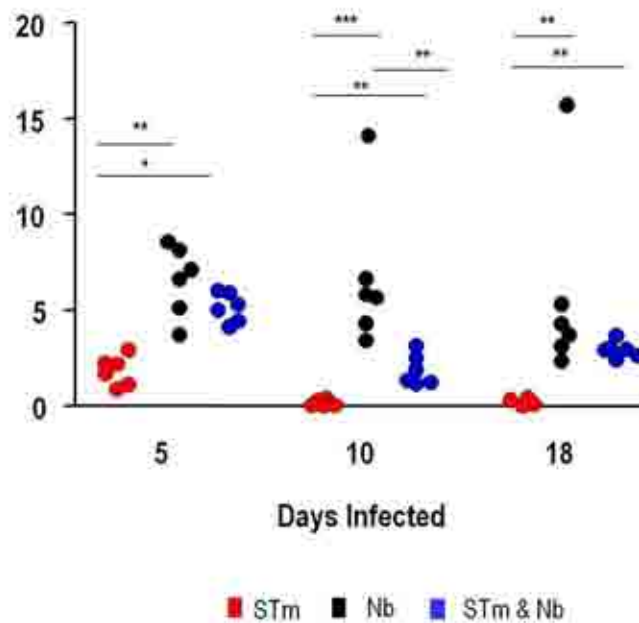


Figure 5.15

Infection with Nb induces IL-10 but STm infection does not: BALB/c mice were infected with either 5×10^5 STm, 500 L3 Nb or both for either 5, 10, or 18 days. Splenocytes were isolated and 2×10^5 cells were re-stimulated in-vitro with anti-CD3 for 72 hours. The supernatants were harvested and levels of IL-10 were quantified by ELISA. Infections with STm and Nb were intraperitoneal and subcutaneous, respectively. The Mann-Whitney Test was used to compare the significance between groups. Groups contained 4-6 mice and experiments were performed at least twice for each time point. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

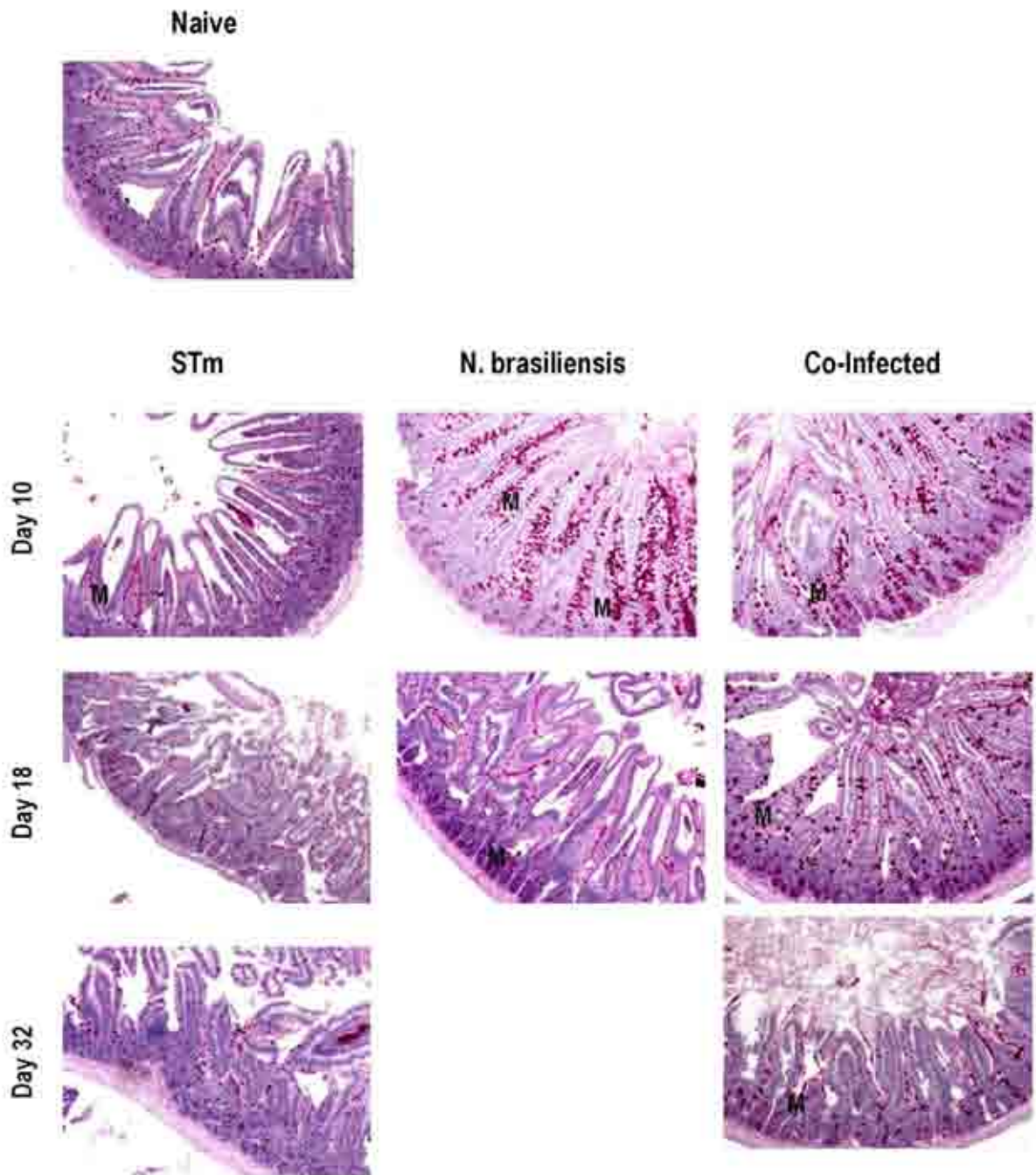


Figure 5.16

Dual infection with STm and Nb is associated with reduced goblet cell hyperplasia: BALB/c mice were infected with either 5×10^5 STm, 500 L3 Nb or both for either 10, 18 or 32 days. Small intestines were removed and a portion of the jejunum was fixed in formalin, sectioned and stained with PAS to detect mucus production (M = mucus-producing cells (stain deep purple)). Images are representative of groups containing 6 mice. Infections with STm and Nb were intraperitoneal and subcutaneous, respectively.

5.4.3 Antibody responses are reduced upon co-infection

Antibody responses were then examined which showed infection with STm, Nb or both pathogens induced pathogen specific IgM responses by day 5 post-infection (Fig 5.17). Nevertheless, close examination showed that co-infection gradually induced lower anti-STm and anti-NES IgM titres in comparison to mice infected with only STm or Nb, respectively. Switched antibody responses were then assessed to ascertain whether appropriate antibody isotypes were produced upon co-infection, IgG2a for STm and IgG1 for Nb. The anti-STm IgG response showed IgG was induced in STm-only and co-infected mice from day 5 onwards and titres were comparable up to day 18. At day 32, anti-STm IgG titres were enhanced in STm-only infected mice relative to co-infected mice (Fig 5.18A). The anti-NES IgG response revealed co-infected mice had a significantly lower titre at day 10 post-infection relative to Nb-only infected mice, but titres were comparable at day 18 (Fig 5.18A).

In terms of antibody isotype, at day 32 co-infected mice induced both an anti-STm IgG1 and IgG2a antibody response. As expected, STm-only infected mice induced IgG2a and titres were comparable to co-infected mice, whilst IgG1 was not detected. Furthermore, both Nb-only and co-infected mice induced an anti-NES IgG1 response relative to mice infected with STm-only, and titres were comparable at the time-points assessed (Fig 5.18 B&C). As an additional control, whether this pattern of switching in co-infected mice was reflected in the spleen by immunohistology was investigated. This revealed a marked induction of IgG1 in Nb-only infected mice at day 10 post-infection, and although IgG1 was detected in co-infected mice, qualitatively it was reduced relative to Nb-only infected mice. As expected, IgG1 was largely undetectable in STm-only infected mice (Fig 5.19A). In terms of IgG2a, as expected Nb-only infected mice did not induce switching to IgG2a whilst IgG2a cells were present in both STm-only and co-infected mice at day 10 post-infection, and at a similar level (Fig 5.19A). Furthermore, spleens isolated from co-infected mice at day 32 post-infection demonstrated induction of both IgG1 and IgG2a, whilst STm-only infected mice showed induction of IgG2a only (Fig 5.19B). Thus, co-infected mice show pronounced switching to both IgG1 and IgG2a as reflected by immunohistology and ELISA.

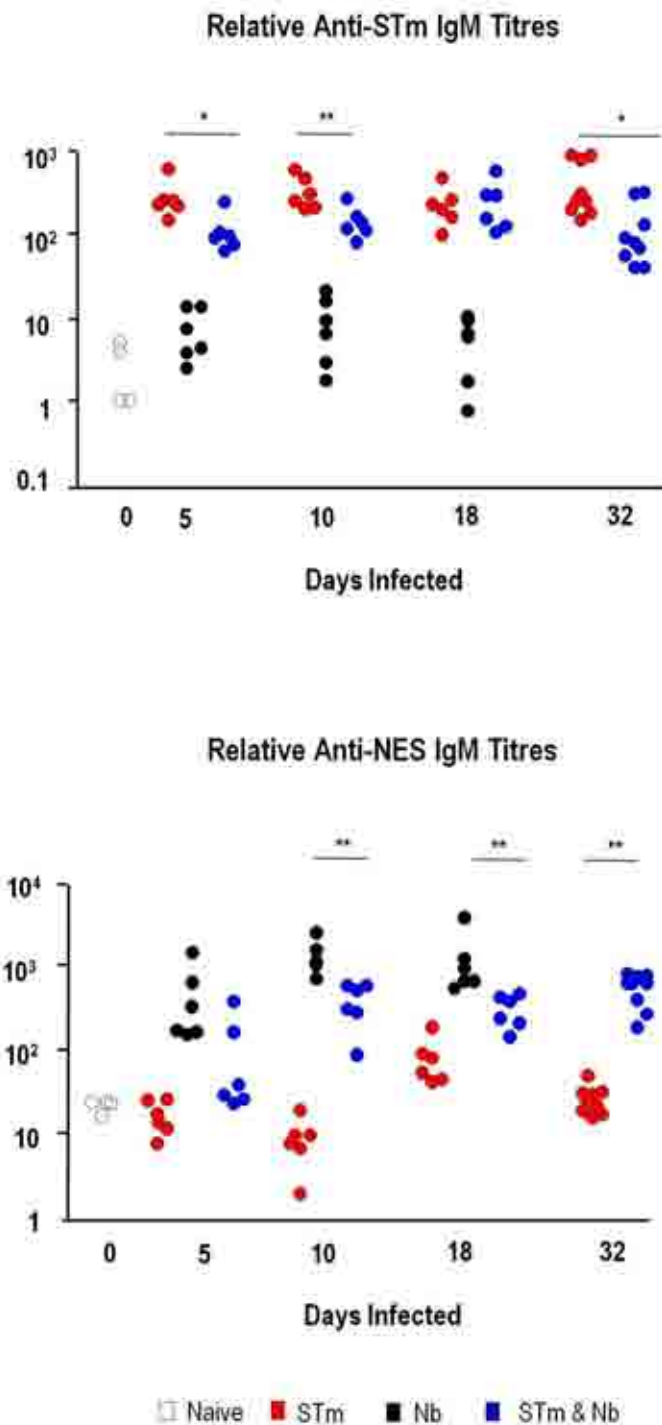


Figure 5.17

Dual infection is associated with lower anti-NES and anti-STm IgM titres: BALB/c mice were infected with either 5×10^5 STm, 500 L3 Nb or both for either 5, 10, 18 or 32 days. Serum anti-STm (upper graph) and anti-NES (lower graph) IgM titres were quantified at each time-point by ELISA. Infections with STm and Nb were intraperitoneal and subcutaneous, respectively. The Mann-Whitney Test was used to compare the significance between groups. Groups contained 4-6 mice and experiments were performed at least twice for each time point except day 32, which was done once. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

