Regulation of Pathogen-Specific and Self-Reactive Antibodies after *Salmonella* Infection

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ABSTRACT

Systemic *Salmonella* Typhimurium infection induces an atypical antibody response in which the extrafollicular and germinal centre reactions develop at discordant rates. Late in the infection, when the bacterial burden is low, germinal centres form and their appearance coincide with the production of high affinity antibodies to the pathogen. Dysregulated or atypical antibody responses represent a potential source of autoantibodies. In this thesis, we show that systemic infection induces anti-pathogen and self-reactive IgM and IgG antibodies. Whilst pathogen- and self-reactive antibodies are induced early after infection, these are induced with differing kinetics, suggesting that they are distinct responses. To characterize and dissect the molecular mechanisms controlling these two responses we assessed the antibody response in a range of genetically altered mice after infection. We identified a role for TLR4 in this process, but surprisingly this is partially dependent upon the genetic background of the murine host. Using classical methods to assess the targets of self-reactive antibodies, we show there is strong reactivity of self-reactive antibodies from wild-type mice with stomach, muscle and liver. Moreover, these antibodies can persist long after infection is cleared from key sites of infection such as the spleen and liver. Our assessments into the mechanisms underpinning the induction of these antibodies suggest that IL-6 from stromal cells can play a role in this response, but ICOSL, TNF, IFN-γ and Th1 responses are dispensable. Critically, we identify that CD80/CD86 are essential for the induction of the anti-self, but not the pathogen-specific response. These data suggest that pathogen-specific and self-reactive antibody responses are independently regulated and thus it may be possible to modulate anti-self-responses without compromising the generation of effective anti-pathogen immunity.

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ABBREVIATIONS

AFC – Antibody Forming Cells
AID – Activation-Induced Cytidine Deaminase
ANA – Anti-Nuclear Antibodies
AP- Alkaline Phosphatase
APC – Antigen Presenting Cell
APRIL – A Proliferation-Inducing Ligand
B Cell - Bone marrow-derived cell
BAFF – B-Cell Activating Factor
BCIP/NBT – 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium
BCL-2 – B-Cell Lymphoma 2
BCL-6 – B-Cell Lymphoma 6
BCR – B-Cell Receptor
BLIMP-1 – B-Lymphocyte Induced Maturation Protein 1
BM – Bone Marrow
BMSU – Biomedical Service Unit
BSA – Bovine Serum Albumin
CCL – Chemotactic Cytokine Ligands
CCR -- Chemotactic Cytokine Receptors
CD – Cluster of Differentiation
CD40L – CD40 Ligand
cDC – Conventional Dendritic Cell
CFU – Colony Forming Unit
CpG – Cytosine-Phosphate-Guanine
CS – Class Switching
CSR – Class Switch Recombination
CTLA-4 – Cytotoxic T-Lymphocyte-Associated Protein 4
DAB – 3, 3'-Diaminobenzidine Tetrahydrochloride
DAMPs – Damage-Associated Molecular Patterns
DAPI – 4', 6-Diamidino-2-Phenylindole
DC – Dendritic Cell
EAE – Experimental Allergic/Autoimmune Encephalomyelitis
EDTA – Ethylenediaminetetracetic acid
EF -- Extrafollicular
EFR – Extrafollicular Response
F - Follicle
Fab – Fragment Antigen Binding
FBS/FCS – Fetal Bovine Serum/ Fetal Calf Serum
Fc – Fragment Crystallisable Region
FDC – Follicular Dendritic Cell
FS/FSC – Forward Scatter
GC – Germinal Centre
Gy – Grays
HEV – High Endothelial Venules

HRP – Horse Radish Peroxidase
i.p. - Intraperitoneal
i.v. – Intravenous
ICOS – Inducible T-Cell Co-Stimulator
ICOSL – Inducible T-Cell Co-Stimulator Ligand
IFN-α – Interferon Alpha
IFN-β – Interferon Beta
IFN-γ – Interferon Gamma
Ig - Immunoglobulin
IL - Interleukin
iNOS – Inducible Nitrix Oxide Synthase
JAK – Janus Kinase
K - Kidney
L - Liver
LB – Luria Broth
LPS – Lipopolysaccharide
M - Muscle
MAMPs- Microbe-Associated Molecular Patterns
MHC – Major Histocompatability Complex
MyD88 - Myeloid Differentiation Primary Response Gene 88
N.I. – Non-infected/Non-immunised
NFκB – Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
NK – Natural Killer
NKT – Natural Killer T
NLRs – NOD-Like Receptors
NMS – Normal Mouse Serum
NOD – Nucleotide binding and oligomerisation domain
NTS – Non-Typhoidal Salmonella
OD – Optical Density
OMP – Outer Membrane Proteins
PAMPs – Pathogen-Associated Molecular Patterns
PBS- Phosphate Buffered Saline
PD-1- Programmed Cell Death Protein 1
pDC – Plasmacytoid Dendritic Cell
PMN – Polymorphonuclear
PNA – Peanut Agglutinin
PRRs – Pattern Recognition Receptors
PSGL-1 – P-Selectin Glycoprotein Ligand-1
RA- Rheumatoid Arthritis
RAG – Recombination-Activating Gene
ReA – Reactive Arthritis
RES – Reticuloendothelial System
RF – Rheumatoid Factor
RNS – Reactive Nitrogen Species
ROS – Reactive Oxygen Species

RP – Red Pulp
rpm – Revolutions per minute
RPMI – Roswell Park Memorial Institute
S - Stomach
SEn – Salmonella enterica serovar Enteritidis
SHM – Somatic Hypermutation
SLE – Systemic Lupus Erythematosus
SLO – Secondary Lymphoid Organ
SPI-1 – Salmonella Pathogenicity Island 1
SPI-2 – Salmonella Pathogenicity Island 2
SS/SSC- Side Scatter
ST – Salmonella enterica serovar Typhi
STAT – Signal Transducer and Activator of Transcription
STm – Salmonella enterica serovar Typhimurium
T – T-Zone
T-bet – T-box expressed in T cells
T Cell – Thymus-derived cell
TCR – T-Cell Receptor
TD – T-Dependent
TfH – T Follicular Helper Cell/ Follicular B Helper T-Cell
Th1 – T Helper 1
Th17 – T Helper 17
Th2 – T Helper 2
TI – T-Independent
TIR – Toll-Interleukin Receptor
TLR – Toll Like Receptor
TNF – Tumour Necrosis Factor
Treg – Regulatory T Cell
TRIF – TIR-Domain-Containing Adapter-Inducing Interferon-β
Vi – Vi Capsular Polysaccharide
WT - Wild Type

1. INTRODUCTION

1.1. A Brief Overview of the Immune System

The immune system is comprised of a network of molecules, cells, tissues and organs that collectively defend the body from pathogens and maintain homeostasis. It can be functionally divided into innate and adaptive immunity but the effective communication between these two responses through cytokines and cell-to-cell contact is essential for effective immune responses.

1.2. Anatomical Barriers

Anatomical barriers such as the skin or gastric mucosa physically prevent pathogens from gaining access to the body. The mechanical action of physical barriers such as the cilia in the epithelium of the nose or lungs can facilitate removal of pathogens from potential sites of entry. In addition, epithelial cells may secrete a range of different antimicrobial substances or chemicals that help kill pathogens that are attempting to colonize the host. Two examples are lysozyme in tears and the low pH of the stomach. However, pathogens have developed a range of different mechanisms to survive and breach these barriers. When this happens the non-specific innate immune response is important for keeping the infection under control, while the highly specific adaptive immune response can develop and eliminate the pathogen (1, 2).

1.3. The Innate Immune Response

When pathogens breach the anatomical barriers to access the body, the innate immune system responds immediately in a non-specific manner to either rapidly eliminate/expel the invading organism or limit the growth of the pathogen. The innate immune system is

essentially composed of a range of different cells that express receptors that recognize evolutionary conserved components of microbes known as Pathogen-Associated Molecular Patterns (PAMPs). Depending on the cell type, the recognition of non-self through PAMPs can result in the production of pro-inflammatory cytokines, phagocytosis, changes in cell migration, expression of co-stimulatory molecules and a range of different forms of cell death such as apoptosis, necroptosis or pyroptosis (3, 4).

1.3.1. Pattern Recognition Receptors

Innate immune cells recognize PAMPs through the action of germline encoded Pattern-Recognition Receptors (PRRs) (5). There is a broad range of different PRRs that will not all be discussed here but they exhibit crosstalk with one another (4, 6). The main ones associated with intracellular bacterial infections are the Toll-Like Receptors (TLRs) and Nucleotide-Binding Oligomerization Domain-Like Receptors (NLRs). However, for the purpose of this thesis we shall focus on TLRs. It should also be noted that in addition to their role in the recognition of PAMPs, some PRRs detect host damage through the binding of Damage-Associated Molecular Patterns (DAMPs).

1.3.2. Toll-Like Receptors

TLRs are a group of germline encoded PRRs that recognise PAMPs and DAMPs. The primary role of these receptors is to initiate inflammatory responses following the detection of components of pathogens, but they may also contribute to a range of other types of responses such as sterile inflammation. These receptors are broadly expressed by immune and non-immune cells (7). There are 13 different TLRs that are known to exist in mammals. Each TLR recognises a selection of different ligands and is located on

either the cell surface or within endosomal compartments. 10 of these TLRs are found in humans while mice have 12. Only TLRs 1-9 are shared by both mice and humans (**Table 1.1**). TLR10 is present in humans but is a pseudogene in mice. While mice express TLR11 which can recognise profilin and has been implicated in the defense against uropathogenic *E.coli*, *Toxoplasma gondi* and *Salmonella* (8-11). All TLRs are type I integral membrane proteins and are members of the Interleukin-1 Receptor family. Each TLR exists as a monomer at steady state and is composed of three regions: a solenoid shaped extracellular ligand binding domain composed of N-terminal Leucine Rich repeats, a transmembrane spanning region and a cytosolic C-terminal intracellular signalling domain known as a Toll IL-1 Receptor Domain (TIR). During ligand binding TLRs can form either homo or heterodimers that brings together the TIR domains that allows the binding of adaptor proteins. This results in the induction of an intracellular signalling cascade down either a MyD88 or TRIF dependent pathway. As a general rule MyD88 dependent signalling induces the production of pro-inflammatory cytokines while TRIF signalling results in the secretion of Type 1 interferons. All TLRs except TLR3 can signal down a MyD88 dependent pathway. In contrast, only TLR3 and TLR4 may signal through a MyD88 independent pathway (12, 13).

Table 1.1: Toll-Like Receptors: Expression, Ligands and Signalling.

TLR	Expression	PAMPs	DAMPs	Signalling Adaptors	Cytokines
TLR1+ TLR2	Cell surface Mo, MΦ, DC, B	Triacylated lipoproteins(Pam3CSK4) Peptidoglycans	(TLR2 DAMPs listed below)	TIRAP, MyD88,	Inflammatory Cytokines
TLR2+ TLR6	Cell surface Mo, MΦ, DC, B & En	Diacylated lipoproteins	Heat Shock Proteins (HSP 60, 70, Gp96), High mobility group proteins (HMGB1), Protecoglycans (Versican, Hyaluronic acid	TIRAP, MyD88,	Inflammatory Cytokines
TLR3	Endosomes B, T, NK, En & DC	dsRNA (poly(I:C) tRNA, siRNA	mRNA & tRNA	TRIF	Inflammatory Cytokines & Type 1 IFNs.
TLR4	Cell Surface & Endosomes Mo, MΦ, DC, B, En and IE	Lipopolysaccharides Porins Paclitaxel	HSP22, 60, 70, 72, Gp96. HMGB1 Proteoglycans (Versican, Heparin Sulfate, Hyaluronic Acid, Fibronectic and Tenascin-C)	TRAM, TRIF, TIRAP, MyD88, Mal	Inflammatory Cytokines & Type 1 IFNs.
TLR5	Cell surface Mo, MΦ, DC and IE	Flagellin		MyD88	Inflammatory Cytokines
TLR7	Endosomes Mo, MΦ, DC, B	ssRNA Imidazoquinolines Guanosine analogs	ssRNA	MyD88	Inflammatory Cytokines & Type 1 IFNs.
TLR8	Endosomes Mo, MΦ, DC, MC	ssRNA Imidazoquinolines	ssRNA	MyD88	Inflammatory Cytokines & Type 1 IFNs.
TLR9	Endosomes Mo, MΦ, DC, B, T cells	CpG DNA CpG ODNs	Chromatin IgG Complex	MyD88	Inflammatory Cytokines & Type 1 IFNs.

IE- Intestinal Epithelium, En-Endothelium Mc-Mast Cell Mo - Monocyte, MΦ - Macrophage, DC – Dendritic Cell, B- B cell, T - T cell. Figures is taken and adapted from (14).

1.3.3. Toll-Like Receptor 4

TLR4 is expressed on the surface of cells and recognises the lipid A portion of LPS from Gram-negative bacteria through the collaborative action of an LPS binding protein (LBP), CD14 and the TLR4-MD-2 complex (**Figure 1.1**). LBP is an acute phase protein found in serum. LBP binds LPS from bacterial membranes or vesicles. This protein then transfers the LPS to CD14, which is expressed on the surface of cells such as macrophages, DCs, B cells and T cells, although it may also be found in a soluble form in the serum. CD14 extracts monomers of LPS from the LBP-LPS complex and transfers it to the TLR4-MD-2 complex (15). MD-2 is a small protein that is permanently bound to the extracellular domain of TLR4 and forms a small hydrophobic pocket that can bind up to 5 acyl chains of lipid A. Lipid A binding to the TLR4-MD-2 complex induces dimerization of TLR4 and initially causes signaling down a MyD88 dependent pathway. The TLR4-MD-2-LPS complex may be taken up into the endosome later on and here it can signal down a TRIF dependent pathway (16).