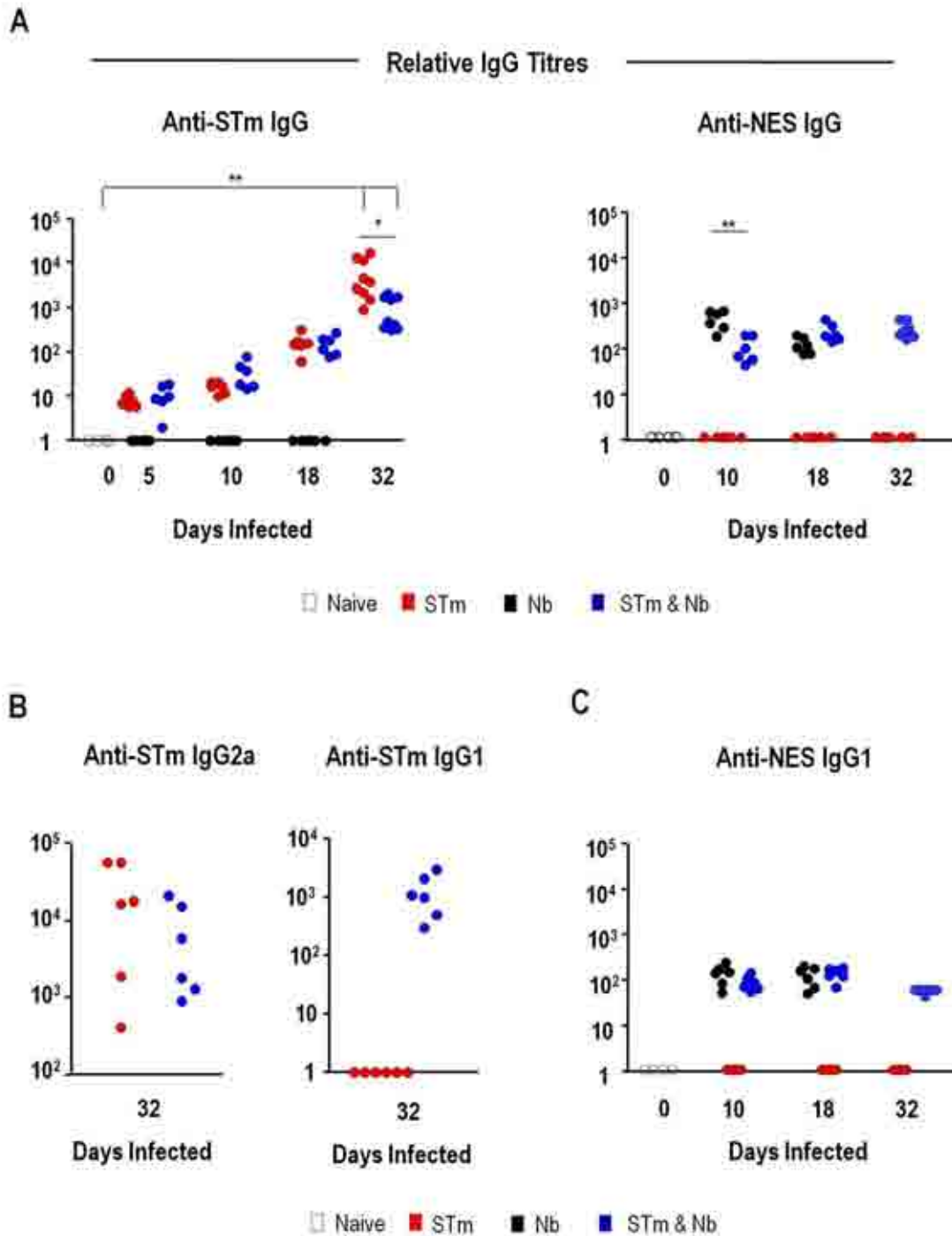


Figure 5.18

Dual infected mice induce both IgG1 and IgG2a antibody responses: BALB/c mice were infected with either 5×10^5 STm, 500 L3 Nb or both for either 5, 10, 18 or 32 days: (A) Serum anti-STm (left) and -NES (right) IgG titres were quantified by ELISA for each time-point. (B) Serum anti-STm IgG2a (left) and IgG1 (right) titres were quantified from mice infected with STm or STm and Nb for 32 days. (C) Serum anti-NES IgG1 titres were quantified from mice infected for 10-32 days. Infections with STm and Nb were intraperitoneal and subcutaneous, respectively. The Mann-Whitney Test was used to compare the significance between groups. Groups contained 4-6 mice and experiments were performed at least twice for each time point except day 32, which was done once. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

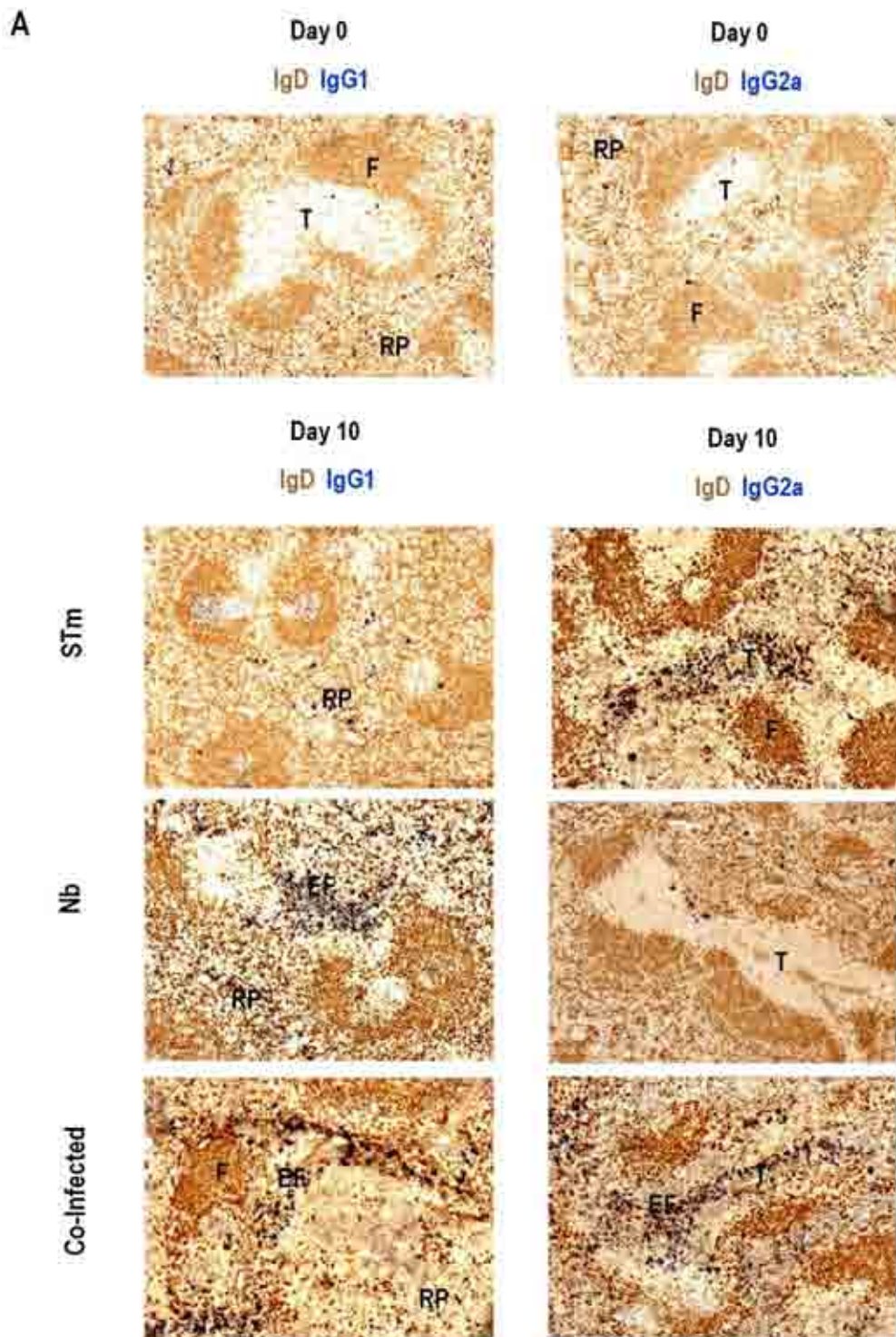


Figure 5.19

Dual infected mice induce both a IgG1 and IgG2a antibody response at day 10 post infection: BALB/c mice were infected with either 5×10^5 STm, 500 L3 Nb or both for either 5, 10, 18 or 32 days. (A) Spleen sections were double-stained for IgD and IgG1 (left) or IgD and IgG2a (right) and switching assessed at day 10 post-infection. Blue cells represent EF plasma cells. F = Follicles, RP = Red Pulp, T = T-Zone, EF = Extra Follicular. Images are representative of groups containing 6 mice from at least two independent experiments. Infections with STm and Nb were intraperitoneal and subcutaneous, respectively.

B

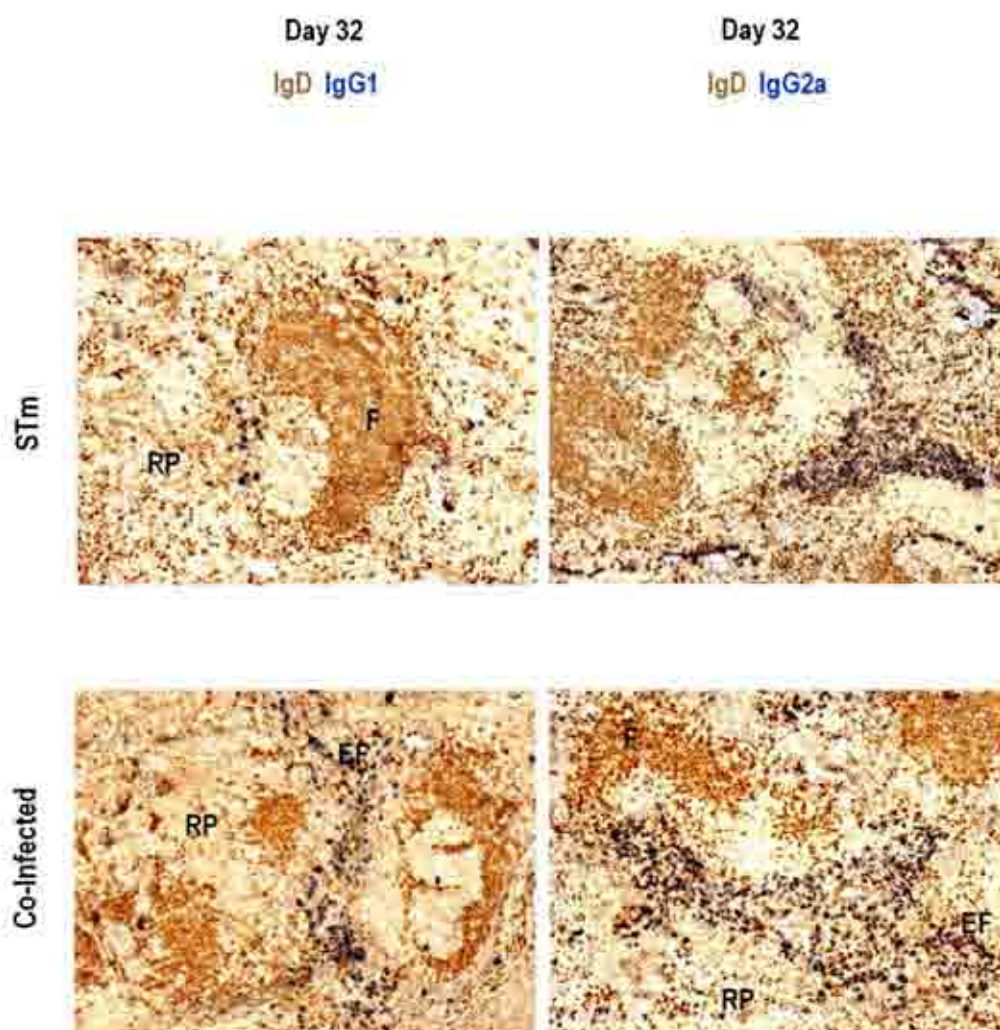


Figure 5.19

Dual infected mice induce both a IgG2a and IgG1 antibody response at day 32 post infection: BALB/c mice were infected with either 5×10^5 STm, 500 L3 Nb or both for either 5, 10, 18 or 32 days: (B) Spleen sections were double-stained for IgD and IgG1 (left) or IgD and IgG2a (right) and switching assessed at day 32 post-infection. Blue cells represent EF plasma cells. F = Follicles, RP = Red Pulp and EF = Extra Follicular. Images are representative of groups containing 6 mice from at least two independent experiments. Infections with STm and Nb were intraperitoneal and subcutaneous, respectively.

Since GCs are a late feature of the STm response (Cunningham et al., 2007) the GC response was examined in co-infected mice. Spleens were sectioned and stained by immunohistochemistry for IgD (to identify follicular B2 cells) and CD3 (to identify T cells) and follicular regions that were IgD⁻ and CD3^{lo} (GCs) were assessed (Fig 5.20). In mice infected with Nb-only GCs were present by day 10 post-infection and as expected, GCs were largely absent from STm-only infected mice and surprisingly, also in co-infected mice. GCs were however, detected at day 32 in both groups. This suggested the GC response in co-infected mice mirrored the STm profile.

These data show co-infection with STm which requires Th1 immunity and Nb which requires Th2 responses, has a negative impact on the host's ability to control either pathogen compared to a single infection. Assessment of immune responses did not identify any obvious correlates for this increased host susceptibility as polarisation to both Th1 and Th2 appeared unchanged. Interestingly, the antibody response was maintained to both pathogens, but was reduced upon co-infection. Furthermore, features of the antibody response were distinct after co-infection as demonstrated by the induction of high titres of both IgG1 and IgG2a to both Nb and STm, respectively.

5.5 Primary Infection with *N. brasiliensis* impairs the efficacy of porin immunisation in a STm vaccine model

As described in Chapter 4 immunisation with porins induces early control of subsequent STm infection which is dependent upon the presence of OmpD specific antibody. Since Nb infection could negatively impact on clearance of STm from the tissues, whether Nb infection also impaired vaccine mediated control of STm infection was investigated. BALB/c mice were infected with 500 L3 Nb larva for 19 days before immunisation with porins for 11 days prior to challenge with 5×10^6 STm for 5 days (Fig 5.21A). As a control, mice were either infected or immunised with Nb or porins respectively, for 11 days prior to challenge with STm. On reflection, a better control group for Nb may have been to infect mice with Nb for 30 days before STm infection to match the Nb-infected and porin-immunised group (Fig 5.21).

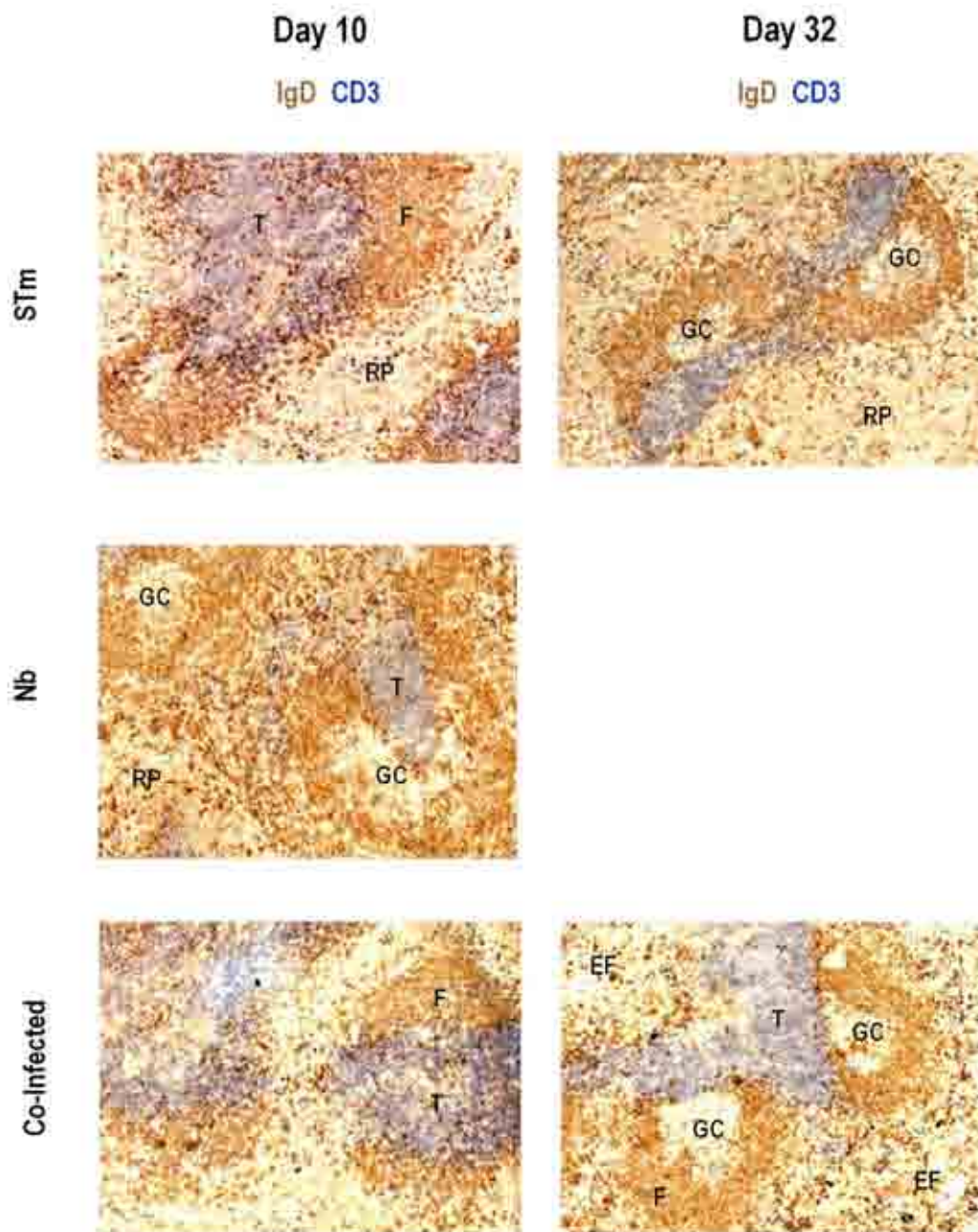


Figure 5.20

Dual infected mice induce a late GC response: BALB/c mice were infected with either 5×10^5 STm, 500 L3 Nb or both for 10 or 32 days. Spleen sections were double-stained for IgD (brown) and CD3 (blue), and germinal centre (GC) responses assessed. GC were identified as areas of the IgD⁺CD3⁻ follicle which were IgD⁺. F = Follicles, RP = Red Pulp and T = T-Zone. Images are representative of groups containing 6 mice from at least two independent experiments. Infections with STm and Nb were intraperitoneal and subcutaneous, respectively.