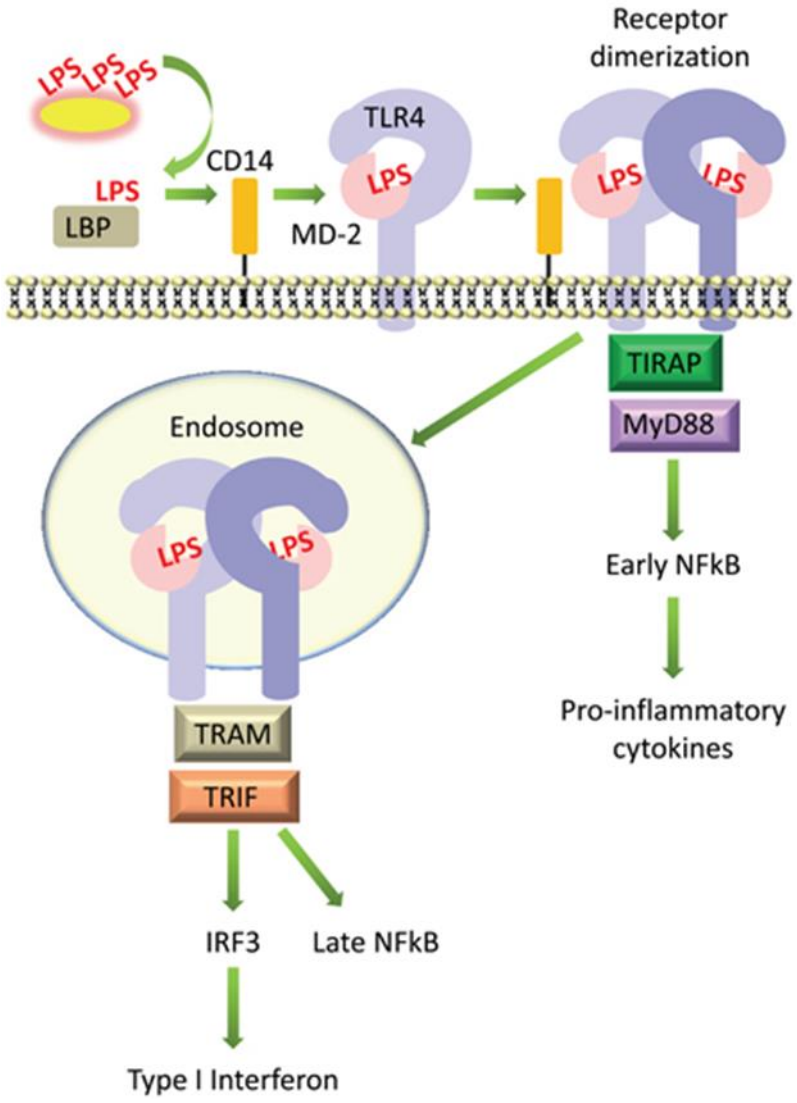

Figure 1.1: Model for TLR4 binding to Lipid A
This figure shows the LPS-CD14-TLR4-MD-2 Complex and the MyD88 dependent signaling and TRIF dependent signaling that take place. See Text for details. Image is taken from (16).

1.3.4. Cells of the Innate Immune System

Immune cells are derived from Haematopoietic Stem Cells (HSCs) located in the medulla of the bone marrow. HSCs can differentiate into either a common myeloid progenitor (CMP) or a common lymphoid progenitor (CLP). The majority of innate cells such as neutrophils, monocytes, macrophages, mast cells, basophils and eosinophils are derived from the CMP, although Natural Killer (NK) cells develop from the CLP (**Figure 1.2**)(2, 17). The main cells involved in defense against infections with bacteria are monocytes, macrophages, neutrophils and NK cells. Once a pathogen gains access to the underlying barriers they are recognized by PRRs expressed on epithelial cells and tissue resident macrophages. The epithelial cells and macrophages in conjunction with complement induce the release of pro-inflammatory cytokines that lead to the recruitment of neutrophils into the tissues from the bloodstream. Neutrophils chemotactically migrate along an IL-8 gradient and following recognition of non-self they, like macrophages, engulf the pathogen through phagocytosis (18, 19). The pathogen is then killed inside the cell when they are exposed to antimicrobial compounds in the endolysosomal compartment. While complement, macrophages and neutrophils attempt to control the growth of the invading organism the dendritic cells (DCs) located in non-lymphoid tissues can begin the process of initiating the development of the adaptive immune response (2, 18).

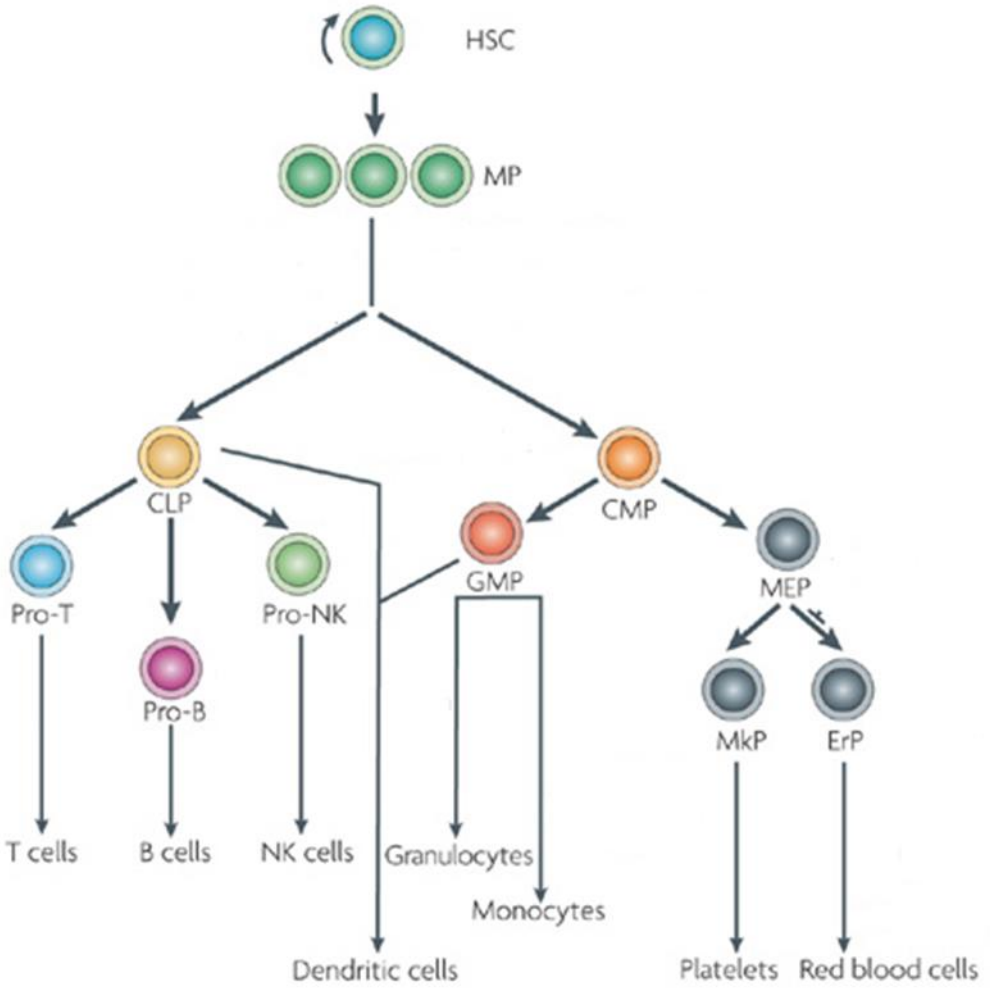

Figure 1.2: Haematopoiesis in the bone marrow.
Self-renewing Haematopoietic Stem Cells (HSC) differentiate into multipotent progenitors (MP) which can then differentiate into a Common Myeloid Progenitor (CMP) or Common Lymphoid Progenitor (CLP). The CMP may further differentiate into a Megakaryocyte-Erythroid Progenitor (MEP) or Granulocyte-Macrophage Progenitor (GMP). MEP cells give rise to platelets and red blood cells while GMP cells produce the granulocytes, monocytes and DCs of the innate immune system. The CLP can differentiate to produce NK cells from the innate system or B and T cells of the adaptive immune system. Figure is taken and adapted from (17).

1.3.5. Dendritic Cells: The link between the innate and adaptive response

All nucleated cells in the body are continually presenting peptides derived from the proteins they synthesis on the surface of the cell in conjunction with Major Histocompatability Complex class I (MHC I) (2). These cells are referred to as non-professional antigen presenting cells. Dendritic cells (DCs) on the other hand are one of three major classes of professional antigen-presenting cells (APC) that specialize in presenting antigen on Major Histocompatability Complex II (MHC II). The other two professional APCs are B cells and macrophages. Only professional APCs can activate naïve $CD4^+$ T Helper cells and of these, DCs are the most efficient at activating T cells in primary immune responses (20). DCs reside in non-lymphoid tissues in an immature state and continually sample antigen from the surrounding environment, process and present it on MHC class I and II. When immature DCs take up antigen that contains PAMPs, the recognition by PRRs results in the activation/maturation of the DC resulting in an increase in the number of peptide:MHC class II complexes displayed on the cell surface alongside the co-stimulatory molecules CD80 and CD86 (2, 21-23). At the same time the chemotactic behavior of these activated DCs changes as they upregulate the expression of CCR7 and migrate to the T-zone of secondary lymphoid organs (SLOs) such as the spleen (**Figure 1.3**), through the binding of CCL19 and CCL21 (24, 25). It is within the T-zone of SLOs that DCs can interact and prime the T cells to start the cell-mediated immune response.

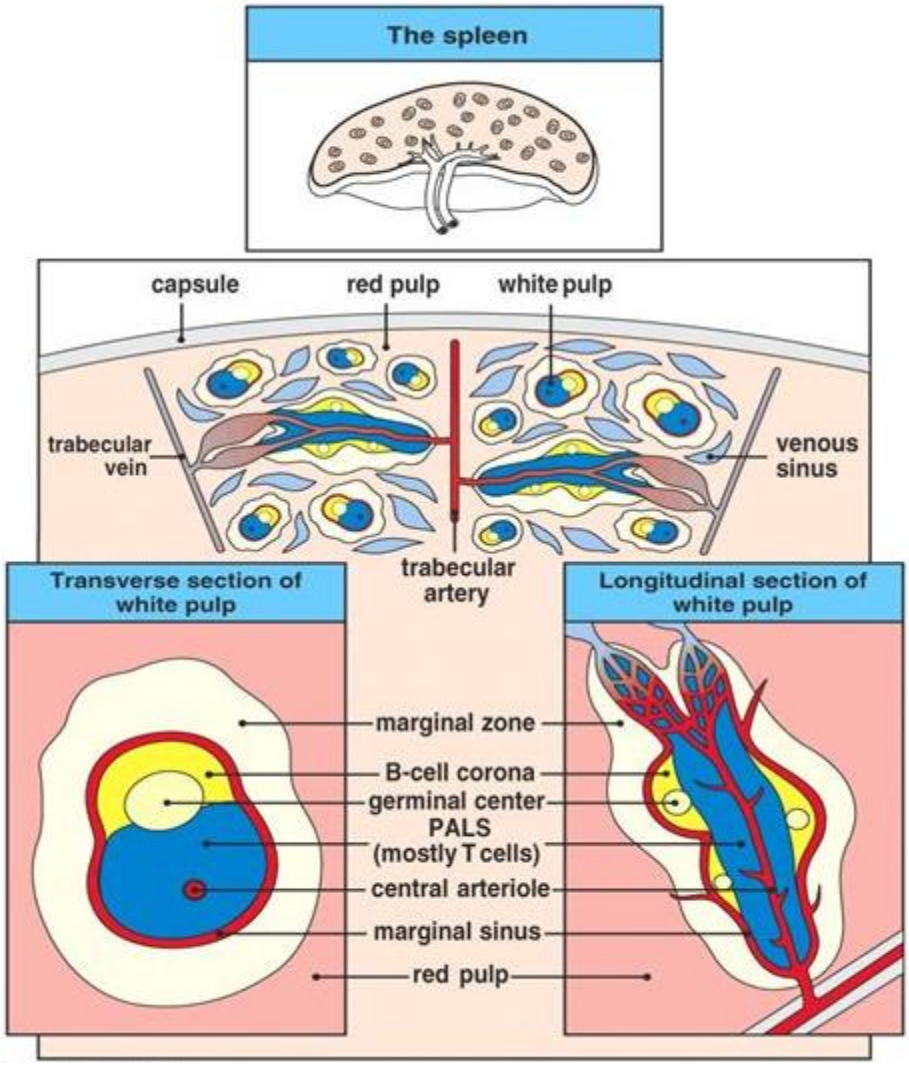

Figure 1.3: Structure of the Spleen
The spleen is an encapsulated secondary lymphoid organ that has a rich blood supply through the splenic artery and splenic vein. Its primary function is to remove senescent erythrocytes and filter the blood, which takes place in an extremely monocyte rich area of the spleen known as the red pulp. The white pulp is interspersed throughout the red pulp and is closely associated with arterioles. A T cell rich zone forms the periarteriolar lymphoid sheath (PALs) that surrounds the central arteriole. The follicles are closely associated with the PALs and are composed of follicular B cells and stromal cells. Marginal zone B cells are located at the edge of B cell follicles where they are stationed between the red pulp and white pulp. It is within the white pulp that adaptive immune responses are initiated. Figure is taken and adapted from (2).

1.4. The Adaptive Immune Response

The adaptive immune response is highly specific to the invading organism and takes one to two weeks to fully develop during a primary infection. Upon secondary exposure, the adaptive response exhibits memory and develops at a faster and greater magnitude than the primary response, thus potentially leading to the rapid elimination of the pathogen (2). B and T cells are the main effectors of this response. Both B cells and T cell precursors are initially produced in the bone marrow from the CLP. B cells undergo positive and negative selection in the Bone marrow to remove most autoreactive cells. In contrast, T cell precursors must undergo maturation and selection in the Thymus to give rise to naïve T cells (26, 27). Autoreactive B and T cells can be further controlled through a range of peripheral tolerogenic mechanisms.

1.4.1. Cell-Mediated Immune Responses

αβ and γδ T cells can be broadly classified as $CD4^+$ or $CD8^+$ T cells. $CD4^+$ T cells are important for secreting cytokines to help other immune cells and for inducing class switching in B cells. $CD4^+$ T cells recognize MHC Class II:peptide complexes (28). While CD8 T cells are important for killing infected cells through recognition of MHC class I: peptide complexes and releasing cytotoxic molecules (29). However, for the purpose of this thesis $CD8^+$ T cells will not be discussed.

1.4.2. Priming of Naïve $CD4^+$ T Helper Cells

Activated DCs that have migrated to the T-zone of SLOs present peptide:MHC class II complexes alongside the co-stimulatory molecules CD80 and CD86. The T Cell Receptor (TCR) of cognate naïve $CD4^+$ T cells recognises the peptide:MHC Class II

complex and upon binding this leads to the delivery of a signal to the naïve $CD4^+$ T cell, commonly referred to as signal 1. A second signal is delivered through the binding of CD80 and CD86 on the APC to CD28 on T cells. This induces the expression of IL-2 and CD40L on the T cell. IL-2 drives the proliferation and expansion of T cells while CD40L can bind CD40 on APCs to induce the release of cytokines. The cytokines from the APC and surrounding environment can provide a third signal to the $CD4^+$ T cells that help drive its differentiation down one of several different effector pathways (2).