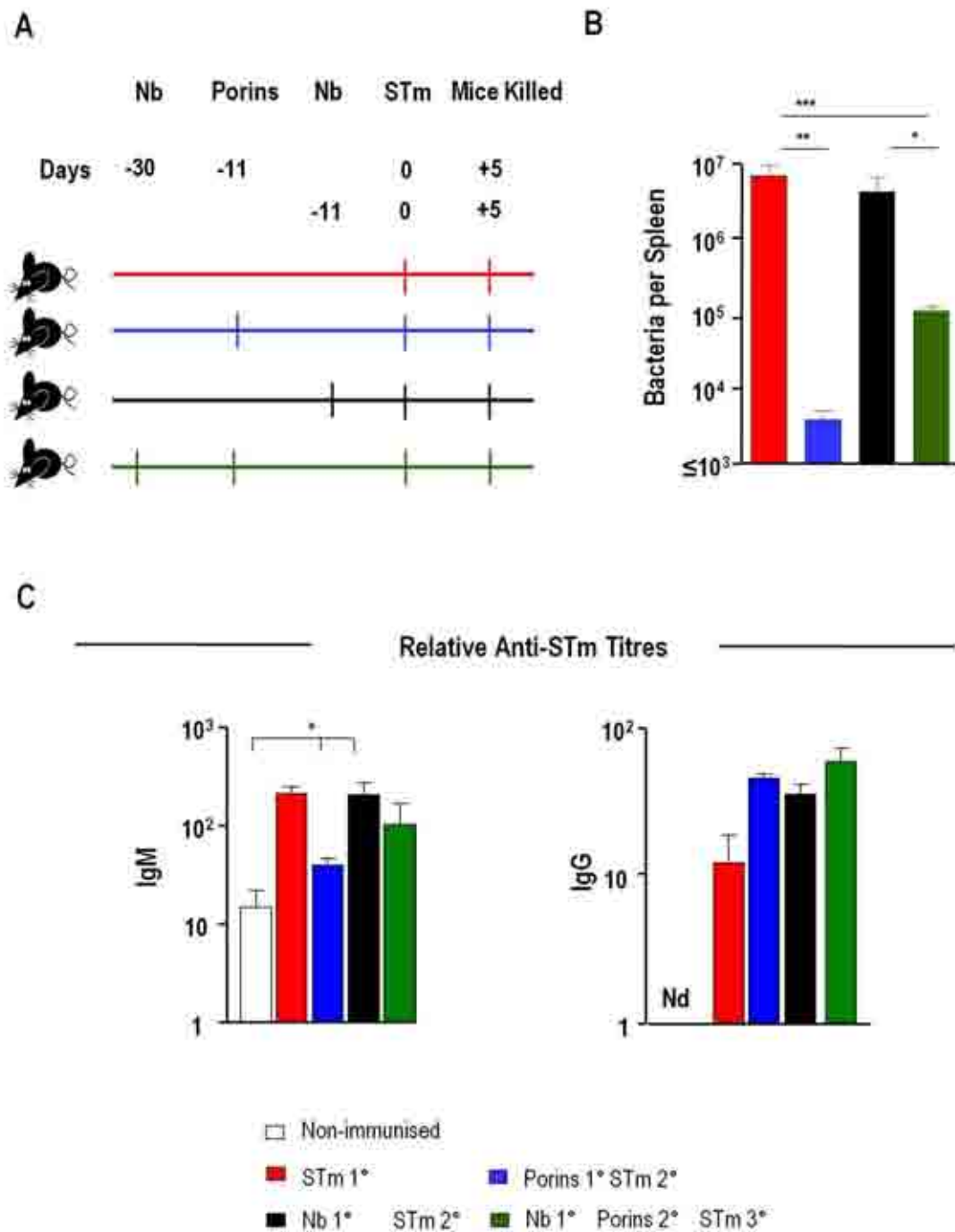


Figure 5.21

Nb priming impairs the efficacy afforded by porin immunisation upon subsequent challenge with STm: (A) BALB/c mice were infected and/or immunised at the time-points outlined with 500 L3 Nb (s.c) or 20 μ g porins (i.p) respectively, prior to challenge with 5×10^5 STm. (i.p) (B) Splenic bacterial CFU were quantified at day 5 post STm infection and (C) serum anti-STm IgM (left) and IgG (right) titres were assessed. The Mann-Whitney Test was used to compare the significance between groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Quantification of the splenic bacterial burden revealed that as expected, mice infected with Nb prior to STm challenge had a comparable bacterial load to NI mice at this time-point. Both porin-immunised and Nb infected-porin-immunised mice had a significantly lower bacterial load compared to NI mice. However, Nb infected-porin immunised mice had a 30-fold greater bacterial burden compared to porin-immunised mice. This suggested Nb infection impaired the benefit afforded by porin vaccination. Interestingly, the anti-STm IgM and IgG responses were broadly equivalent between the groups, although porin-immunised STm-infected mice had a marginally lower IgM response yet the greatest level of protection (Fig 5.21B). This suggests that since there is antibody present but less protection in the Nb infected-porin-immunised mice, that the difference is likely to involve killing of STm rather than antibody, and the profile of responding macrophages should be considered.

5.6 Discussion

In this study we show that an infectious history with Nb or STm prior to subsequent challenge with STm or Nb respectively, or co-infection with both these pathogens can impact on the development of immunity and control to each respective pathogen. Furthermore, a history of Nb infection markedly impacted upon the success of vaccination with porins. Although the concept of dual-infection is not novel, previous studies have largely focussed on the effects of concurrent infection when both pathogens occupy the same anatomical site (Chen et al., 2005; Mansfield et al., 2003). Whilst helminths and enteric bacteria may colonise the same sites, this may not necessarily occur at the same time. For STm colonisation occurs through the gut, whereas for Nb, the gut is at the end stage of infection and is important for transmission to new hosts. For reasons of time and simplicity it was not possible to study all combinations of antigen encounter which is an important future aim. Compartmentalisation of pathogens was chosen because severity of STm infection is associated with systemic infection, and it is clear that adaptive cells can migrate within a few days of immunisation to secondary lymphoid tissues or effector sites such as the liver, after primary immunisation with alum-precipitated proteins or with STm

(Kool et al., 2008; Luther et al., 2007; Serre et al., 2009)(and unpublished observations from work by J. Hitchcock).

Co-infection of STm and Nb has been studied previously (Sitepu et al., 1984), but this study did not address the impact of STm or Nb relative to each other or their influence on the immune response, but the outcome of infection to STm or Nb in mice selectively reared for high or low immune responsiveness to *Nematospiroides dubius*. This revealed high-immune responders (mice bred which display increased resistance to infection) of *N. dubius* were more susceptible to STm and more resistant to NB. Furthermore, it has been demonstrated that primary infection in rats with flukes (*Fasciola hepatica*) exacerbated the severity of subsequent infection with *S. Dublin* (Aitken et al., 1978), whilst primary *Ostertagia ostertagia* (Nematodes which infect cattle) infection did not influence subsequent challenge with *S. Dublin* in cattle (Aitken et al., 1984). The immunological detail from these earlier studies is limited, largely since they did not specifically aim to examine the immune response to STm and Nb relative to each other, and as such our data provides the first example of such a study.

More recent STm and helminth co-infection studies have had inconsistent findings. For instance, primary infection of pigs with the strongyloide nematode *Oesophagostomum* spp, before challenge with STm, revealed no enhancement of STm infection or effect on the helminth using a range of helminth doses (Steenhard et al., 2006) however, STm infection was enhanced at the highest dose of helminth given (2500 worms given 14 times over a period of 7 weeks) (Steenhard et al., 2002). These studies suggest that helminth infection may not always influence the course of subsequent STm infection.

In the model used in these studies Nb is expelled 6-7 days before mice were challenged with STm. Despite this, the influence of Nb infection remains detectable more than 40 days after initial Nb infection. This suggests that the increased susceptibility to STm does not require a continual helminth infection but can be driven by pre-exposure to Nb. This is not surprising, since infections with Nb can elicit persistent effects in the host that remain long after worms are expelled, such as Th2 differentiation or

effects on macrophages, this is likely in part, at least to be related to the deposition of large amounts of antigen by parasite moulting and secretions (Arizono et al., 1996; Marsland et al., 2008), and Nb antigen is likely to persist for longer than worms are detected.

In contrast to this persistent effect of Nb on STm clearance, our data also indicates that after host resolution of STm infection the influence on Nb clearance was largely absent, with any delayed worm expulsion requiring STm to still be present in the host. It was demonstrated that direct interaction between enteric microflora (including STm) and macrofauna (intestinal dwelling helminths) can be beneficial for persistence of *Trichuris muris* in the host through impairment of Th2 immunity (Hayes et al., 2010). Our model shows that even in the absence of direct interaction, bacterial and helminth co-infection can have a marked effect on host immunity, impairing control of either pathogen.

In addition to examining the impact of infectious history, we found that simultaneous co-infection markedly affected clearance of each pathogen. This reflects earlier studies which demonstrated synergistic interactions between nematodes and bacteria can produce significant disease and pathology relative to single infection (Mansfield et al., 2003). Under all conditions assessed Nb infection did not influence STm clearance until after the first week of infection, when adaptive immunity is required. It was possible that the presence of two strongly polarising challenges could lead to an inappropriately activated immune response, resulting in ineffective immunity to each pathogen, yet Th1 responses were largely normal in co-infected mice, as were Th2 responses. This was surprising since natural exposure to helminths has been shown to dampen Th1 responses to other infections (Correale and Farez, 2009; Zacccone et al., 2003). Conversely, several malarial-helminth co-infection models have shown helminth elicited Th2 responses are reduced upon co-infection (Buendia et al., 2002; Hoeve et al., 2009). Nevertheless, in other systems Th1 and Th2 responses have been shown to develop in parallel in the same LN (Toellner et al., 1998) and DC co-pulsed with Th1 and Th2 antigens can induce appropriate polarised Th responses (Cervi et al., 2004).

It is possible that the T cell response may be defective in aspects other than polarisation, such as the impaired migration of T cells to sites of infection, inefficient or inappropriate interactions between T cells and macrophages, or macrophages failing to respond to T cell instructions. This may be because of different states of macrophage activation due to the influence of Nb exposure (discussed below). Due to time constraints we have not looked at in vitro interactions between T cells and macrophages, and this is currently being addressed by Dr. William Horsnell in Cape Town. Furthermore, we did not look at chemokines or migration profiles, and since differences in chemokine expression can further define Th1 and Th2 polarity (Serre et al., 2008) it is an important area for future study.

Effective resolution of Nb requires IL-13 signalling and activation of the STAT6 pathway (Barner et al., 1998; McKenzie et al., 1999; Urban et al., 1998) which is associated with induction of goblet cell hyperplasia. In the main, the IL-13 response in STm-primed-Nb-infected mice and co-infected mice was relatively normal despite the pronounced delay in worm expulsion. Surprisingly, these mice did show a clear reduction in goblet cell hyperplasia. Thus, it could be speculated that impaired Nb expulsion in co-infected mice and STm-primed Nb-infected mice may be related to this reduced mucus response. It would be interesting to assess whether the delay seen in Nb expulsion is exacerbated in the absence of IL-13 signalling. This may help identify the mechanism for the reduced mucus response upon co-infection.

In addition to the reasons already listed, other mechanisms may be at play, such as differences in macrophage populations and the influence of regulatory T cells. Regulatory T cells (Groux et al., 1998; Levings et al., 2001) and B cells (Yanaba et al., 2008) are potent sources of IL-10. In this study, the IL-10 profile varied depending upon the antigen combination but generally having Nb increased the levels of IL-10 from splenocytes. Due to time constraints detailed assessment of regulatory cells was not possible but is important for future analyses. Indeed, helminth induced T cell regulatory populations can suppress Th1 proliferative responses in vitro (Tetsutani et al., 2009; Wammes et al., 2010) and

regulatory B cells can suppress T cell proliferation (Gray and Gray, 2010). IL-10 protein levels were lower in co-infected mice at day 10 relative to Nb-only infected mice and it is possible the reduced IL-10 in co-infected mice may be important for the observed phenotype, possibly through the action of regulatory cells. Therefore, identification of the source of IL-10 is important for future studies.

The antibody profile of Nb-primed and STm infected mice showed induction of both anti-STm and anti-NES IgM and IgG antibody. Furthermore, the anti-STm antibody profile in co-infected mice showed switching to both IgG1 and IgG2a, whereas mice infected with STm-only showed induction of IgG2a, but not IgG1. Antibody is dispensable for clearance of primary STm (Mastroeni et al., 2000; McSorley et al., 2000; Mittrucker et al., 2000) and since Th1 priming to STm was normal, this suggests the impaired capacity to clear STm may involve inappropriate polarisation of other cells, such as macrophages. Macrophage function can vary significantly depending on the T cell response induced. In this study there was insufficient time to assess macrophage populations in detail but this is an important future aim since selective polarisation to CAM and AAMs can influence pathogen expulsion (Zhao et al., 2008). It is possible that AAMs induced in response to Nb impair the host's ability to induce an effective CAM response to STm. Furthermore, it is unclear whether AAMs or CAMS can be reprogrammed to a classical or alternative phenotype. Indeed, AAMs have been shown to revert to a phagocytic role upon exposure to *Leishmania mexicana* (Mylonas et al., 2009). However, in the presence of *Neisseria meningitidis* the AAM phenotype is stable (Varin et al., 2010) suggesting this plasticity may be pathogen-specific.

Nb infection significantly reduced the efficacy of porin immunisation against STm. Immunomodulatory effects of helminths on vaccination against bacterial pathogens have been documented before in response to immunisation with cholera toxin (CTB) (Harris et al., 2009), and tetanus toxoid vaccination (Sabin et al., 1996). Antibody to STm can function through the activation of complement, neutralisation or opsonisation. In the mouse model antibody-mediated opsonisation and cell killing is probably more

important than cell-free killing by complement (Siggins et al., 2011). Antibody was induced in all groups of mice, but anti-IgM was marginally lower in porin-immunised and STm-infected mice, which had the greatest level of protection, whilst anti-STm IgG titres were similar in all groups. This suggests that the reduced efficacy of porin-vaccination is likely to be independent of the ability to induce antibody, but may be related to the ability of macrophages and neutrophils after Nb-infection to kill the bacteria, due to alterations in the macrophage population. This is supported by a recent opsonisation experiment carried out by Dr. William Horsnell in Cape Town in which STm opsonised with STm-specific sera had a markedly impaired capacity to control infection in mice previously infected with Nb (data not shown) compared to naive mice. This suggests that cell-dependent complement function needs to be investigated.

Simultaneous exposure to helminths and other pathogens are common in developing countries. Given the striking phenotype we found and the knowledge that both NTS and hookworm infections are clinically relevant and endemic to SSA, it is surprising that studies have not addressed these pathogens together in the context of host immunity. We have demonstrated that infectious history can imprint the host response to subsequent immune challenges, and furthermore, helminth infection impaired the efficacy of vaccination demonstrating a complicated relationship between the host and pathogen. This has important implications for vaccine efficacy and administration, and we suggest immunological background of target populations and their infectious history should be taken into careful consideration, when designing mass vaccine strategies.

6.0 Final Discussion and Future Work

A major obstacle in developing a suitable vaccine for NTS has been a poor understanding of the nature of immunogenic moieties associated with protection. Micro-organisms contain a mosaic of antigens and as we have shown here the elicited immune response can be complex. However, surface components are at the interface between the bacteria and the host and represent ideal targets for protective immunity. Thus, a better understanding of immunity to infection, and the processes of immunity, is revealed through studying responses to microbial antigens. Our studies on the immune response to core bacterial structures embedded in the OM or attached to the surface of STm, has important implications in general for immunity against GN bacteria as these structures are largely conserved.

T cells are important for clearance of STm infection within macrophages, but in SSA NTS infections also usually manifest in the blood, and predominantly in children under the age of two when anti-STm antibody is absent. Antibody can contain bacteraemia and restrict the level of initial colonisation (Cunningham et al., 2007; Mastroeni et al., 1993; McSorley and Jenkins, 2000). Therefore, the benefit afforded by humoral immunity can be immediate. We found that despite the induction of potent antibody responses, the capacity of antibody to influence STm infection was not apparent upon immunisation with either flagellin or LPS (data for LPS not shown), whilst immunisation with porins conferred substantial benefit. The lack of antibody-mediated protection by anti-sFliC antibody in the mouse was supported by our recent work on human responses to NTS in collaboration with Dr. Cal MacLennan (MacLennan et al., 2010). In this study, antibodies to flagellin had no clear protective role against infection whilst antibody to LPS could inhibit bacterial killing. However, antibodies to porins could direct complement-mediated killing of STm.

Optimum killing of some GN bacteria requires participation of the terminal components of the complement system through formation of a pore, termed membrane attack complex (MAC) (Peitsch and Tschopp, 1991). It is likely that structures distal from the cell wall such as flagella or LPS O-chains are

not efficient targets of antibody, possibly because they do not allow MAC to develop sufficiently close to the body of the cell. Indeed, reducing the length of the O-antigen is sufficient to trigger bactericidal killing by complement and can impact upon interactions between macrophages and STm (Murray et al., 2006). Nevertheless, we have identified that antibody to surface-localised OmpA is not protective in the mouse model, and antibody to the exposed trimeric autotransporter adhesin, SadA can induce limited antibody-mediated protection in the mouse model, but is not an ideal dominant protective antigen (data not shown). Therefore, it is possible that additional factors other than proximity to the cell wall are involved in determining whether antibody responses will be beneficial. In terms of OmpA it is likely that its abundance in the OM may serve as an immune decoy.

In terms of antibody function, complement fixing bactericidal antibody has been documented to be protective in children with NTS in SSA (MacLennan et al., 2008). However, the benefit afforded by antibody in STm is not limited to its capacity to induce bactericidal killing, as the ability of neutrophils to induce oxidative burst and thus, killing of STm has been shown to correlate with the presence of anti-STm opsonising antibody and C3 deposition on bacteria, indicating a requirement for both antibody and complement (Gondwe et al., 2010). This suggests that the ability to induce protective antibody is likely to be more important rather than the mechanism by which antibody may act. Although it is important to note that cell-free complement-mediated killing is not effective in the mouse unlike humans and antibody responses in the mouse may understate the effectiveness of antibody in humans (MacLennan et al., 2008; Siggins et al., 2011) and this should be considered when interpreting and reaching conclusions from antibody-related studies.

T cells are rapidly activated after STm infection but they are not effective at controlling infection during the first week, and only contribute to immunity once infection is already well established. Because the clearance of STm is protracted in the face of potent T cell activation this suggests that it may be unwise to focus simply on vaccines that evoke CD4 T cell-mediated benefit without antibody. Furthermore,

developing a vaccine to drive a particular T cell response to STm may be unnecessary, as we found that the T-helper response to sFliC and by implication other subunit vaccines, may change during the course of infection if encountered in a different context. This T cell flexibility after infection of vaccinated animals has been described in other systems (Divekar et al., 2006; Wang and Mosmann, 2001). Therefore, for NTS vaccination inducing T cell help for antibody switching is more important than directing a particular effector T cell subset.

Immunisation with porins could moderate infection with STm via the early induction of antibody. Furthermore, porins have the additional capacity to induce T cell help which makes it an ideal target for the basis of a subunit vaccine. Flagellin has been an attractive and ideal candidate for protective immunity due to its auto-adjuvant properties, but it may not be an ideal choice for a subunit vaccine against STm due to its failure to promote protective antibody responses. Nevertheless, it is tempting to speculate that rather than focussing on the immunomodulatory effects of flagellin when given alone, it may be wise to exploit flagellin as an adjuvant when conjugated to known protective antigens against NTS. Therefore, it seems plausible that a porin-flagellin conjugate vaccine may prove to be highly efficacious against STm. This has the potential to induce an early protective anti-porin antibody response which could be augmented by flagellin, and this may limit colonisation and restrict bacterial dissemination before flagellin-specific T cells can clear residual intracellular infection.

Many pathogens gain access to the host after interactions with the mucosal epithelium followed by penetration of the mucosal barrier, typical of a natural STm infection. The mucosal barrier provides a major site for initial antigen presentation and T cell activation. Therefore, it seems plausible that vaccine design for STm should strive to induce mucosal antibody responses which may act as soon as STm infiltrates the intestinal mucosa and this may act to restrict the immediate dissemination of STm. Indeed, secretory IgA has the capacity to protect mice against STm (Michetti et al., 1992). Nevertheless, we found that after intraperitoneal flagellin immunisation bacteria were still able to colonise systemic sites

following oral infection and this was only modestly reduced in non-lymphoid tissues. Furthermore, we have recently found that colonisation of aflagellated and flagellated STm after oral infection is comparable (data not shown). Thus, we propose mucosal immunity may not be important for immune responses to flagellin in isolation. Although this is not surprising since flagellin is compartmentalised by intracellular bacteria which reduce antigen availability and presentation by antigen presenting cells (Alaniz et al., 2006).

To assess whether the route of immunisation can impact on the efficacy of porin immunisation we have altered the route of administration, and have found no differences in bacterial colonisation upon subsequent challenge of mice (intraperitoneal) immunised with porins intravenously or intraperitoneally (data not shown). In the future we are hoping to look into oral immunisation or porins followed by oral challenge with STm. Furthermore, data from our lab has found immunisation of mice with the Vi antigen sub-cutaneously, intravenously or intraperitoneally does not substantially influence the antibody response (data not shown). This suggests that antibody responses to un-conjugated subunit fragments may not be influenced by the initial route of administration due to their capacity to invoke systemic responses. Indeed, Vi antigen is administered systemically and induces protective immunity. Nevertheless, we acknowledge that our studies need to encompass oral infection with STm.