1.4.3. $CD4^+$ T-Helper Cell Subsets

The information provided by the types of PRRs engaged and the cytokines released from the innate immune response modulate the phenotype of the DC which directs the polarization of the effector T cell that is produced in order to ensure the most effective response develops. Primed $CD4^+$ T Cells can differentiate into multiple T cell subsets including Th1, Th2, Th17, TfH and iTregs (**Figure 1.4.**). Th1 cells differentiate in response to IL-12 and IFN-γ, which induce signaling through STAT 4 and STAT 1, respectively. As a consequence the transcription factor T-bet is expressed and promotes the differentiation of Th1 cells that are classically considered to be effective at dealing with intracellular pathogens through the production of IFN-γ. In mice, secretion of IFN-γ can induce B cells to class switch to IgG2a/c and IgG2b. On the other hand IL-4 promotes STAT6 signalling and the expression of the transcription factor GATA-3 that drives the formation of Th2 cells (30). These cells are commonly associated with combating extracellular or parasitic infections through actions of IL-4, IL-5 and IL-13 which can induce IgG1 and IgE class switching. The cytokines TGF-beta, IL-6 and IL-23

drive the expression of RORyt that enables the differentiation of Th17 cells that are effective at inducing protective inflammatory responses at mucosal sites using IL-17A-F and IL-22. TfH cells develop in response to IL-6, IL-21, CD40L and ICOS signaling that induce the expression of the transcription factor BCL-6. This downregulates CCR7 and promotes the expression of CXCR5 and PD-1. CXCR5 enables the TfH cell to migrate to T-B borders or B cell follicles where it can facilitate the development of extrafollicular or germinal centre responses, respectively. The expression of PD-1 on TfH cells allows inhibitory signals to be delivered in order to regulate both itself and the B cell response (31). In contrast, $CD4^+$ T cells may also differentiate into regulatory T cells following exposure to TGF-beta and IL-2. This promotes the expression of the transcription FOXP3. Regulatory T cells can suppress inflammatory responses through the production of IL-10 and may also induce tolerance through the action of co-inhibitory molecules such as CTLA-4 or ligands for death receptors such as FASL. However, it is important to note that effector T cell subsets are not fixed and that there is extensive plasticity exhibited by them throughout an immune response(32). In addition, more than one T cell lineage is induced to the same antigen in the same SLO throughout an immune response (33).

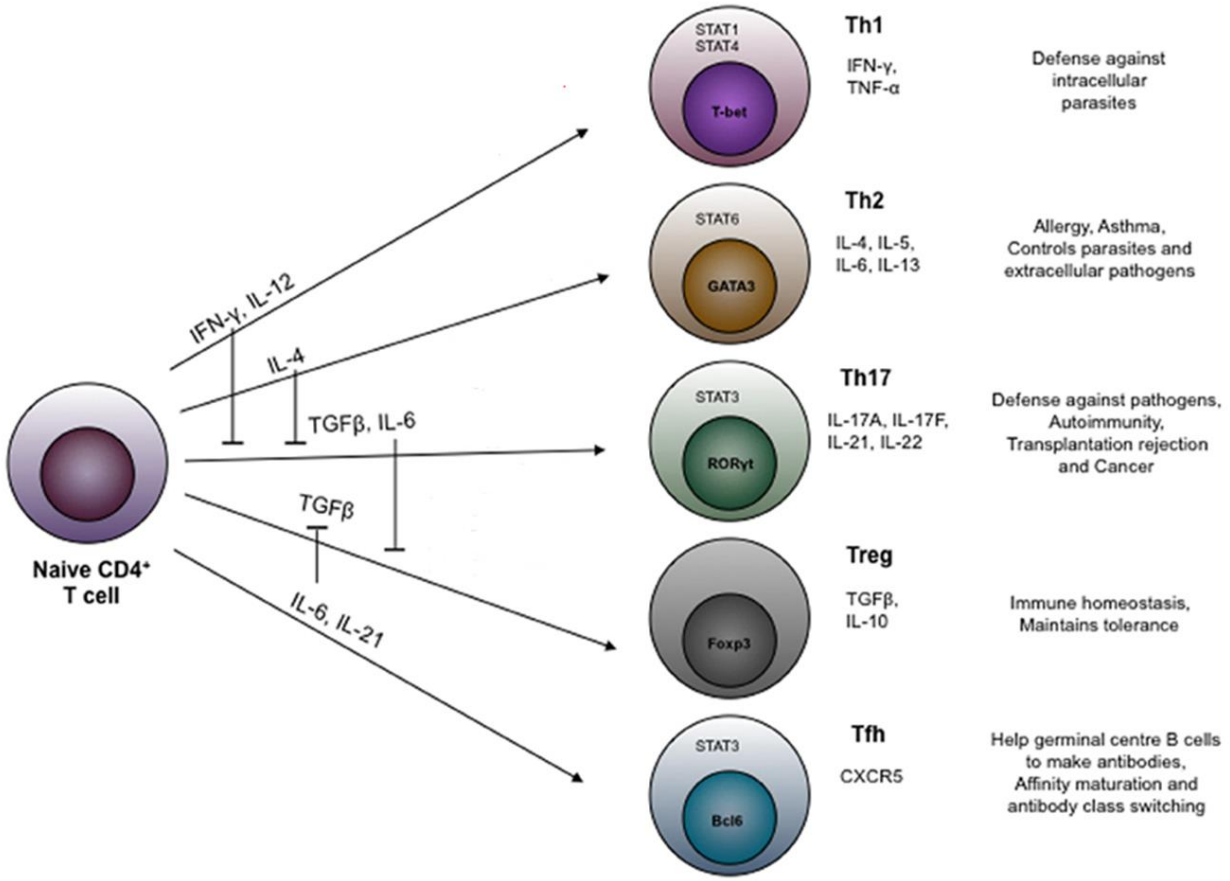

Figure 1.4: CD4⁺ T Cell Subsets
Following the activation of naïve CD4⁺ T cells through the presentation of MHC Class II:peptide complexes in conjunction with co-stimulatory molecules and cytokines, these may differentiate into several different T cell subsets that are more efficient than others at particular effector mechanisms. In addition, the differentiation of one T cell subset may inhibit the differentiation of another subset. This is is observed in Th1 and Th2 cells. Figure is taken and adapted from (34).

1.4.4. B Cell-Mediated Immune Responses

There are three types of mature B cells found in mice known as Follicular B cells, Marginal Zone B cells and B1 B cells (**Figure 1.5**) (35). Follicular and Marginal zone B cells are referred to as B2 cells. Follicular B cells circulate through the body or reside within B cell follicles in SLOs. In contrast, MZ B cells in mice are non-circulating B cells that remain stationed at the interface of the red and white pulp border in the spleen (36). B1 cells are initially produced during infancy and colonise the peritoneum and pleural cavities (37). B1 cells are unique in that they replenish themselves through self-renewal rather than being continually reproduced from the bone marrow. In addition, B1 cells can be further divided into B1a and B1b cells in mice. However, the existence of these cells in humans is controversial (38). Follicular B cells are largely involved in T-dependent antibody responses, while MZ and B1 cells can participate in T-Independent antibody responses which are typically restricted to extrafollicular foci (39) .

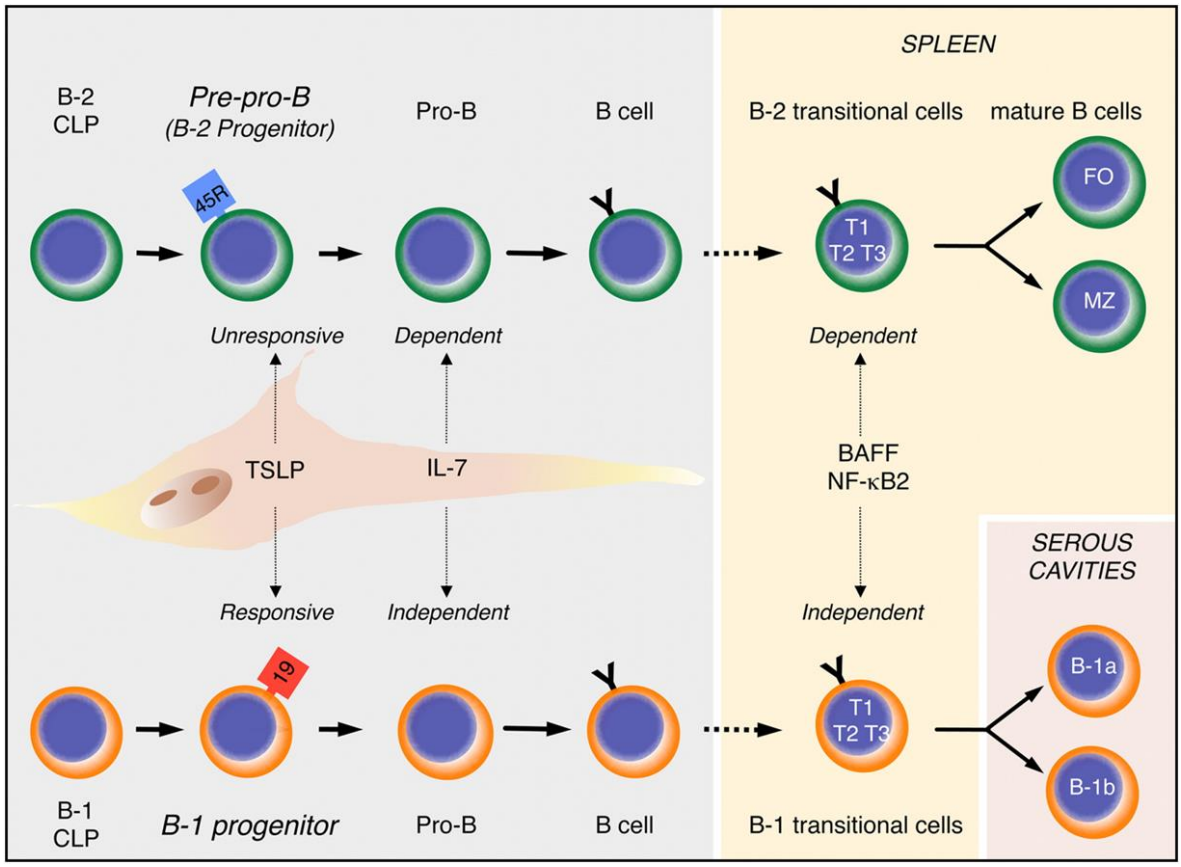

Figure 1.5: B-1 and B-2 cell Development.
In the bone marrow the CLP can differentiate into either an early thymic progenitor, B-1 progenitor or Pre-Pro B cell. B-2 cells develop from a pre-pro-B cell which differentiates through a Pro and Pre B cell stage to become an immature B cell. Immature B cells express surface IgM as a BCR and migrate from the bone marrow to secondary lymphoid organs such as the spleen. Here they undergo further differentiation and maturation through B-2-transtional cells 1-3 which can then become either a mature re-circulating follicular B cell ($IgM^{lo}IgD^{Hi}CD19^{+}B220^{+}CD5^{-}CD23^{Hi}CD21^{lo}$) or non-re-circulating Marginal Zone B cells ($IgM^{Hi}IgD^{Lo}CD19^{+}B220^{+}CD5^{-}CD23^{lo}CD21^{Hi}$). B-1 cells differentiate from the B-1 CLP in the bone marrow. This differentiates through a Pro and Pre B cell stage and also express surface IgM BCR in an immature stage. Immature B-1 cells mature through several transitional stages in the spleen to give rise to either B1a or B1b cells that colonize the serous cavities. B-1 cells are identified as $IgM^{hi}IgD^{lo}CD19^{+}B220^{lo}CD23^{-i}CD21^{lo/int}$ that can be divided into B1a ($CD5^{+}$) and B1b cells ($CD5^{-}$). Figure taken from (40).

1.4.5. Development of T-Dependent Antibody Responses

The majority of B cells in humans or mice are mature naïve re-circulating B cells and can be found passing through the blood, lymph and follicles of SLO (41). In response to BCR-antigen binding these cells migrate to the interfollicular zone of SLOs through CCR7 (binds CXCL21) and CXCR5 (binds CXCL13) dependent chemotaxis. B cells then become fully activated by interacting with their cognate T_H cells through the TCR:MHC Class II-Peptide Complex, CD40L:CD40 plus various cytokines and their receptors (42, 43). Fully activated B Cells may then migrate to extrafollicular foci and primary follicles to establish an extrafollicular antibody response or form germinal centres (GCs) (**Figure 1.6**). The exact signals that dictate which pathway an activated B cell proceeds down are not clear. However, it should be noted that there has been some literature suggesting that the affinity of the primary interaction between a BCR and antigen may influence this (44, 45).