STm is highly complex and we have focussed on inducing protective T and B cell responses, but this may not be sufficient when the host is harbouring an infection with an unrelated pathogen. Ultimately, in terms of killing of STm we may have to consider whether the appropriate macrophage population is induced. Our dual infection study implies that the macrophage population is altered upon co-infection and this can impact on the capacity to phagocytose STm (data not shown). Furthermore, when identifying candidate vaccines the combination in which vaccines are administered should be considered. Co-immunisation of STm and sFliC negatively impacted on the benefit afforded to STm upon subsequent challenge, and more striking, immunisation with porins in mice previously infected with

N. brasiliensis significantly reduced the efficacy of vaccination. Since helminths and invasive NTS are clear problems in SSA, this is likely to mean such factors need to be considered before vaccination against NTS in endemic regions.

In closing, in terms of vaccines against invasive STm an effective vaccine should encompass induction of protective antibody responses, T cell responses (induction of Th1 may not be a prerequisite) and appropriate macrophage populations. Identifying targets and the mechanisms by which they act are the first step. Porins are ideal candidates because they have the capacity to reduce bacteraemia and more importantly, invoke T cell help which has the capacity to confer long-lasting benefit.

Future Directions

The flagellin project has been advanced by Dr. Adriana-Flores Langarica. In particular, aspects of the Th2 response associated with flagellin immunisation have begun to become better understood, and assessment of gut-associated lymphoid tissues post-oral immunisation with flagellin has revealed discrete changes in T cell populations. Furthermore, studies on porin immunisation have been advanced by Dr. Jennifer L. Marshall to investigate other protective antigens. Finally, Dr. William Horsnell has continued to study the dual infection model in Cape Town, and begun to characterise the balance between AAM and CAM and evaluate their resistance/sufficiency to infection during co-infection. This has already revealed impaired opsonisation may be critical for the delayed clearance of STm and Nb upon dual challenge. Nevertheless, multiple questions still arise from each study and in particular I would like to assess the following aspects:

Immune response to flagella and flagellin:

Studies using the aflagellated strain of STm did not find any differences between the flagellated and aflagellated strain of STm in terms of bacterial colonisation upon primary infection when given intraperitoneally, and now by Dr. Adriana-Flores Langarica upon oral challenge. I would like to further

assess the immune response directed against the aflagellated strain to ascertain properties of flagella, especially during secondary responses. Specifically, it would be interesting to gain insight into STm infection in the absence of flagella expression in a TLR5 deficient mouse.

Flagella is a known virulence factor in *P. aeruginosa* infections (Arora et al., 2005; Montie et al., 1982), and antibody induced to flagella is protective against pneumonia (Campodonico et al., 2010). In contrast, for STm the data is conflicting. Some studies suggest expression of flagella is critical for virulence (Fournier et al., 2009; Zeng et al., 2003) whilst other studies have demonstrated that neither flagella nor components of the flagellar synthetic apparatus are necessary for pathogenicity (Carsiotis et al., 1989; Lockman and Curtiss, 1990; Schmitt et al., 2001). These latter results are consistent with our study with the aflagellated strain of STm. Attempts to prime mice with flagella and subsequently challenge with STm or aflagellated STm to assess whether flagella can protect relative to flagellin have been made, but this has been held back due to limitations in obtaining a truly-pure preparation of polymeric surface-isolated flagella, and it is an area I would like to further pursue.

Antibody Responses to STm:

Infection with STm is orally acquired and protection against ST is mediated by mucosal and serum antibodies (mucosal IgA and serum IgG), as well as cell-mediated immunity (Salazar-Gonzalez et al., 2004). Since porins can confer benefit it would be interesting to assess whether oral immunisation can confer additive benefit upon subsequent oral STm challenge.

The impact of Dual Infection:

Infectious history or co-infection may influence the specificity of the T cell response, and a proportion of the effector T cells may be specific for the primary pathogen. Moreover, the ratio of effector T cells in co-infected mice may be altered. Thus, I would like to assess the antigen-specificity of the responding T cells to ascertain whether a mixed T cell response is induced upon co-infection.

Furthermore, during co-infection STm was administered intraperitoneally and Nb sub-cutaneously to assess the influence of infection when these pathogens occupy distinct niches. Intestinal sites are initially colonised upon oral administration before systemic dissemination, at least after infection of mice with strains of STm that are virulent. It is important we examine the immune response in Nb-infected mice subsequently infected orally with virulent STm to see potential interactions in their native immunological niches (gut) and assess whether mucosal control is influenced by infectious history. Additionally, sites of interaction between the two pathogens where T cell priming may be initiated should be investigated, such as the mediastinal and mesenteric lymph nodes. Looking at these sites may identify site-specific differences in DC populations.

Appendix A – Buffer Recipes

This Appendix contains recipes for all the buffers and reagents used in this project which have not already been outlined. All buffers were filter sterilised with a 0.45 µm filter (Millipore, MA, USA) prior to use and stored at 4°C, unless stated otherwise.

Buffers used for protein purification (Discussed in 2.2.3)

The following buffers were used for purification of porins from STm and ST by Dr. Cristina Gil-Cruz. All buffers were prepared in MilliQ water.

Wash Buffer

50 mM Tris.HCl (pH 7.4)

Solubilisation Buffer (For Solubilisation of Inner Membrane)

50 mM Tris.HCl (pH 7.4)

2% Sodium Dodecyl Sulphate (SDS)

Nikaido Solubilisation Buffer (For Solubilisation and Extraction of the Outer Membrane)

50 mM Tris.HCl (pH 7.4)

1% Sodium Dodecyl Sulphate (SDS)

3.25 mM EDTA

0.05% β2-mercaptoethanol

Nikaido Purification Buffer (For Purification of OMPs using the Sephacryl S-200 Column)

50 mM Tris.HCl (pH 7.4)

0.5% Sodium Dodecyl Sulphate (SDS)

3.25 mM EDTA

200 mM NaCl

Buffers used for Cytokine ELISAs (Discussed in Section 2.8.3)

The following buffers were used for cytokine ELISAs. They were prepared in 1X PBS which was made up in MilliQ water.

1 X Phosphate Buffered Saline (PBS) pH 7.4

8.0 g NaCl

0.2 g KCl

1.44 g Na₂HPO₄

0.24 g KH₂PO₄

Weigh out chemicals and dissolve in 900 ml of MilliQ water and adjust pH to 7.4. Bring up to final volume of 1 L.

ELISA Blocking Buffer

20 g Powder Milk

0.2 g NaN₃

Weigh out chemicals and dissolve in 900 ml of 1xPBS, bring up to final volume of 1L.

ELISA Coating Buffer

0.2 g NaN₃

Weigh out NaN₃ and dissolve in 900 ml of 1xPBS, bring up to final volume of 1 L and store at room temperature.

ELISA Dilution Buffer

10 g BSA

0.2 g NaN₃

Weigh out chemicals and dissolve in 900ml of 1xPBS, bring up to final volume of 1 L.

ELISA Washing Buffer (20 X)

20 g KCl

20 g $\text{KH}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$

800 g NaCl

50 ml Tween-20

100 ml 10% NaN_3

Weigh out chemicals and dissolve in 4.5 L MilliQ water. Add 50 ml of Tween-20 and 100 ml of 10% NaN_3 . Bring final volume to 5 L and store at room temperature. Dilute 1:20 in MilliQ water for a 1X working concentration.

ELISA Substrate Buffer (for alkaline phosphatase conjugates)

0.2 g NaN_3

97 ml diethanolamine

0.8 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$

Dissolve chemicals in 700 ml of MilliQ water. Add 97 ml of liquefied diethanolamine and adjust pH to 9.8. Bring the final volume to 1L.

Buffers used for Antibody ELISAs (Discussed in Section 2.10)

The following buffers were used for antibody ELISAs. They were made just prior to use and not stored long-term, except for the Carbonate Coating Buffer which was stored at 4°C.

ELISA Carbonate Coating Buffer

0.83 g Na_2HCO_3

1.42 g NaH_2CO_3

0.1% NaN_3

Weigh out chemicals and dissolve in 400 ml MilliQ water, bring the final volume to 500 ml.

ELISA Blocking Buffer

1% Bovine Serum Albumin (BSA)

Weigh out BSA and dissolve in 400 ml of 1xPBS, bring the final volume to 500 ml.

ELISA Wash Buffer

0.05% Tween-20 in 1X PBS

ELISA Dilution Buffer

0.05% Tween-20

1% Bovine Serum Albumin (BSA)

Weigh out BSA and dissolve in 400 ml of 1xPBS, add Tween-20 and bring the final volume to 500 ml.

Buffers used for Immunohistochemistry (Discussed in Section 2.11.1)

The following buffers were used for immunohistochemistry and were stored at room temperature.

Tris Buffer pH 7.6

1.0 L of 200 mM Tris Base

1.5 L of 154 mM Physiological NaCl

1.0 L of 0.1 N HCl

Tris Buffer pH 9.2

As above but at pH 9.2. Initially pH 200 ml to give pH 9.2 using HCl (dropwise with 1M or greater) and make up to desired volume with NaCl.

Peroxidase Substrate

Dissolve a single 3-3'-diaminobenzidine tetrahydrochloride (DAB) tablet (Sigma Aldrich, Dorset, UK) in 15 ml Tris Buffer pH 7.6. Filter to 10 mls and add 1 drop of hydrogen peroxide (Sigma Aldrich). A single drop was added to each section until the desired level of positivity was reached, whereupon slides were washed.

Alkaline Phosphate Substrate

Alkaline phosphatase activity was detected using naphthol AS-MX phosphate and Fast Blue salt. Dissolve 8 mg of Levamisole (Sigma Aldrich) in 10 ml Tris buffer (pH 9.2) to block endogenous phosphate activity. In a fume hood dissolve 4 mg of naphthol AS-MX phosphate (Sigma Aldrich) in 380 μ l dimethyl-formamide in a glass bottle. Add this to the middle of the levamisole solution. Add 10 mg 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.

Buffers used for Periodic Acid Schiff's (PAS) Staining (Discussed in Section 2.11.2)

Reagents used for PAS Staining

Fixative

40 ml Formaldehyde

Add 40 mls Formaldehyde to 960 ml 1X PBS and store at room temperature.

Periodic Acid Solution

0.1 g Periodic Acid in 100 ml dH₂O

Schiff's

1.0 g Parasaniline (Sigma Aldrich)

1.0 g NaCl

2.0 g Activated Charcoal

20 ml of 1 N HCl

Dissolve reagents in 800 ml 1X PBS and add 20 ml HCl. Bring up to final volume of 1 L and store at room temperature.