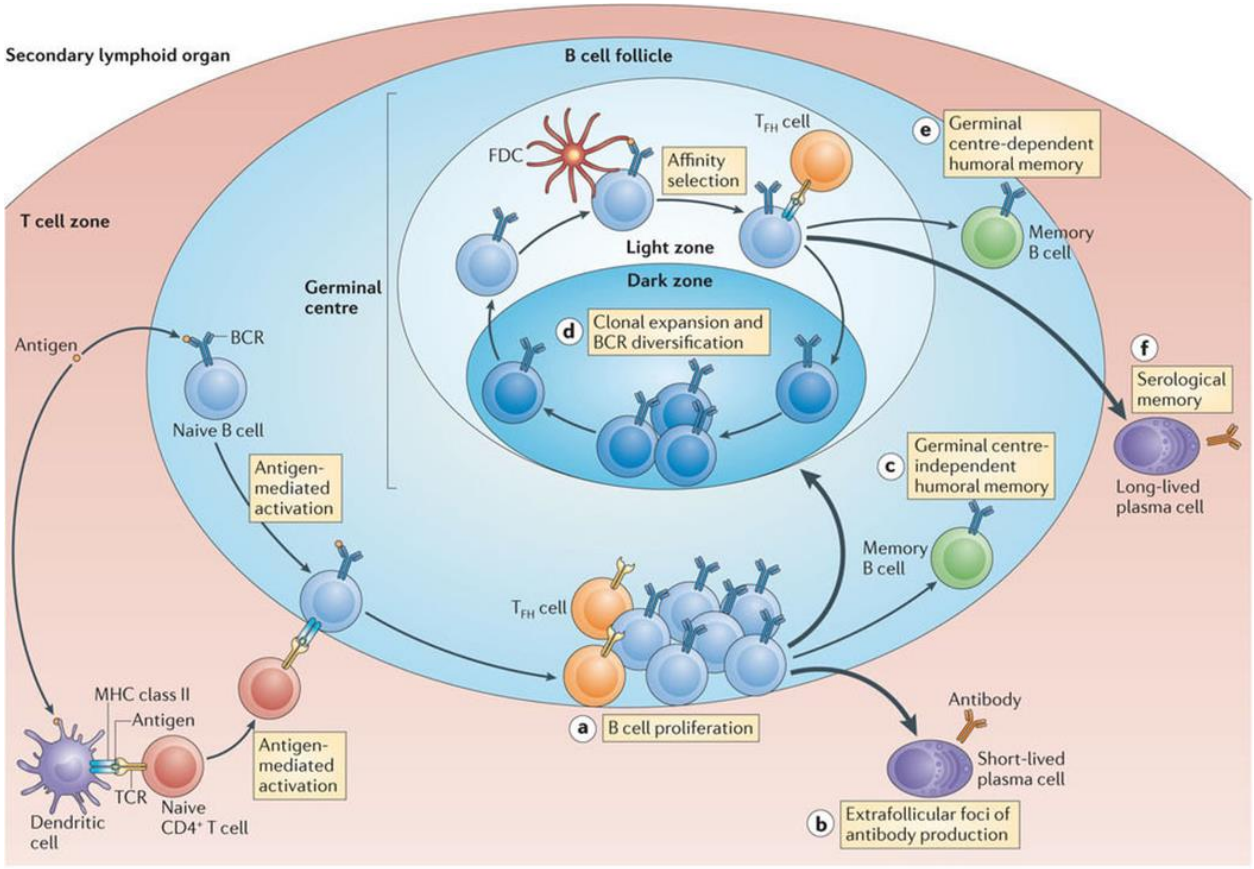

Figure 1.6: T-Dependent Antibody Responses

APCs present antigen and prime Naïve CD4+ T cells within the T zone. These activated CD4 T cells migrate to the T-B interface and interact with a cognate B cell that has cross-linked antigen through their BCR and presented parts of the antigen on MHC class II. A) Both T and B cells deliver signals to one another and proliferate at the T-B interface. B) Some B cells exit the follicle and migrate to extrafollicular foci where they differentiate into plasmablasts or short lived plasma cells and secrete low affinity antibodies. The majority of these cells die after 3 days. Alternatively, C) B cells may differentiate into memory B cells or migrate to B cell follicles to form germinal centres. The germinal centre is composed of dark and light zones. D) In the dark zone germinal centre B cells proliferate and mutate the BCR through somatic hypermutation. These cells then migrate to the light zone and undergo selection on the basis of the affinity of their BCR for antigen held on FDCs and ability to present and compete for survival signals from TfH cells. The B cells can then repeat this process to undergo iterative cycles of somatic hypermutation and affinity maturation. E) Germinal centre B cells may eventually differentiate into memory B cells or F) form plasma cells that can migrate to the bone marrow and become long lived. Germinal centre derived cells typically produce high affinity class switched antibodies, in contrast to extrafollicular responses. Figure is taken from (46).

1.4.6. Extrafollicular Antibody Response

Follicular or MZ B cell blasts exit the interfollicular zone after two cell cycles and migrate to the red pulp of the spleen or the lymph nodes medullary chords. At these sites they form extrafollicular foci, undergo expansion, and differentiate into low/moderate affinity antibody secreting plasmablasts through the upregulation of Blimp-1 and inhibition of PAX-5 (39). In response to protein antigen adsorbed in alum, plasmablasts are generally short lived and predominantly produce IgM antibody by 3-5 days after antigen exposure. As the EFR develops the plasmablasts may undergo class switch recombination to produce other isotypes. Normally, in mice the type of isotype secreted is influenced by the polarity of the interacting T_H cell or surrounding inflammatory environment. For example, the strong T_H1 response generated against *S*Tm promotes the development of IgG2a and IgG2b plasmablasts by 5-7 days post-infection, that in turn persists until clearance (47). Some plasmablasts may then further differentiate into plasma cells through the support of $CD11c^{Hi}$ DCs and increased expression of Blimp-1 and XBP-1 (39). A minority of these plasma cells might be able to extend their survival by accessing the stroma, but the majority will eventually undergo apoptosis (39). However, a very small number of these cells can become memory B cells or long lived plasma cells.

1.4.7. Germinal Centre Response

Germinal centres are microenvironments that develop within follicles of SLOs to specifically give rise to memory B cells and plasma cells that can secrete high affinity class switched antibody (42). B cells that enter these microenvironments are characterised by the upregulation of the transcriptional repressor known as B cell

lymphoma 6 (BCL-6) and downregulation of EBI-2 (48). This simultaneously suppresses differentiation into plasmablasts and facilitates the migration of B cells to the primary follicle (42, 43). In the follicle, activated B cells rapidly expand thus forcing out resident B cells to the periphery. This results in the formation of the mantle zone and the secondary follicle. Following several days of proliferation, the germinal centres can be distinguished as being composed of both a dark zone and a light zone. The dark zone is composed of rapidly dividing B cell blasts that are $CXCR4^{Hi}CD83^{low}CD86^{low}$ (42, 43). The light zone is less densely packed and has a stromal network of FDCs supporting interactions between themselves, $CXCR5^{+}$ B cells and Tfh cells (49). In the dark zone, dividing B cells undergo somatic hypermutation in their IgV region through the expression of activation induced cytidine deaminase (AID), whilst in the light zone B cell clones are selected on the basis of their affinity for the antigen which in turn leads to enhanced acquisition of survival signals from TfH cells (50, 51). B cells that acquire these survival signals are positively selected and become either memory B cells or plasma cells that can secrete high affinity class switched antibodies. Plasma cells that migrate to the bone marrow following the germinal centre reaction are typically high affinity and can live for many years through close association with CXCL12 producing stromal cells and eosinophils that secrete survival molecules such as IL-6, APRIL and BAFF (52-54) (43, 55).

1.4.8. T-Independent Antibody Responses

Antibody responses that develop in the absence of T cell help are referred to as T-Independent (TI) antibody responses. These responses develop faster than TD antibody responses and can provide an immediate source of protection through the production of low affinity germline encoded polyreactive antibodies (56). Classically there are considered to be two types of TI responses on the basis of whether or not an antigen induces an antibody response in CBA/N mice. These mice are deficient in Bruton tyrosine kinase (Btk) and can make antibody responses against TI-1 antigens, but not TI-2 antigens (57-59). TI-1 antigens are B cell mitogens that can directly engage both the BCR and TLRs on B cells. Examples of these are LPS, CpG and viral RNA (60). Excess amounts of molecules like LPS can induce non-specific polyclonal B cell activation in mice without binding to the BCR (59, 61). In contrast, TI-2 antigens are most frequently composed of structures with regular repeating patterns that can induce extensive cross-linking of the BCR. Examples of these are capsular polysaccharides from bacteria such *Haemophilius Influenzae* and *Streptococcus pneumonia*, but other examples are viral capsids (62). TI responses play an important role in protection, particularly in infants. In humans, MZ B cells are important for responses against TI-2 antigens and provide protection against *S.pneumoniae* infections. However, the source of these types of antibody responses may be derived from both MZ and B1 cells in mice. B1b cells have been shown to mount IgM antibody responses against *Borrelia hemsi*,many pathogen components including, STm and ST in mouse models of infection (63-67). B1a cells may also produce antibodies in the absence of external antigens (68). These natural antibodies are typically IgA, IgG3 or IgM mediated responses (68, 69).

1.5. An Introduction to *Salmonella*

Salmonella is a genus of facultative intracellular anaerobic Gram-negative bacteria that were first discovered in 1885 by Theobald Smith after he isolated them from pigs presenting with swine fever. This genus of bacteria were named after his chief, Dr Daniel E. Salmon (70). These motile rod shaped bacteria are a member of the Enterobacteriacea family and are known to infect both animals and humans following ingestion of contaminated liquids or food (71). This complex group of bacteria can be divided into two species known as *S. bongori* and *S. enterica*. *S. bongori* is known to primarily infect cold blooded organisms such as lizards, while *S. enterica* typically colonises humans (72). Unlike *S. bongori, S. enterica* can be further classified into one of six subspecies: *arizonae* (IIIa)*, diarizonae* (IIIb)*, enterica* (I)*, houtenae* (IV)*, indica* (VI) and *salamae* (72-74). Genomic analysis/serotyping of *Salmonella* has given rise to approximately 2600 serovars of which over 50% belong to S.*enterica* subspecies *enterica* (74, 75). The identification of these serovars is achieved through the Kaufmann-White scheme(76) and works on the basis of the type of O (LPS O Chain), H (Flagellin) and K (Polysaccharide capsule) antigen that each bacterium expresses (77, 78). This in turn has led to the identification of four key clinically relevant serovars that are responsible for most global cases of human salmonellosis: Typhi, Paratyphi, Enteriditis and Typhimurium (73). The serovars Typhi and Paratyphi are responsible for enteric fever in humans, while Enteriditis and Typhimurium are the main cause of Non-Typhoidal Salmonellosis (NTS).

1.6. Disease burden of Non-Typhoidal Salmonellosis

Salmonella infections are one of the most common causes of bacterial gastroenteritis worldwide. Salmonellosis thus represents a major cause of morbidity and mortality (79). However, the global distribution of this varies depending on the economic state of the country. In developed countries infection with NTS typically presents as a self-limiting gastroenteritis that can resolve within 2-7 days. These patients may present with abdominal pain, diarrhea, nausea and fever. However, they rarely require treatment with antibiotics. In rare cases, this infection may spread systemically in susceptible individuals such as young children, the elderly and immunocompromised. In contrast, in developing countries patients often present with no symptoms other than fever. This lack of symptoms is a feature that is usually only seen in the immunocompromised (80-82). As a consequence patients in developing countries have a higher risk of bacteremia and life threatening septicaemia. This is particularly evident in Sub-Saharan Africa in HIV infected adults and children under the age of 2. Evidence of this is that globally there are 3.4 million cases of iNTS per annum, yet over 2.5 million of these are in Africa with 4100 deaths per year (**Figure 1.7**). In addition, there is a 20-25% mortality rate each year out of the 500 cases/ 100,000 children infected under the age of 2 (80). There are some reports suggesting the overall case fatality rate for adults and children is between 22-47% even after antibiotic treatment. The systemic spread of NTS throughout the host is therefore associated with a greater severity of disease.

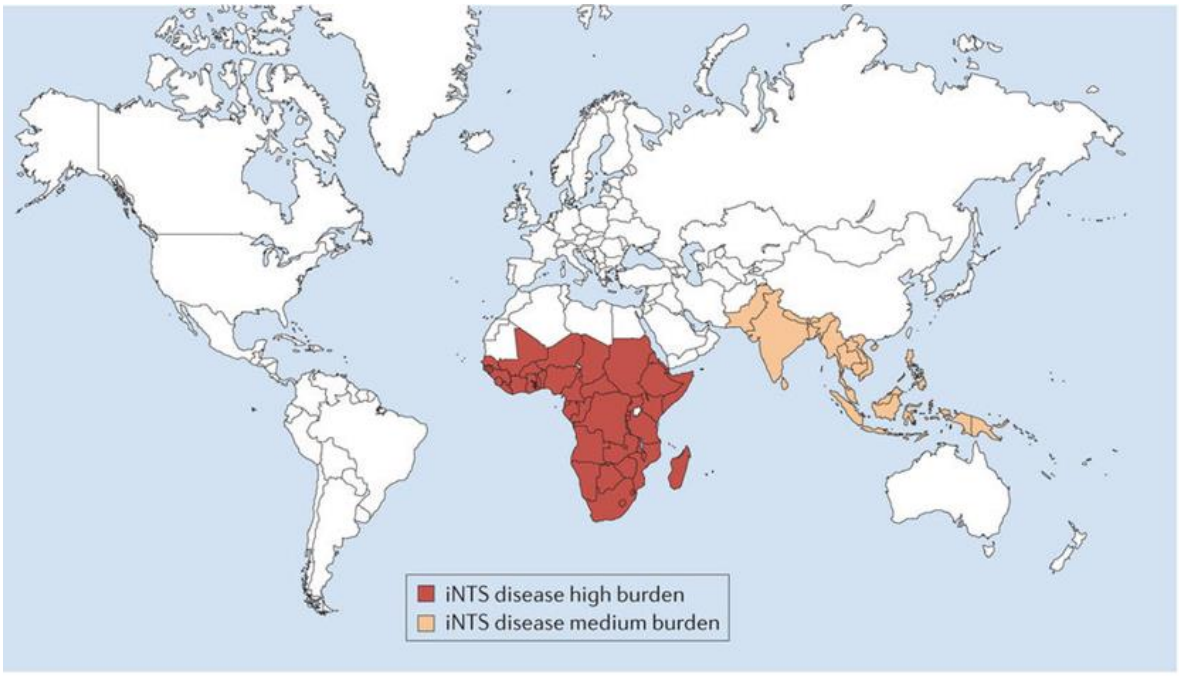

Figure 1.7: Global Distribution of invasive Non-Typhoidal Salmonellosis
This figure shows the geographical distribution of invasive Non-Typhoidal Salmonellosis across the globe. The majority of invasive disease is observed in Sub-Saharan Africa and South-East Asia. Figure is taken from (83).

1.7. Composition of the Cell Wall

Salmonella is a Gram-negative bacterium and as a consequence has a cell wall composed of both an inner and outer membrane that are separated by the periplasmic space. The outer membrane is an asymmetric bilayer composed of an inner and outer leaflet. The inner leaflet, much like the inner membrane, is made of phospholipids. In contrast, the outer leaflet is largely formed from lipopolysaccharides (LPS). A range of other molecules can also be found in the outer membrane such as porins and flagella (84-86). The expression of LPS, porins and flagella on the surface of the bacterium makes them key targets for recognition by the innate and adaptive immune system (**Figure 1.8**.).

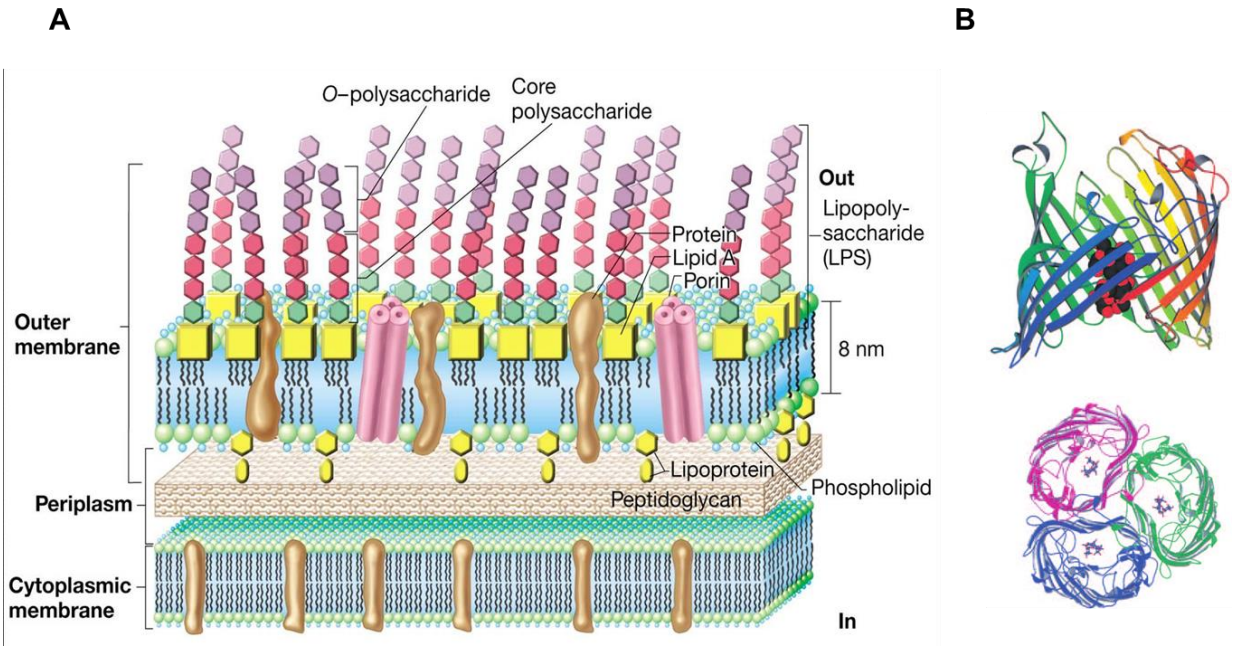

Figure 1.8: Cell Wall of Gram-Negative Bacteria
A) Structure of the Cell Wall of Gram Negative Bacteria. Taken from (87).
B) β-barrel structure and trimeric form of the porin ScrY from STm. Taken from (88).

1.7.1. Lipopolysaccharides

Lipopolysaccharides (LPS) form the outer leaflet of the outer membrane of Gram-negative bacteria. LPS can be divided into three parts: Lipid A, a Core polysaccharide and the O-antigen repeats (89, 90). Lipid A is an amphiphilic glycoprotein that is most commonly composed of six hydrophobic long chained fatty acids that are connected to two glucosamine residues that are linked together by a β-1,6 glycosidic bond (91). The Lipid A portion of LPS is responsible for anchoring this molecule to the outer membrane of the cell wall and its structure is highly conserved. However, lipid A is also responsible for most of the toxic properties of LPS (92). The core polysaccharides are directly attached to lipid A through the inner core, and an outer core connects these to the O-antigen repeats. Both the core region and O-antigen repeats are composed of negatively charged surface exposed carbohydrates that exhibit a high degree of variation between different species and serovars. This diversity may enhance survival or virulence through changes in the structure, length or biochemical properties of LPS. In addition, bacteria can actively modify LPS throughout an infection in order to avoid recognition by the immune system. For example, Salmonella can modify the acetylation pattern of lipid A which might allow it to evade recognition by TLR4(93).

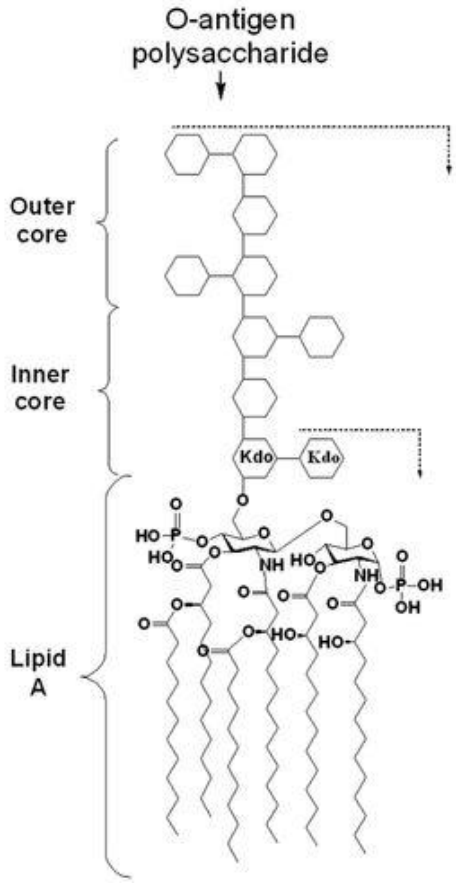

Figure 1.9: Structure of LPS
LPS is composed of three regions known as Lipid A, the Core oligosaccharide and the O antigen polysaccharide. Lipid A is embedded in the outer membrane and alongside the core oligosaccharide form Rough LPS. If these two regions also have an O-chain these collectively form smooth LPS. The lipid A structure is highly conserved and normally has between 4-6 acyl chains. In contrast, the core oligosaccharide and O-antigen polysaccharide varies in composition of sugars, chemical modifications and length of these chains(94). Figure is taken and adapted from (95).

1.7.2. Flagella and Flagellin

Salmonella expresses peritrichous flagella that can allow it to swim, tumble and exhibit chemotactic behavior. The expression of flagella is important for competing for nutrients and is associated with the induction of acute colitis (96, 97). Flagella are composed of three distinct parts known as the basal structure, the hook and the filament. The basal structure is embedded in the inner and outer membrane. It is composed of a Type 3 secretory system (T3SS) needle that is complexed with several different rings that collectively act as a proton driven rotary motor to turn the flagella clockwise or counterclockwise (98, 99). At the outer membrane the hook connects the basal structure to the filament. The filament is composed of several long chains of flagellin monomers, which are exported to the surface of the outer membrane through the T3SS. The flagellin monomers are linked together by covalent bonds to form protofilaments that can be over 10 um in length (99). Approximately 11 protofilaments make up a single flagellum. However, the monomeric composition of the protofilaments can vary. For example, in STm there are two different flagellin molecules that can be expressed, FliC and FljB. Through gene variation STm express FliC or FljB,, but not concurrently(100). Both flagellin monomers are functional and immunogenic. Nevertheless, *in vivo* mouse models of oral infection suggest that most strains of STm express FliC during systemic infection(101). Flagellin is recognized and targeted by both the innate and adaptive immune system during infection with STm (102-106).

1.7.3. Outer Membrane Proteins/Porins

Porins are the most abundant protein found in the OM of Gram-negative bacteria. There are approximately 100,000 porins expressed per cell of STm (107). Porins are trimeric β-barrel structures that span the outer membrane and can exists in homo or heterotrimeric forms (108). Porins allow the passive diffusion of small molecules through the water filled channel of this complex. They are therefore important for the acquisition of nutrients although they have also been associated with antibiotic resistance and virulence (107). A single monomer is typically an oval shape and is composed of 16 β-strands that are connected by extraplasmic loops and periplasmic turns (109). The exact composition can vary with different sizes or numbers of loops and surface charges being reported. Changes in the composition can alter substrate specificity. For example, 16 β-stranded porins are typically associated with passive diffusion of small inorganic molecules, while porins with fewer or more loops such as ScRy, from STm, can allow selective uptake of specific substrates such as sucrose(109, 110). In the OM of STm the main porins that can be found are OmpC, OmpF and OmpD. OmpC and OmpF are expressed in the OM of STm, ST and E.coli. In contrast, OmpD is expressed in all serovars of *Salmonella*, except ST (111).

1.8. Salmonella Entry & Colonization

Following ingestion, *Salmonella* can survive the hostile environment of the gut and reach the terminal ileum. Here the bacterium can enter the host through exploiting a range of virulence factors that enable it to invade both phagocytic and non-phagocytic cells (112). One portal of entry is through epithelial cells. *Salmonella* utilises fimbriae to adhere to

the epithelial cells and induces its uptake through T3SS-1 (encoded by SPI-1) mediated secretion of effector proteins into the cytosol (71). Alternatively, DCs can extend between epithelial cells to sample antigens from the gut and directly engulf Salmonella. A more common and successful route of invasion is through the Microfold (M) cells of the Peyers Patches. M cells are more vulnerable to pathogens than enterocytes as they lack a glycocalyx. In addition, they are continually sampling gut antigens and transcytosing them to the underlying lymphoid tissue (113). As a consequence M cells rapidly translocate *Salmonella* across the epithelial barrier allowing them to access the underlying mucosa-associated lymphoid tissues. The innate immune system helps restrict the dissemination of the bacteria and limit its growth to the gastric mucosa. This is associated with a rapid influx of granulocytes/neutrophils. In contrast, this inflammation is less pronounced in enteric fever. However, in immunosusceptible hosts the innate immune response may fail to achieve this and the pathogen can spread to the mesenteric lymph nodes, then to the blood and from here it can disseminate to various organs such as the spleen, liver and in some cases, the gallbladder and bone marrow, in order to establish a systemic infection (114).

1.9. Innate Immune Response to STm

TLR5 is expressed on the basal membrane of gastric epithelial cells. When STm breaches the gut barrier it is initially recognized through the binding of TLR5 to flagellin. This induces NFkB signaling that results in the release of pro-inflammatory cytokines such as IL-1β, IL-6 and IL-8. In addition, complement activation leads to the production of chemotactic molecules and inflammatory mediators. Neutrophils and macrophages

are recruited to the site of infection/inflammation through the action of IL-8 and C5a (115). These cells recognize the bacteria through TLR2, TLR4 plus TLR5 and attempt to control the growth of the pathogen by phagocytosing the bacteria and destroying it through the actions of ROS and RNI (116, 117). However, attempts to destroy the internalised bacteria are impaired as TLR signalling promotes acidification of the phagosome, which in turn induces the upregulation of SPI-2 and the expression of the T3SS-2 (118, 119). This molecular syringe enables *Salmonella* to secrete effector molecules into the cytosol that hijack the phagosome and prevent its fusion with the lysosome. As a consequence a *Salmonella* containing vacuole (SCV) forms which provides an intracellular niche for bacterial replication and survival (118). Hiding within macrophages and migratory DCs allows *Salmonella* to escape the innate inflammatory response at the gut and facilitates the dissemination of the bacteria to systemic sites (120). Once the bacteria colonises organs like the spleen, liver and gallbladder there is a rapid recruitment of DCs, monocytes, macrophages, neutrophils and NK cells (121). PMNs initially form inflammatory foci that attempt to limit the further spread of the bacteria. The release of TNF from PMNs promotes the formation of macrophage-rich granulomas that inhibit further dissemination of the bacteria. IL-12, IL-15 and IL-18 are predominantly made by DCs and macrophages (115, 122). These cytokines, alongside TLR4 activation, promote the release of IFN-γ from neutrophils, macrophages and NK cells that induces the killing of intracellular *Salmonella* by increasing the rate of phagolysosomal fusion and the production of ROS and RNI (123, 124). Despite the effectiveness of these molecules the innate immune system can only restrict the growth of the bacteria during a systemic infection.

1.10. Cell-Mediated Immune Response to *S*Tm

Although the innate response may control *S*Tm infection in healthy individuals, it only helps limit the growth of bacteria in both mice and immunocompromised patients. In order to eliminate these intracellular reservoirs a strong cell mediated immune response is required. Clearance of a primary infection is dependent upon T_H1 $CD4^+$ T Helper cells and their production of IFN- γ. Evidence of this is that T-bet, TCR alpha-beta and MHC Class II-deficient mice cannot clear attenuated *Salmonella* infections (125-127). In addition, infecting mice deficient in IFN-γ receptor or simultaneous administration of anti-IFN-γ leads to a greater susceptibility to infection and death (127). The exact role of IFN-γ throughout a *S*Tm infection is complex, but in conjunction with TNF, it is known to induce expression of iNOS and NADPH oxidase in macrophages. These enzymes catalyse the production of ROS and RNS, which alongside T_H1 dependent activation of macrophages, results in the disruption of the SCV and the destruction of intracellular *S*Tm (128-131). The development of a strong Th1 response thus enables clearance of *S*Tm around 5 weeks after infection (47). Production of IFN-γ by $CD4^+$ T cells might not be the only method of killing, as in one report *Salmonella* infected mice that have received anti-IFN-γ antibodies administered 16 days post-infection can clear infection at a similar rate to non-treated mice (132). However, studies in patients with IFN-γ receptor or IL-12 receptor deficiencies show such individuals are more susceptible to *Salmonella* infections, thus supporting a crucial role for Th1 cells (133, 134). $CD8^+$ T Cells and γδ-T cells are not required for clearance of a primary infection with attenuated strains of STm (135). Although it should be noted that both B cells and $CD8^+$ T cells may also contribute to immunity albeit to a lesser degree (127).

1.11. B cell response to Salmonella

B cells and antibody can help protect against secondary *Salmonella* infection, but not during a primary response to systemic infection. Although B cells are not required for clearance of a primary infection under model circumstances, the extrafollicular response helps limit the extracellular spread of *Salmonella* and thus reduces the levels of bacteraemia (47, 136, 137). Absence of B cells has been associated with an impaired production of Th1 cells in response to secondary infection, which implies that they are involved in both the development and maintenance of *Salmonella*-specific T cells independent of antibody (138, 139). This suggests that B cells have a role in *Salmonella* infections by presenting antigen and secreting cytokines. Moreover, B cells contribute to recall in response to oral challenge in an antibody independent manner (140). Despite this the main protective role of B cells in *Salmonella* infection is to produce antibody to a variety of antigens such as LPS, flagellin and various outer membrane proteins such as porins (141). Although antibodies to these are not necessary for protection against primary infections with attenuated *Salmonella*, antibodies have been shown to protect against secondary infection with virulent strains (137, 142). In C57BL/6 mice, it has been shown that both EF and GC derived IgG2a/c helps prevent *S*Tm from infecting splenic macrophages (47). As a consequence both humoral and cell mediated immune responses are required for protection against *Salmonella* infections. Evidence supporting this is that the transfer of both serum and T cells into naïve mice is required for protection (125, 138, 143). Antibodies therefore help protect by reducing the amount of bacteria that spread systematically. This is further supported by epidemiological data from Malawi that reports that children are more likely to develop iNTS between 6-36

months of age when maternal antibodies wane (81). However, in order to develop vaccines that induce production of protective antibodies to *Salmonella*, the mechanisms behind how antibody responses are generated must first be understood.

1.12. Kinetics of the EF and GC response during primary infection with STm

Traditionally, the development of the EF and GC response has been investigated largely in the context of proteinaceous antigens which in turn are polarized towards a T_H2 immune response (144). Under these conditions the two phases of the antibody response develop in parallel (145). As a consequence GCs form alongside the EFR by Day 7 and produce plasma cells and memory B cells by Day 14 (144). In the context of an infection with live attenuated *S*Tm, the development of the GC response is delayed in the spleen. For example, the Cunningham laboratory have shown that *S*Tm induces a rapid and extensive extrafollicular response that is dominated by B1b-derived IgM antibodies at Day 3, but after this it switches to IgG2a in a TD manner with IgG2a and IgG2b isotypes dominating from Day 7 onwards (47, 67). This EFR persists until the infection is cleared. In contrast, the formation of germinal centres is impaired as they do not form until 5 weeks after infection (47, 146). This delay in GC formation appears to be related to the bacterial burden. Evidence of this is that treatment of STm infected mice with antibiotics leads to a reduction in splenic bacterial numbers and this coincides with the appearance of germinal centres 20 days post-infection (47). This suggests that *Salmonella* or components of the bacterium may be inhibiting the formation of germinal centres. Atypical antibody response could represent a potential source of self-reactive antibodies.