Mayers Haemalum

1.0 g Haematoxylin (BDH, VWR International, PA, USA)

50 g Ammonium Alum (Merck Chemicals, Nottingham, UK)

0.2 g Sodium Iodate (Merck Chemicals, Nottingham, UK)

1.0 g Citric Acid (Merck Chemical, Nottingham, UK)

50 g Chloral Hydrate (BDH, VWR International, PA, USA)

40 ml Formaldehyde (Sigma Aldrich)

Dissolve reagents in 800 ml 1X PBS and add 40 ml formaldehyde. Bring up to final volume of 1 L and store at room temperature.

Appendix A1 – PAS Staining

This Appendix contains the method used by the Department of Histology, Groote Schuur Hospital, Cape Town for PAS staining of jejunum sections as outlined in 2.11.2 and discussed in Chapter 5.

Preparation of sections for PAS Staining

Wax Embedding

Jejunum tissue was stored in 4% phosphate-buffered formalin and tissues were dehydrated in an automated processor before embedding in wax as indicated:

70 % alcohol		30 min
96 % alcohol	(twice)	45 min
100 % alcohol	(twice)	45 min
Xylol	(twice)	60 min
Wax (55°C-60°C)	(twice)	45 min with vacuum

Wax-embedded tissues were sectioned at 5-7 µm with a microtome, mounted onto glass slides and fixed by incubation at 37°C overnight. Slides were heated at 60°C for 2-18 hours to remove the wax and sections were then re-hydrated and stained in PAS as follows:

Periodic Acid Schiffs (PAS) Staining

Xylol (twice)	30 min
100 % alcohol	1 min
96 % alcohol	1 min
70 % alcohol	1 min
Water	1 min

Once the sections were hydrated in water they were and oxidised in 1% aqueous periodic acid for 5 – 10 min. They were then washed for 10 min in running H₂O, and stained in Schiff reagent for 15 minutes, washed for 5 minutes, counter-stained in Mayer's haemotoxylin, washed for 5 minutes, dehydrated and mounted with cover slips.

Appendix B – DNA Specific Techniques Used

This Appendix details methods used to generate and screen the sFliC Δ TLR5 construct (Described 2.2.3.2), and the technologies used by Dr. RA Kingsley to generate the STm strains used.

Appendix B1 DNA Manipulation

B1.1 Electrotransformation

Plasmid DNA harbouring mutations of interest were transformed into electrocompetent SL3261 cells. Briefly, a single colony of SL3261 was incubated overnight in LB at 37°C with aeration (180 rpm). Following incubation the culture was diluted 1:10 in fresh LB and incubated until mid-exponential phase (OD₆₀₀ 0.6-0.8). Cells were harvested at 5000 x g for 10 minutes and washed in ice-cold MilliQ water before further centrifugation. Cells were then washed in 10% sterile ice-cold glycerol and pelleted once again before being washed a second time in glycerol to obtain electrocompetent cells. Electrocompetent STm (10^8) was mixed with plasmid DNA (1 μ g) and chilled on ice for 1 minute. Cells were transferred to the bottom of a cold electroporation cuvette and placed between the electrodes of a BioRad Electroporator. The cuvette was pulsed once at 2.5 kV, set as 200 Ω and 25 μ F. LB was added and the suspension was transferred to an eppendorf and incubated aerobically at 37°C for 1 hour. Cells were plated on LB-agar supplemented with appropriate antibiotics, and incubated overnight at 37°C.

B1.2 GeneEditor Mutagenesis

The GeneEditor in vitro Mutagenesis System (Promega, Southampton, UK) was used to delete the putative TLR5 binding motif of flagellin (Andersen-Nissen et al., 2005). This system utilises two oligonucleotides: a selection and a mutagenic oligonucleotide. The selection oligonucleotide is provided to introduce mutations in the β -lactamase gene of the target DNA, and confer new resistance to the GeneEditor™ antibiotic selection mix. The mutagenic oligonucleotide is designed to create the desired mutation. The following primer was designed for use with the GeneEditor™ System P5'---AACGAAATCAACAACAACCTGCAGTCTGCTAACAGCACCAAC---3'. The primer was complementary

to sequences coding for residues 89-96 within the TLR5 ligating motif of flagellin. It was designed to incorporate a region of mismatch located in the centre through providing 17-19 perfectly matched nucleotides on either side or a deletion of the mismatched region.

Deletion of the putative TLR5 ligating motif was achieved as per manufacturer's guidelines. In brief, plasmid DNA pET22b⁺ FliC Xho1 was denatured under alkaline conditions allowing hybridisation of the mutagenic and selection oligonucleotides, enabling synthesis of the mutant strand which links the two oligonucleotides. The resulting plasmid was transformed into a repair minus (muts) E. coli strain (BMH 71-18) to avoid repair of the desired mutation, and incubated overnight in the GeneEditor™ antibiotic selection mix. Positive transformants were isolated and transformed into BL21 for subsequent protein expression (Invitrogen, Paisley, UK). The resulting construct (pET22b⁺ FliC Xho1 TLR5_{Δ89-96}) was isolated using the Wizard Plus SV Minipreps DNA Purification System (Promega, Southampton, UK), and the mutation confirmed by PCR under standard thermal cycle conditions.

B1.3 Construction of SL3261 Strains

B1.3.1 Construction of Phase-Locked STm Strains

Phase-locked strains of STm incapable of switching their flagellin genes were constructed as in (Ikeda et al., 2001) through disruption of the *hin* gene, which encodes a recombinase required for inversion of the DNA segment upstream of the *fliB/fliA* operon. Therefore, when the DNA is locked in the ON position FliB is exclusively expressed, whereas only FliC is expressed when DNA is locked in the OFF position. The SL3261 *aroA* ON (RAK 121) and SL3261 *aroA* OFF (RAK 123) strains were generated by transduction of the kanamycin resistance gene using bacteriophage P22 from SL3201 ON *fliC::kan* and SL3201 OFF::Kan respectively into SL3261 by Dr. RA Kingsley (Wellcome Trust, Sanger Institute, Cambridge). The source strains for the phase locked mutants were kindly provided by Dr. Alison O'Brien (Uniformed Services, University of Health Science). The genotype of the strains was confirmed by PCR.

B1.3.2 Construction of Aflagellated SL3261

A strain in which both the *fliB* and *fliC* genes were deleted and replaced with an aminoglycoside phosphotransferase gene (*aph*) and chloroamphenicol acetyltransferase (CAT) cassette, respectively was constructed using red recombinase methodology (Datsenko and Wanner, 2000) by Dr RA Kingsley (Wellcome Trust, Sanger Institute, Cambridge).

Briefly, the *fliC* mutation was constructed using oligonucleotide primers A and B to amplify the *cat* gene from pKD3. The *fliB* mutation was constructed using oligonucleotide primers C and D to amplify the *aph* gene from pKD4 (Primer sequences are outlined in Table B1). The purified PCR products were electrotransformed into STm SL1344 expressing λ -red recombinase from pSIM18, and kanamycin resistant transformants were selected, one of which was designated SW552 (bearing the *fliC* deletion) and SW550 (bearing the *fliB* deletion). For construction of an aflagellate strain containing an additional *aroA* mutation in order to attenuate virulence, the Δ *fliB*::*aph* was transferred to STm SL3261 *aroA* by P22 transduction and the resulting strain was designated SW556. The Δ *fliC*::*cat* was transferred to STm SW556 by P22 transduction and the resulting strain was designated SW564. The expected genotype of strain SW564 was confirmed using oligonucleotide primers 5' *tatcatttacagccatacattcc* 3' and 5' *gataaggaagaatttagtcgc* 3', and 5' *ggctccggaattaaaaaggc* 3' and 5' *caagggttacggtgagaaac* 3' that amplify the entire *fliB* or *fliC* locus, respectively, and motility was tested using semi-solid agar (containing 0.3% agar), where the absence of swimming in the aflagellated strain was compared against the swimming observed with SL3261 (Fig B1).

B1.3.3 Construction of OMP-deficient SL3261 Strains

B1.3.3.1 Omp D Deficient STm (RAK 126)

The transposon Tn10 belongs to a group of mobile genetic elements which encodes tetracycline resistance. P22 transduction was used to create the OmpD-deficient SL3261 strain (RAK 126) by Dr. RA Kingsley (Wellcome Trust). Briefly, the host bacterial cell BRD455 in which OmpD was disrupted by

Tn10 insertion (Tn10::tet^R) was infected with phage P22 (Dorman et al., 1989; Meyer et al., 1998). After infection the host plasmid harbouring the insertional mutation was integrated in place of the viral genome as a linear piece of DNA into the phage head to create a transducing particle. Upon lysis transducing particles were separated from virions and used to infect SL3261, where the insertional mutation was re-circulated by homologous recombination thus transferring the Tn10::tet^R insertion in OmpD BRD455 into SL3261. The resulting genotype was confirmed by amplification of the entire OmpD locus by PCR, whilst phenotypic assessment was confirmed by polyacrylamide gel electrophoresis (PAGE) analysis of cell envelopes isolated from the strain to confirm the absence of OmpD.

B1.3.3.2 Omp R Deficient STm (RAK 083)

The OmpR-deficient SL3261 strain (RAK 083) was constructed using red recombinase mutagenesis methodology (Datsenko and Wanner, 2000) by Dr. RA Kingsley (Wellcome Trust). Briefly, the selectable antibiotic resistance gene *aph*, encoding kanamycin resistance that is flanked by FRT (FLP recognition target sites) was amplified from pKD13 by PCR using the following primers (5'-GGATCGTCTGCTGACCCGTGAATCTTTCCATCTCATGGGTGTAGGCTGGAGCTGCTTC-3' and 5',-GTCTGAATATAACGCGGATGTGCCGGATCTTCTTCTTCCACATTCCGGGGATCCGTCGACC3'). The primers were designed to bear extended nucleotides at their 5' that were homologous to selected regions upstream and downstream to the target gene (OmpR) to be deleted. The purified PCR products were electrotransformed into STm SL1344 expressing λ -red recombinase from pSIM18. Kanamycin-resistant transformants were selected and the resistance gene was then eliminated by using a helper plasmid encoding the FLP recombinase, which acts on the directly repeated FRT sites flanking the resistance gene to create directed disruption of the gene. The Δ ompR::aph was transferred to STm SL3261 by P22 transduction and the genotype of the strain was confirmed by amplification across the entire OmpR locus by PCR, whilst phenotypic assessment was confirmed by PAGE analysis of cell envelopes isolated from the strain to confirm the absence of OmpC and OmpF.