1.13. Autoimmunity

Autoimmune diseases occur when B and T cell responses attack the host's tissues and fail to be switched off. There are currently over 80 known autoimmune diseases that are organ-specific or systemic. In some developed countries, autoimmune diseases are one of the leading causes of morbidity and mortality (147, 148). The exact cause of autoimmune diseases are unknown as there are multiple factors involved such as the genetics and environment that can modulate the immune system and alters ones susceptibility to developing these conditions. For example, certain genetic HLA alleles can decrease or enhance the likelihood of an individual's cells to present self-peptides. While variations in molecules/receptors that are associated with tolerogenic mechanisms can impair the ability of the immune system to turn off autoreactive responses or enhance the number of autoreactive cells in an individual's immune repertoire. Evidence for the role of genetics in autoimmune disease includes females being more likely to develop conditions such as multiple sclerosis (MS) and systemic lupus erythematosus (SLE) more frequently than males (149). In addition, diseases like Type 1 diabetes have a genetic origin as they run in families (150). However, genetics alone are not responsible for the increasing prevalence or development of autoimmune diseases. For example, there is a low concordance rate in monozygotic twins in many different autoimmune diseases (151-153). Epidemiological studies have also shown that the global incidence of autoimmunity is increasing so quickly that genetics factors cannot account for this (154, 155). There are a wide range of potential environmental stimuli that can enhance or decrease the likelihood of developing autoimmune disease. Examples of these are infections, diet, stress, smoking, occupation and climate (156).

For instance, in the northern hemisphere the incidence of autoimmune disease is higher in the north than it is in the south. For example, in the Mediterranean the risk of developing MS and type 1 diabetes is lower than in northern Europe (157, 158). Many Infections with viruses and bacteria are associated with the induction and exacerbation of autoimmune disease, but not all infections have a negative impact. Helminths for instance are linked with protecting against autoimmune disease (159). Therefore, infections can have a modulatory effect on the risk and outcomes of autoimmunity. Nevertheless, autoimmunity in the context of an ongoing infection is not well studied, particularly in animal models, and therefore our understanding of this relationship is still in its infancy.

1.13.1. Infections and the Induction of Autoimmunity

All healthy individuals have a considerable number of autoreactive B and T cells within their immune repertoires. The prevalence of autoreactive immune cells is a product of imperfect central plus peripheral selection/tolerogenic mechanisms and a requirement for a degree of self-reactivity in order to maintain enough antigenic diversity to respond against invading pathogens. Infections with viruses and bacteria can initiate autoimmune responses or exacerbate existing autoimmune condition during an infection through several antigen-specific and non-specific mechanisms (**Figure 1.10.**) (160, 161). However, the initiation of an autoreactive immune response alone does not result in persistent autoimmune disease. For example, infection with *Mycoplasma pneumoniae* can induce the production of pathogen-specific antibodies that cross-react with erythrocytes and promote their destruction. This can sometimes result in autoimmune

haemolytic anaemia (162, 163). However, this disease typically resolves after 3 weeks. Prolonged inflammatory responses are often required for the development of full blown autoimmune disease. Evidence of this is seen in mice with a *spin* mutation as they develop spontaneous inflammation in the bone marrow, foot pad, lungs and salivary glands. This response does not develop without microorganisms (164). Infections alone may or may not directly trigger autoimmune disease, but they can also potentially enhances ones susceptibility to developing them by increasing the levels of autoreactive immune cells or autoantibodies.

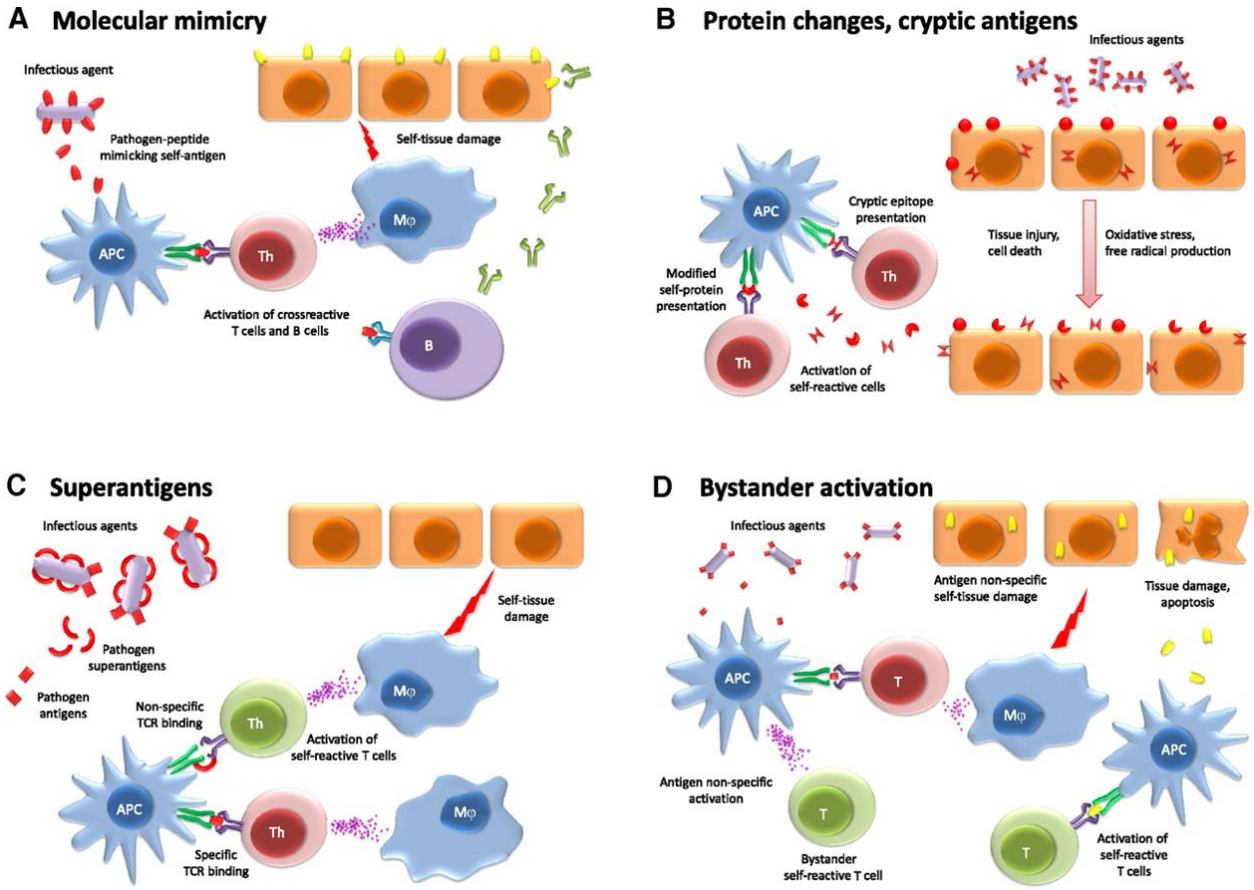

Figure 1.10: How infections can trigger autoreactive immune responses

- **A)** Molecular Mimicry – Foreign antigens that have a similar amino acid sequence or conformation to those in host tissues can activate the adaptive immune response thus leading to the production of antibodies that cross-react with self and induce tissue damage.
- **B)** Inflammation from pathogens can alter transcription, post-translational modifications, antigen processing and antigen presentation. This can lead to the expression of antigens that are not normally presented to self thus resulting in the activation of autoreactive T and B cells.
- **C)** Infections can release superantigens which bind TCRs and induce T cell activation regardless of the antigen specificity. Large populations of T cells can be activated which may or may not induce autoimmune responses.
- **D)** Non-specific activation of T cells due to the surrounding inflammatory environment combined with release of self-antigen due to tissue damage can promote epitope spreading and autoimmune responses.

  Figure is taken from (160).

1.14. Mouse Model of *Salmonella* Infection

S.Typhi does not infect the majority of animals, except humans and primates. However, STm can infect mice and induce systemic disease through dissemination to a range of different organs such as the MLN, spleen, liver and bone marrow (165). Infection of mice with STm therefore provides a model of infection of Typhoid and iNTS. Virulent STm can be used at low doses to infect innately resistant mice to study the innate immune response but these effectively control and clear the infection without the adaptive immune response (166). The majority of genetic knockout mice are generated on an innately susceptible background such as a C57BL/6J or BALB/c genotype. Approximately 50% of these susceptible mice die within one week of infection following inoculation with less than 10 organisms of virulent *Salmonella* (167). This is because they have a G169D point mutation in Natural Resistance Macrophage Protein 1 (Nramp-1), a divalent cation pump, which is important for phagolysosomal killing in macrophages (168). As a consequence they cannot be used to study the development of adaptive immune response to Salmonella. In order to circumvent this problem an attenuated strain of STm, known as SL3261 can be used. This strain cannot metabolise aromatic amino acids and therefore grows at a slower rate than its virulent counterpart (169). Infection of susceptible mice with SL3261 induces a systemic immune response in several different sites that requires innate immunity to control the infection early on, and Th1 cells for the clearance of the bacteria between three to five weeks of infection. This therefore provides a good model to study the action of B cells, T cells and the inflammatory response at several different sites of infection. An i.p. model of infection is used throughout this thesis since administration of SL3261 through the oral route is

subject to large variation in bacterial numbers due to an impaired ability to colonise and infect the mice.

1.15. Aims of this Thesis

TLRs represent an important link between the innate and adaptive immune response. The role of these receptors has been well characterized in innate cells and in response to model antigens. Reports on the exact function of these throughout live infections have been varied, particularly within the context of B cell responses. Some groups have reported that TLRs can influence the speed and strength of antibody responses while others have shown they do not (170-172). In addition, TLRs have been linked to the development of autoimmune diseases (173). Infection with STm induces both a TI and TD antibody response (47, 66). TLR4 is important for the induction of TI-1 responses to LPS and Porins (59, 174, 175). In addition, it has been reported to influence migration of B cells to germinal centres and *in silico* models have suggested that it is crucial for the effective generation of these responses (176, 177). The role of TLR4 in the innate immune response to STm is well characterized, but not in the context of B cell responses. Given that porins and LPS are major components of the outer membrane of STm it was expected that the knowledge acquired from studying this receptor and ultimately how the antibody response is regulated would aid the development of better vaccines or immunotherapies. As a consequence the original aim of this project was to assess the role of TLR4 in the germinal centre and antibody response to STm. However, given differences observed in strain behavior between genetic backgrounds (Chapter 3) and the unexpected relationship between anti-self and anti-*Salmonella* antibodies, the

original project changed to address the hypothesis that: 'Anti-Self antibodies and anti-*Salmonella* antibodies are co-regulated'. The aims of the project were:

A) Chapter 3 - Investigate the role of TLR4 and the host genetic background in the kinetics and magnitude of the antibody response after infection with STm.

Given the detection of self-reactive antibodies in WT mice infected with STm in Chapter 3 and the absence of these in TLR4$^{-/-}$ mice this lead to a change in the aims to address the following:

B) Chapter 4 - Characterise the kinetics of the pathogen-specific and anti-self antibody response induced throughout infection with STm.

C) Chapter 5 - Determine if the production of pathogen-specific antibodies and self-reactive antibodies are co-regulated or independently regulated.

2. MATERIALS & METHODS

2.1. Materials

All reagents and antibodies used were bought from Sigma-Aldrich (Dorset, U.K.) and e-Bioscience respectively, unless otherwise stated. Contents of both all media and buffers are listed in **Appendix A**.

2.2. Mice

All mice were age (6-12 weeks) and sex matched. BALB/c and C57BL/6J mice were purchased from HO Harlan OLAC Ltd (Bicester, U.K) and used as wild-type (WT) controls. $TLR4^{-/-}$ mice on a BALB/c or C57BL/6J background were provided by Dr Andrea Mitchell (University of Birmingham, UK) and Professor Clare Bryant (University of Cambridge, UK) respectively. All transgenic mice used throughout this thesis are listed in **Table 2.1** and were bred and maintained under specific-pathogen free conditions at either the Biomedical Service Unit (BMSU, University of Birmingham) or University of Cambridge. All *in vivo* experiments were performed in accordance with Home Office regulations and with ethical approval from the host institution.

Table 2.1: Genetically Modified Mice

Mouse Strain (Genetic Background)	Phenotype	Provider/Origin
TLR4$^{-/-}$ (BALB/c)	Deficient in TLR4. Cannot recognise and signal in response to TLR4 ligands	Provided by Dr Andrea Mitchell (University of Birmingham, UK). Original Source: OrientalBioService
TLR4$^{-/-}$ (C57BL/6J)	Deficient in TLR4. Cannot recognise and signal in response to TLR4 ligands	Provided by Professor Claire Bryant (University of Cambridge, UK). Original Source: (178)
IFN-γ$^{-/-}$ (C57BL/6J)	Cannot produce IFN- γ.	The Jackson Laboratory Original Source: (179)
T-bet$^{-/-}$ (C57BL/6J)	Impaired development of Th1 cells.	The Jackson Laboratory Original Source: (180)
IL-6$^{-/-}$ (C57BL/6J)	Do not produce IL-6.	Charles Rivers Laboratories Original Source: (181)
p55$^{-/-}$p75$^{-/-}$ (C57BL/6J)	Deficient in TNFR1 (p55) and TNFR2 (p75). Cannot bind and signal in response to TNF.	Provided by Dr Jorge Caamaňo (University of Birmingham, UK) The Jackson Laboratory Original Source: (182)
ICOSL$^{-/-}$ (C57BL/6J)	Deficient in ICOSL and as a consequence cannot signal through ICOS.	Provided by Dr David Withers (University of Birmingham, UK) Original Source: (183)
CD80$^{-/-}$CD86$^{-/-}$	Deficient in CD80 and CD86	Provided by Dr David Withers

(C57BL/6J)	leading to impaired signalling.	(University of Birmingham, UK) Original Source: (184)
TCR-βδ$^{-/-}$ (C57BL/6J)	Deficient in αβ and γδ T Cells.	The Jackson Laboratory Original Source: (185)

2.3. Preparation of Bone Marrow chimeras

Mouse serum was kindly provided by Dr Ruth Coughlan and Dr Ewan Ross. All bone marrow chimaera experiments were made and performed by Dr Ruth Coughlan between September 2008-2011 and Dr Ewan Ross at the University of Birmingham. Detailed descriptions of these experiments can be found in her thesis (186). In brief, C57BL/6J and IL-$6^{-/-}$ or IFN-$\gamma^{-/-}$ mice were irradiated with 9 Grays of γ-radiation. Bone marrow cells from either C57BL/6J, IL-$6^{-/-}$ or IFN-$\gamma^{-/-}$ mice were prepared by flushing both the tibia and femur with 10 mL of RPMI 1640 supplemented with 10% FCS and 1% Penicillin/Streptomycin (R10) media into a falcon tube. Cells were then pelleted by centrifugation at 350 x g for 4 minutes and then re-suspended in sterile PBS and counted. A 200 µL volume of cells were then injected i.v. into the recipient/irradiated mice to give a total of 10^7 cells per mouse. Mice were then left to recover for 12 weeks before being immunised i.p. with either PBS or SL3261 as described in **section 2.4**.

2.4. Preparation of SL3261 and Infection Protocol

In order to study the adaptive immune response to STm an attenuated *aro*A deficient strain known as SL3261 was used. SL3261 grows slower than other strains of STm due to its inability to metabolise aromatic amino acids (169). Colonies of SL3261 were grown by streaking STm on agar plates and incubating them overnight at 37° C. A single SL3261 colony was then used to inoculate 10 mL of sterile LB and was grown in a 37° C rotary incubator at 180 rpm. Bacterial growth was measured through UV-Visible light spectroscopy and then harvested at an absorbance of 600 nm by centrifugation for 5 minutes at 6000 x g. The bacteria were washed and diluted in sterile PBS to form a final

concentration of 5 x 10^5 CFU of SL3261 per 200 µl. Mice were then immunised *i.p.* with 200 µl of PBS or infected with 5 x 10^5 CFU of SL3261.

2.5. Experimental End-point

At the end of the experiment mice were anesthetised with Isoflurane and between 0.5-1 mL of blood was acquired through cardiac puncture. Mice were sacrificed through cervical dislocation in accordance with the approved schedule 1 methods. Blood was then transferred into 1 mL Eppendorf tubes and stored in ice before being centrifuged at 6000 x g for 10 minutes in order to separate serum from blood. Serum was then aliquoted into Eppendorf's and stored at -80 ° C.

2.6. Bacterial culture from infected organs

The kidneys, liver and spleen were removed from infected mice after they were sacrificed by cervical dislocation. Organs were weighed and partitioned into three parts for histology, flow cytometry and bacterial culture. The portion of the organs for bacterial culture were homogenised through a 70 µm cell strainer into 1 ml of RPMI 1640 Medium (RPMI). The cell homogenate was then serially diluted (1/10,1/100 and 1/1000) in RPMI. Sterile LB agar plates were then inoculated with 100 µL of cell homogenate and evenly distributed with a cell spreader. Plates were incubated overnight at 37 ° C and the number of colony forming units (CFU) was enumerated. The total number of CFU per organ was calculated using the following equation: Total CFU = CFU per plate x 10 x Dilution Factor x (Organ Mass/Bacterial Culture Mass).

2.7. Histology

Spleens were prepared for immunohistochemistry and stained as previously described (102, 187, 188).

2.7.1. Preparation and sectioning of organs for Immunohistochemistry

Spleens were frozen in liquid nitrogen and sliced into 5 µm sections using a cryostat. Sections were fixed in acetone at 4 °C for 20 minutes and then air dried for 10 minutes, before being stored in polythene bags at -20 ° C until required.

2.7.2. Immunohistochemistry

Spleens sections were defrosted for 30 minutes at RT and removed from the polyethylene bags and placed in Tris buffer pH 7.6. Primary antibodies were diluted in Tris buffer pH 7.6. Slides were then incubated with 75µL of primary antibody (**See Table 2.2**) for 1 hour in a humidity chamber at RT. Excess liquid was removed and slides were washed for 5 minutes in Tris buffer pH 7.6. Secondary antibodies were then adsorbed with 10% mouse serum for 30 minutes and then diluted to the working concentration with Tris buffer pH 7.6. Slides were incubated with 75 µL of secondary antibody for 1 hour in a humidity chamber. They were then washed in Tris buffer pH 7.6 for 5 minutes. Secondary antibodies were either biotinylated or conjugated to horse radish peroxidase. In order to attach an enzyme to the biotinylated antibodies an alkaline phosphatase complex (AB) was prepared using a VectaStain ABC kit (Vector Laboratories LTD, UK) by incubating streptavidin (1/100) with biotinylated AP (1/100) in Tris buffer for 30 minutes at RT. Slides were incubated for 30 minutes in a humidity chamber with 75 µL

of streptavidin-AP complex. Slides were then stained using diaminobenzidine tetrahydrocholoride (substrate for horse radish peroxidase) and AS-MX phosphate with Fast Blue salt plus levisamole (substrate for alkaline phosphatase). To stop the staining reaction slides were washed in Tris buffer pH 7.6 and then dH_2O. Finally the slides were air dried and then mounted in VectaMount (Vector Laboratories LTD, UK). Images were taken using a Leica DM6000B microscope and LAS X software.

Table 2.2: Antibodies used for Immunohistochemistry

Antibody	Conjugate	Stock Concentration (mg/mL)	Dilution	Source
Primary Antibodies				
Goat anti-Mouse IgG2c	Alkaline Phosphatase	1	1:100	Southern Biotech
Hamster anti-Mouse CD3	None	0.5	1:300	BD Biosciences
PNA	Biotin	5	1:100	Vector Laboratories
Rat anti-Mouse CD3	None	0.25	1:500	AbD Serotec
Rat anti-Mouse IgD	None	0.5	1:500	BD Biosciences
Rat anti-Mouse IgG2b	None	0.25	1:200	AbD Serotec
Rat anti-Mouse IgM	None	0.25	1:500	AbD Serotec
Sheep Polyclonal anti-Mouse IgD	None	None – 2mL	1:1000	AbCam
Secondary Antibodies				
Donkey anti-Sheep IgG (H+L)	Horseradish Peroxidase	0.8	1:100	Jackson Laboratories
Polyclonal Rabbit anti-Rat Immunoglobulins	Biotin	0.85	1:600	Dako
Polyclonal Rabbit anti-Rat Immunoglobulins	Horseradish Peroxidase	1.3	1:50	Dako
Goat anti-Hamster IgG (H+L)	None	1.5	1:100	Vector Laboratories

2.7.3. Plasma Cell Counting

All splenic IgM^+ and $IgG2c^+$ plasma cells were viewed down a light microscope at a 100 x magnification and counted within a $1cm^2$ eyepiece graticule. The total number of cells per graticule was enumerated across 6 different parts of the spleen in order to cover the whole tissue. Average number of splenic IgM^+ or $IgG2c^+$ plasma cells per 1 cm^2 was calculated and plotted using GraphPad Prism 7.0.

2.8. Flow Cytometry

All antibodies used for extracellular and intracellular staining can be found in **Table 2.3**.

2.8.1. Preparation of single cell suspensions

Spleens were mashed through a 70 µm cell strainer into R10 medium (RPMI + 10% FBS + 1% Pen/Strep) and the homogenate was pelleted in a centrifuge at 375 x g for 4 minutes. Cells were then re-suspended in 500 ml of ACK lysis buffer for 3-5 minutes in order to deplete erythrocytes. Cells were then washed, counted on a haemocytometer and then diluted so that the same number of cells (typically 2-3 x 10^6 cells / well) could be plated in a 96 well V bottom plate (Corning Life Sciences, Netherlands).

2.8.2. Extracellular Staining

Splenocytes were washed in FACs buffer, centrifuged and incubated on ice with antibodies to CD16/CD32 (e-Bioscience) for 15 minutes in order to block Fcγ receptors I and II. Cells were then washed in FACs buffer before being incubated on ice for 20 minutes with 75 µL of antibodies targeted against various surface markers, (**Table 2.3**) in order to identify different lymphocyte populations. Samples that were only stained for

extracellular markers were then fixed in FACs buffer with 0.01% PFA and acquired (See 2.8.4).

2.8.3. Intracellular Staining

Following extracellular staining and fixation with 0.01% PFA, cells were intracellular stained for plasma cells (**2.8.4**). For intracellular staining of cytokines/transcription factors please see **Section 2.8.5**.

2.8.4. Intracellular Staining of Plasma Cells

Cells were incubated with cytofix/cytoperm (BD Biosciences) for 20 minutes at 4 °C in order to fix and permeabilise the cells. Samples were then washed in 1 x Permwash (BD Biosciences) and incubated with antibodies for 30 minutes at 4 °C to stain intracellular molecules. Samples were then washed twice in permwash and re-suspended in FACs buffer and transferred to FACs tubes for data acquisition (See 2.8.4).

2.8.5. Ex-*vivo* re-stimulation and Intracellular Staining for cytokines/transcription factors

A 48 well flat-bottomed plate was coated with or without 10 µg/mL of anti-CD3 (BD Biosciences, UK) overnight at 4°C. The following day excess antibodies were removed and plates were washed twice with 500 µL per well of sterile PBS. Splenocytes prepared in R10 media were added in a 500 µL volume to the well in order to give a final concentration of 6 x 10^6 cells per well. Splenocytes were then co-stimulated with or without 1 µg/mL of anti-CD28 alongside plate bound anti-CD3 for 6 hours at 37°C, 5% CO_2. For the last 2 hours of the incubation 10 µg/mL Brefeldin A was added to each well

in order to stop the secretion of cytokines. Cells were then extracellular stained and fixed in PFA as mentioned in **section 2.8.2**. Intracellular staining was carried using an eBiosciences Transcription Factor Staining kit and in accordance with the manufacturers guidelines.

Table 2.3: Antibodies for Flow Cytometry

Antibody Target (Fluorophore)	Stock Concentration (mg/ml)	Dilution	Source (Clone)	Application/Staining Panel
Fcγ Receptor Blocking				
CD16/CD32 (None)	0.5	1:150	eBioscience (24G2)	Blocks Fcγ receptors II and III.
Ex-*vivo* Re-Stimulation				
Anti-CD3	1	1:100	eBioscience (17A2)	Stimulate T Cells
Anti-CD28	1	1:1000	BD Bioscience (JJ319)	Co-stimulate T Cells
Extracellular Staining				
B220 (Brilliant Violet 510)	0.2	1:250	Invitrogen (RA3-6B2)	B Cells GC B Cells T Cell Panels Plasma Cells
B220 (PE-Texas Red)	0.2	1:250	BD Biosciences (RA3-6B2)	B Cell Panel
CD3 e (FITC)	0.5	1:100	eBioscience (145-2C11)	GC B Cells T Cell Panels
CD3 (Pacific Orange)	0.2	1:100	Biolegend (17A2)	B Cell Panel
CD4 (eFluor450)	0.2	1:100	eBioscience (RM4-5)	T Cell Panels
CD5 (PE-Cy5)	0.2	1:100	BD Biosciences (53-7.3)	B Cell Panel
CD8 α (APC)	0.5	1:300	eBioscience (CT-CD8a)	T Cell Panels
CD11b (APC)	0.2	1:1000	eBioscience (M1/70)	B Cell Panel
CD11c (PE-Cy7)	0.2	1:300	BD Biosciences (HL3)	T Cell Panels
CD19 (APC)	0.2	1:100	BD Biosciences (1D3)	Plasma Cells
CD21 (FITC)	0.5	1:100	BD Biosciences (76G)	B Cell Panel

CD23 (PE)	0.2	1:100	BD Biosciences (B3B4)	B Cell Panel
CD38 (PE-Cy5)	0.2	1:200	eBioscience (90)	GC B Cell Panel
CD44 (PerCP-Cy5.5)	0.2	1:200	eBioscience (IM7)	T Cell Panels
CD62L (PE)	0.2	1:200	eBioscience (MEL-14)	T Cell Panels
CD138 (PE)	0.2	1:200	BD Biosciences (281-2)	Plasma Cells
FAS (PE-Cy7)	0.2	1:300	BD Biosciences (Jo2)	GC B Cell Panel
GL7 (eFluor450)	0.2	1:150	eBioscience (GL-7)	GC B Cell Panel
IgD (eFluor450)	0.2	1:200	eBioscience (11-26c)	B Cell Panel
IgM (PE-Cy7)	0.2	1:200	eBioscience (II-41)	B Cell Panel
NK1.1 (PE-Cy7)	0.2	1:300	BD Biosciences (PK136)	T Cell Panels
PD1 (PE)	0.2	1:200	BD Biosciences (J43)	T Cell Panels
TCR-β (PerCP-Cy5.5)	0.2	1:300	eBioscience (H57-597)	GC B Cell Panel
Intracellular Staining				
IFN-γ (APC)	0.2	1:200	BD Biosciences (X.MG1.2)	T Cell Panels
IgG2a (FITC)	1	1:500	Southern Biotech	Plasma Cells
IgG2b (FITC)	1	1:500	Southern Biotech	Plasma Cells
IgG2c (FITC)	1	1:500	Southern Biotech	Plasma Cells
IgM (PE-Cy7)	0.2	1:500	eBioscience (II/41)	Plasma Cells
T-bet (PE-Cy7)	0.2	1:300	eBioscience (eBio4B10)	T Cell Panels
TNF (PE)	0.2	1:200	eBioscience (MP6-XT22)	T Cell Panels

2.8.6. Data Acquisition and Analysis

Data was acquired on a CyAn ADP flow cytometer (DakoCytomation) and analysed using FlowJo 8.7 or 9.3.2 software (TreeStar).

2.9. Antigens

All antigens were prepared in coating buffer for ELISA or ELISPOT applications. TLR grade smooth form LPS derived from *Salmonella* Typhimurium was purchased from Enzo Life Sciences LTD, UK. Porins from *Salmonella* Typhimurium were kindly provided by Professor Constantino López-Macías (Mexican Institute for Social Security, Mexico) and the extraction and purification of these have been described elsewhere (189). Outer membrane proteins from *Salmonella* Typhimurium (SL3261) were isolated in house according to the protocol outlined in **section 2.9.1**.

2.9.1. Preparation of *Salmonella* Typhimurium Outer Membrane Proteins (OMPs)

SL3261 was streaked on an LB agar plate and incubated at 37°C overnight. The following day 10 mL of LB broth was inoculated with a single colony of SL3261 and incubated overnight at 37°C and a speed of 180 rpm. Cells were pelleted through centrifugation at 10,000 x g for 4 minutes at 4 °C. Pellets were then re-suspended in 50 mL of 10mM Tris Buffer pH 7.4. Cells were pelleted again and re-suspended in 50 mL of 10mM Tris Buffer pH 7.4 with 2mM phenylmethylsufonyl fluoride in order to inhibit serine proteases. Bacterial cells were then lysed through exposure to 15,000 psi in a C3 Homogenizer. Supernatants were collected and unbroken cells were removed through centrifugation at 6,000 x g for 10 minutes at 4 °C. The cell membrane and cytoplasmic

contents were separated through centrifugation at 30,000 x g for 90 minutes at 4 °C. Pellets were re-suspended in 20 mL of 10mM Tris buffer pH 7.4 with 2% (v/v) Triton X-100 and incubated for 15 minutes at 25°C in a water bath to solubilise the inner membrane. In order to separate the outer membrane (OM) from the inner membrane (IM), samples were spun at 30,000 x g for 90 minutes at 4 °C. The supernatant containing the IM was then discarded and the pellet that contains the OM was re-suspended in 1mL of 10 mM Tris Buffer pH 7.4 and then centrifuged at 6,000 x g for 10 minutes. The OM fraction was washed 2 more times and the composition confirmed by SDS PAGE. Finally the concentration of outer membrane proteins (OMPs) was assessed by using the Bicinchoninic acid assay (Thermo Fischer Scientific, UK) in accordance with the manufacturer's guidelines.