Table B1: Primer Sequences Used to create the SW564 strain:

Name	Sequence	Use
A	5'-gcttgcgctgccttgattgtgtaccacgtgctggtgaatcaatcgccgga-TGTGTAGGCTGGAGCTGCTTCG-3'	Amplify cat to delete flhC
B	5'-cgtgggcaacagcccaataacatcaagttgtaattgataaggaaaagatcCATATG AATATCCTCCTTAG-3'	
C	5'-tgaaaaagccccgaattcacggggctgaataaaaacgaaataaattGTGTAGGCT GGAGCTGCTTCG-3'	Amplify aph to delete fljB
D	5'-tcatggaggattgctttatcaaaaacctccaaaaggaaaatttCATATGAATATC CTCCTTAG-3'	

Figure B1:

Swimming Motility Assay: The motility of STm and aflagellated STm (SW564) was assessed using semi-solid agar. STm or aflagellated STm were harvested at late-log phase (OD_{600 nm} of 1.4) and washed. The STm strains were injected just below the surface of semi-solid agar (containing 0.3% agar) and incubated at room temperature overnight. Following incubation the swarming from the point of inoculation was used as a marker of motility.



STm



SW564 – Aflagellated STm

Appendix C – Protein Specific Techniques Used

This Appendix describes technologies used during the purification and screening of protein immunogens used in this study. Additionally, the method used by Dr. Cristina Gil-Cruz to purify porins from STm and ST is outlined alongside the detailed methods used to functionally characterise them.

C1 Qualitative and Quantitative Assessment of LPS and Proteins

C1.1 Limulus Amoebocyte Lysate (LAL Assay)

The E-Toxate (Limulus Amoebocyte Lysate) assay was used to detect bacterial Endotoxin from purified proteins as per manufacturer's guidelines (Sigma Aldrich, Poole, UK). Briefly, a series of Endotoxin standards were created ranging from 400 EU/ml to 0.015 EU/ml. The standards and the sample of interest were diluted 1:1 with a pre-supplied working reagent and incubated for 1 hour at 37°C. After incubation the presence of LPS was qualitatively determined by the formation of a gel. The level of endotoxin was quantified by determining the greatest dilution, and therefore, lowest concentration of Endotoxin standard required to form a gel using a standard curve.

C1.2 Protein Quantification

The concentration of purified protein was determined using the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, USA) which was adapted for microplates. In brief, 50 µl of the purified protein and diluent were mixed with 200 µl of the BCA working reagent (BCA and copper complex) and incubated at 37°C for 30 minutes. Albumin protein standards were run alongside. After incubation, the colour change observed upon the simultaneous reduction of copper in the working reagent upon protein binding, and the chelating of BCA by this resulting cuprous ion was read at 550 - 570 nm using an Emax Precision Microplate reader (Molecular Devices). A standard curve of absorbance was constructed against known concentrations of the protein standards which was used to determine the concentration of the protein of interest.

C1.3 Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis

Purified proteins and the fractions collected during purification were analysed by Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Briefly, protein was mixed with SDS loading buffer which was made as a 6x concentrated stock as outlined in Table C1. Samples were heat-denatured at 95°C for 10 minutes prior to separation, unless stated otherwise. Precast gradient gels (4-20%) were largely used (Thermo Fisher Scientific, USA) and samples were separated in Tris-HEPES-SDS electrophoresis buffer at a constant voltage of 150 volts for 40 minutes, alongside a protein marker (Fermentas: PAGE RulerPlus). When a greater degree of separation was required, proteins were separated using 15% polyacrylamide gels which were run in Tris-glycine-SDS electrophoresis buffer at a constant current of 30 mA for 1 hour. Proteins were stained with GelCode Blue Reagent (Thermo Fisher Scientific) as per manufacturer's recommendations. The composition of the buffers used for SDS-PAGE and Immunoblotting are outlined in Table C1.

C1.4 Immunoblotting

Immunoblot analysis was performed using gradient polyacrylamide gels. Gels were run as described and proteins were electrophoretically transferred to Polyvinylidene Fluoride (PVDF) transfer membranes (Immobilon-Millipore) in transfer buffer at 35 mA for 1 hour using a semi-dry blotting system (Bio-Rad Laboratories, Hemel Hempstead, UK). Membranes were blocked overnight and extensively washed before endogenous HRP was eliminated using SG substrate (Vector Laboratories, Peterborough, UK) as per manufacturer's instructions. Briefly, membranes were incubated with HRP-conjugated goat-anti-mouse IgM (Southern Biotech, AL, USA) for 40 minutes at room temperature on a platform shaker, before incubation with SG substrate for 30 minutes. The SG substrate was then discarded and the membranes were rinsed twice in dH₂O to stop the reaction. Membranes were further blocked for 1 hour and then washed. Primary monoclonal antibodies or sera were added and blots incubated overnight. After overnight incubation membranes were washed and probed with HRP-conjugated goat-anti-mouse IgG or IgM secondary antibodies (Southern Biotech, AL, USA). Membranes were washed before

developing using Supersignal Chemiluminescence (Thermo Fisher Scientific, USA). All washing, blocking, and probing steps were carried out in wash buffer (Table C1). Blocking was done in 20% (w/v) skimmed milk powder (Marvel, UK) whilst antibodies were added in a solution containing 5% (w/v) skimmed milk powder. Overnight incubation of membranes were at 4°C under gentle agitation whilst shorter incubations were for 2 hours at room temperature and under gentle agitation.

C2 Isolation of Purified Porins for Immunisation (Described in 2.2.3)

Purified porins from ST (strain ATCC 9993) or STm (strain ATCC 14028) were extracted by Dr. Cristina-Gil Cruz through repeated extraction with SDS. In brief, ST or STm were incubated in minimal salts medium (MAM) containing 0.1% yeast extract, 0.5% glucose and 0.1% MgSO₄ at 37°C with aeration (200 rpm) until late log phase (OD_{λ600nm} of 1). Cells were then diluted 1:10 in 1.5 L MAM and further incubated until late log phase.

Cells were harvested at 6000 x g for 15 minutes at 4°C and washed in wash buffer. Cells were then re-suspended in wash buffer and disrupted using a French pressure cell at 20, 000 psi. Un-broken cells were removed at 6000 x g for 20 minutes at 4°C and the lysate was spun at 30, 000 x g for 40 minutes at 4°C. The resulting envelope fraction was re-suspended in 100 ml solubilisation buffer and incubated for 2 hours with aeration (120 rpm) at 37°C. The soluble inner membrane fraction was then separated from the insoluble outer membrane (OM) by centrifugation at 30, 000 x g for 45 minutes at 4°C. This extraction step was performed twice. Pelleted cells (OM envelope) were re-suspended in Nikaido buffer and incubated for 1 hour at 37°C with aeration (120 rpm) before centrifugation at 30, 000 x g for 1 hour at 20°C. The resulting supernatant isolated after this third extraction contained the OM fraction. The composition of all the buffers used for the purification of OMPs is outlined in Appendix A.

Final purification was achieved by FPLC gel filtration on a Sephacryl S-200 column with Nikaido's purification buffer. Fractions with an optical density 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 (Thermo Fisher Scientific). The LAL assay showed LPS contamination to be 0.06 EU/480 µg 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.

C3 Biophysical Assessment

C3.1 Analytical Ultracentrifugation (AUC)

Porins were structurally assessed by sedimentation velocity using analytical ultracentrifugation (AUC) to assess whether they adopted an oligomeric conformation through monitoring their rate of movement in response to centrifugal forces. In brief, purified porins were extensively dialysed in conjunction with Dr. Karina-Tveen Jensen against PBS (pH 7.4) containing 0.1% SDS at 20°C. After dialyses the porins were subjected to sedimentation velocity by Drs. Karina-Tveen Jensen and Tim Dafforn. AUC sedimentation velocity was performed at 37°C using PBS (pH 7.4) containing 0.1% SDS (buffer reference) with a ProteomeLab XL-I analytical ultracentrifuge protein characterisation system (Beckman Coulter). The protein sample and reference buffer were centrifuged at 20,000 rpm in a Ti-50 rotor and absorbance measured at 280 nm. The data was analysed using the software Sedit (Schuck, 2000).

C3.2 Circular Dichroism (CD)

Purified porins were assessed using circular dichroism (CD) spectroscopy to assess whether they were folded. Porins were extensively dialysed in conjunction with Dr. Karine-Tveen Jensen as with AUC assessment. Far UV CD measurements were performed on a JASCO J-715 spectropolarimeter with a 1 mm path length cell, 2 nm bandwidth, 1 nm increments, 100 nm/min scanning speed and a 2 second response time by Drs. Karina-Tveen Jensen and Tim Dafforn. The scanning mode was continuous. Twenty scans were averaged and the resulting spectrum was corrected for buffer contribution. Data was interpreted using standard methods (Kelly et al., 2005).

Table C1: Buffers used for SDS PAGE and Immunoblotting

Buffer	Composition	Volume	Use
SDS Sample Buffer (6X)	500 mM Tris HCl (pH 6.8) 12% (w/v) SDS 25% (v/v) Glycerol 5mM EDTA 0.2% (v/v) Bromophenol Blue (From 0.1% stock)		Sample preparation
10X Tris-HEPES-SDS Electrophoresis buffer	100 mM Tris Base (pH 8.3) 100 mM HEPES (electrophoresis grade) 3 mM SDS		Electrophoresis of 4-20% gradient gels
Resolving Buffer	20% (w/v) Sucrose 1.5 M Tris Base (pH 8.8) Acrylamide 10% (w/v) SDS 10% (v/v) APS (Ammonium Persulphate) N, N, N', N' N- tetramethylethylenediamine (TEMED)	1.25 ml 1.25 ml 2400 µl 50 µl 12.5 µl 5 µl	Preparation of one 15% Resolving Gel
Stacking Buffer	Water 0.3 M Tris HCl (pH 6.8) Acrylamide 10% (w/v) SDS 10% (v/v) APS (Ammonium Persulphate) N, N, N', N' N- tetramethylethylenediamine (TEMED)	944 µl 789 µl 267 µl 40 µl 10 µl 2.1 µl	Preparation of one 4% Stacking Gel
10X Tris-Glycine-SDS Electrophoresis buffer	250 mM Tris Base (pH 8.3) 1.92 M Glycine (electrophoresis grade) 1.0% (w/v) SDS		Electrophoresis of 15% polyacrylamide gel
Transfer Buffer	25 mM Tris HCl (pH 8.3) 150 mM Glycine 20% (v/v) Methanol 0.1% (w/v) SDS		Transfer of gels to PVDF membrane
Wash buffer	100 mM Tris Base (pH 7.5) 1.0 M NaCl 1.0% (v/v) Tween 20		Washing, blocking and probing stages of immunoblotting

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