2.10. B cell Enzyme-Linked ImmunoSpot (ELISPOT)

LPS or porins were prepared in coating buffer at a concentration of 5 µg/mL and MultiScreen 96 well filtration plates were coated with 50 µL per well. Plates were then incubated overnight at 4 °C. Plates were washed with PBS to remove unbound antigen and blocked with R10 media for 1 hour at 37 °C to reduce non-specific binding. Cells from the spleen or bone marrow were prepared in R10 media and plated in triplicate at a concentration of 250,000 cells per well. Plates were then incubated for 6 hours at 37 °C and 0.05% CO_2, to stimulate antigen secreting cells. Following incubation the plates were washed with 0.05% PBS-Tween in order to lyse cells and remove non-specific binding. LPS or porin specific antibodies were then detected using a goat anti-mouse

IgM or IgG antibody conjugated to Alkaline phosphatase at 1/1000 in ELISPOT diluent (Thermo Fisher Scientific). The plates were then incubated for 1 hour at 37 °C and then washed three times in 0.05% PBS-Tween and once in PBS. SIGMA FAST BCIP/NBT tablets were each diluted in 10 mL of dH_2O and spots were developed for 5-8 minutes. The reaction was stopped by washing plates with dH_2O. Plates were then left to dry and the number of spots counted using an ELISPOT plate reader.

2.11. Enzyme-Linked Immunosorbent Assay (ELISA)

In order to measure the relative titres of various isotypes of *S*Tm specific antibodies produced in response to infection with SL3261, MaxiSorp Nunc flat-bottom 96 well plates (Thermo Fisher Scientific) were coated with 5 µg of STm outer membrane proteins (OMPs) overnight at 4 °C. Plates were then washed in 0.05% PBS-Tween-20 and blocked with 1% BSA for 1 hour at 37 °C. Following incubation, plates were washed in 0.05% PBS-Tween-20. Plates were then incubated for 1 hour at 37 °C with sera that was serially diluted 4 x in 1% BSA made in 0.05% PBS-Tween-20. Plates were then washed and incubated for 1 hour at 37 °C with either IgM (1/2000), IgG (1/1000), IgG1 (1/1000), IgG2a (1/1000), IgG2b (1/1000) or IgG3 (1/1000) goat anti-mouse antibodies conjugated to alkaline phosphatase (AP) (SouthernBiotech, USA). To detect antibody titres the plates were washed and then incubated at 37 °C with Sigma-Fast p-Nitrophenylphosphate tablets. The conversion of this substrate to a chromogenic product by AP was then detected by measuring the absorbance of each well at 405 nm using an Emax Precision Microplate reader.

2.12. Mouse Heart, Liver and Kidney ELISA

The heart, liver and kidneys were removed from a non-immunised (N.I.) mouse and mashed through a 70 µm cell strainer into R10 medium (RPMI + 10% FBS + 1% Pen/Strep). The homogenate was then centrifuged at 375 x g for 4 minutes. Cells were then re-suspended in 1 mL of ACK Lysis buffer for 5 minutes and then centrifuged. Pellets were re-suspended in 20 mL of R10 medium and 100 µl of cells was added to each well of a MaxiSorp Nunc flat-bottom 96 well plate (Thermo Fisher Scientific). These were then incubated overnight at 4 °C. All remaining ELISA steps were followed as mentioned in **Section 2.11**.

2.13. Fluorescence Microscopy

NOVA Lite Rat Liver, Kidney and Stomach (Inova Diagnostics) or HEp-2 slides were kindly provided by Dr Fabrina Gaspal of the Lane Group (University of Birmingham, UK) or Dr Tim Plant (University of Birmingham, UK). Slides were air dried in the bag for 30 minutes until they reached room temperature (RT). They were then blocked with 10% goat serum for 10 minutes at RT. Slides were washed in PBS and all sera were diluted either at a 1/10 or 1/20 in PBS. Slides were incubated with sera for 30 minutes at RT. They were then washed in PBS and incubated with either a Goat F(ab')2 Anti-Mouse IgG (H+L), Human Ads FITC conjugate (1/100) (Invitrogen) or one of the other antibodies listed in **Table 2.4** for 30 minutes at RT. Finally slides were washed in PBS, cell nuclei were stained with DAPI (Invitrogen) and slides mounted in prolong gold (Invitrogen). Slides were then wrapped in aluminium foil and stored at 4 °C until

required. Images were taken at a 10 x magnification using a Leica DM6000B wide field epifluorescence microscope and Leica LAS X software (Zeiss, Germany).

2.14. Statistical Analysis

The non-parametric Mann-Whitney U test was used to test for statistical significance. All p values were calculated using GraphPad Prism 7.0 and only values <0.05 were considered statistically significant. P values are represented as follows: * $P \leq 0.05$, **$P \leq 0.01$ or ** $P \leq 0.01$.

3. CHAPTER 3 – DIFFERENTIAL ROLE OF TLR4 AND THE GENETIC BACKGROUND OF THE HOST IN THE IMMUNE RESPONSE TO *SALMONELLA* TYPHIMURIUM

3.1. INTRODUCTION

TLR4 is a key component of the innate and adaptive immune system. It is expressed on a wide range of cells and is involved in many different processes such as the production of pro-inflammatory cytokines, recruitment of immune cells, antigen processing, antigen presentation, phagocytosis, immunosuppression, wound healing and the development of adaptive immune responses (190).

In the innate immune response, the role of TLR4 in the defense against Gram-negative bacteria has been well characterized (191-193). For example, C3H/HeJ mice, which are known to have impaired TLR4 signaling (194), are resistant to endotoxic shock, but have a significantly higher mortality rate following infection with STm and display a greater susceptibility to infection with *Neisseria meningitidis* (178, 195-197). In addition, it has been shown that TLR4 and MyD88 signalling can control the growth of virulent STm *in vivo*, at times when innate immunity is vital (116). The key role of this receptor can be further demonstrated by the fact that following infection with STm, TLR4 can compensate for a deficiency in TLR2 (198), TLR5 (199) and TLR9 (83, 200). However, it should be noted that the exact significance in humans it not well defined and that GWAS looking at how TLR4 polymorphisms influence susceptibility to enteric fever are contradictory (83, 201, 202). Despite the crucial role of TLR4 in the innate immune

response to *in vivo* models of infection with STm, the role of this receptor in adaptive immunity is less clear.

Clearance of STm infections requires T cells and in particular Th1 cells that through production of IFN-γ can activate infected macrophages to kill intracellular bacteria (135, 203). TLR4 agonists such as LPS and MPL have largely been shown to induce Th1 polarised immune responses in many different contexts (204). In addition, LPS has been reported to induce non-cognate production of IFN-γ from *Salmonella* experienced $CD4^+$ T Cells (205). Despite this, normal $CD4^+$ and $CD8^+$ T cell responses have been reported in the absence of TLR4 (206) and MyD88 signaling (207).

Reports on the role of TLRs in the antibody response to *Salmonella* or antigens derived from this pathogen have been conflicting. Loss of TLR signaling has been associated with an inability to establish long lived plasma cells after viral infections (208), yet greater protection is observed after virulent challenge with *Salmonella* (206, 207). On the contrary, $MyD88^{-/-}$ B cell chimaeras reportedly have weaker antibody responses to *Salmonella* but in these mice all TLRs except TLR3 are affected (209). TLR4 has been reported to be required for optimal antibody responses to *S*.Typhi porins (174). Its expression on B cells has been found in adoptive transfer experiments to also be important for primary IgM responses (174). However, others have observed normal IgM and IgG antibody responses in TLR2, TLR4 and MyD88 deficient mice following oral (210) or intravenous infection with *Salmonella* (206).

Antibody responses to STm are atypical compared to those formed against model antigens such as alum-precipitated proteins. Previous work from the Cunningham lab has shown that the extrafollicular and germinal centre response after *Salmonella* infection develop at different times (47). In addition, the extrafollicular antibody response is known to be a source of both T-independent and T-dependent derived low/moderate affinity antibodies. While at later time-points the germinal centre gives rise to high affinity antibody secreting cells in a T-dependent manner. A single cell of STm is estimated to contain between 2-4 million molecules of LPS (211) and approximately 200,000-400,000 porins (107). Given that LPS and porins are well-known TLR4 agonists and induce T-independent antibody responses it was decided to investigate this further (59, 67).

The aim of this chapter was to investigate the role of TLR4 in the primary antibody response to systemic infection with STm.

3.2. RESULTS

3.2.1. Absence of TLR4 impedes clearance of the attenuated strain of *Salmonella* Typhimurium, SL3261

Since TLR4 can help restrict the *in vivo* growth of both attenuated and virulent strains of *Salmonella* Typhimurium (116) in the innate phase, we hypothesized that TLR4$^{-/-}$ mice would exhibit poorer control of STm infection compared to WT mice. To test this, BALB/c and TLR4$^{-/-}$ mice were infected with 5 x 10^5 SL3261. The bacterial burden and splenic mass was assessed at various time points. The bacterial burden was at its highest in the spleen one week after infection and decreased with time to less than 2000 CFU after 35 days in WT mice. After one week of infection the number of bacteria in the spleen was slightly higher in the TLR4$^{-/-}$ mice. Splenic bacterial numbers were significantly greater in the TLR4 knockout compared to the WT, both 14 and 21 days post infection. The bacterial burden was marginally higher in the TLR4$^{-/-}$ mice after 35 days of infection (**Figure 3.1.A**).

After STm infection higher bacterial numbers in the spleen are normally associated with a greater spleen size. The spleen size of the WT mice and TLR4$^{-/-}$ mice increases until it peaks at day 21 and then declines to 0.19-0.46 grams, 35 days post-infection. Nevertheless, in the first two weeks after infection the TLR4$^{-/-}$ mice have a spleen approximately half the size of the WT mice, despite having a higher bacterial burden (**Figure 3.1.B**). Therefore, TLR4 either directly or indirectly influences the kinetics of splenic expansion.

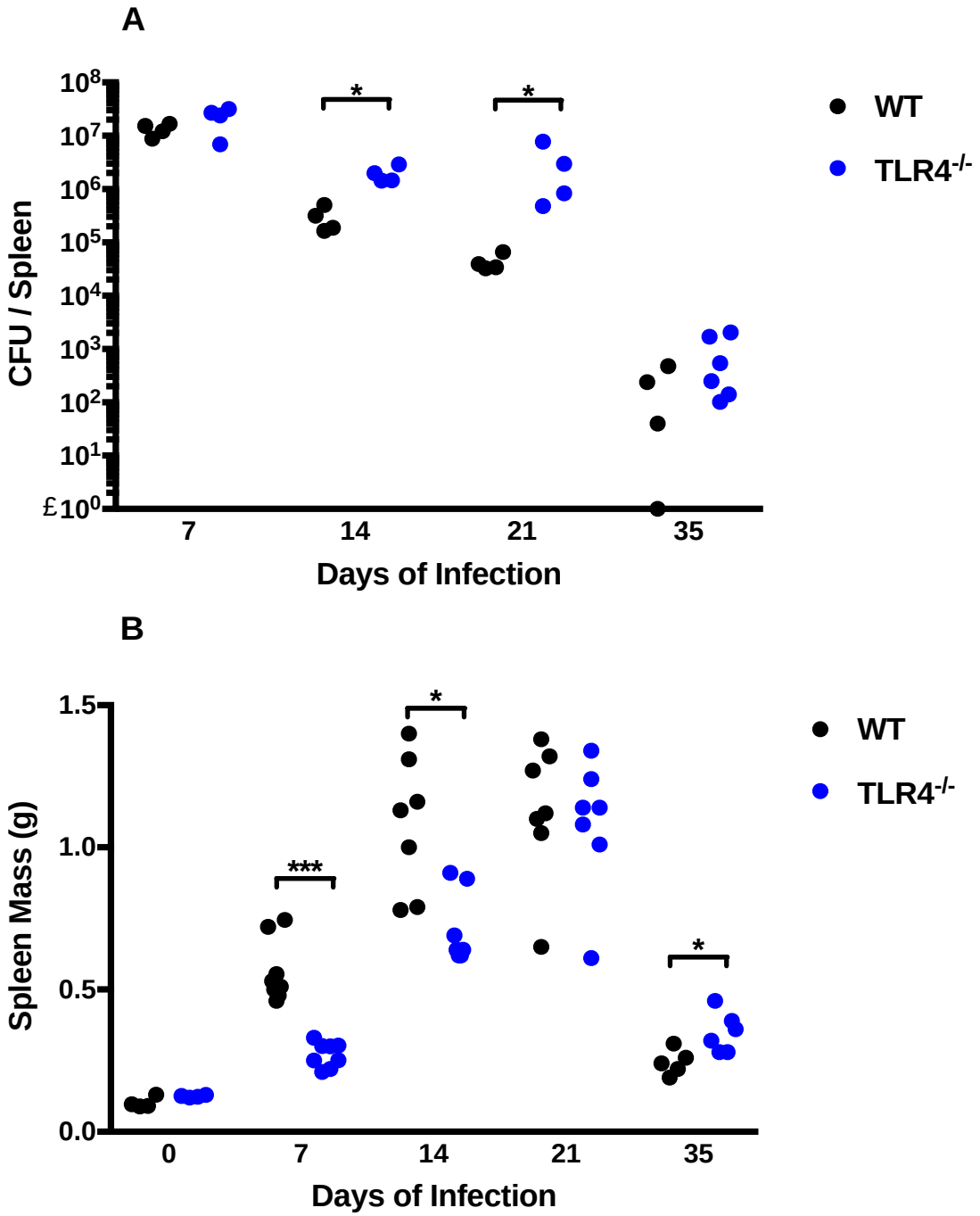

Figure 3.1: Bacterial burden and spleen mass of TLR4$^{-/-}$ mice following infection with STm. BALB/c and TLR4$^{-/-}$ mice were injected with PBS or infected *i.p.* with 5 x 10^5 SL3261 and sacrificed at 7, 14, 21 & 35 days post-infection. **A)** Bacteria were cultured from the spleen at various time points and the total number of colony forming units (CFU) was calculated. **B)** Spleen mass. Each point represents a single mouse. Data is either (**A**) representative or (**B**) pooled from two independent experiments with 3-6 mice per group per time point per experiment, for all time points, except 35 days post-infection. * P<0.05, **P<0.01 & ***P<0.001.

3.2.2. TLR4 is required for extrafollicular IgG2a class switching in response to STm.

The extrafollicular antibody response to STm is known to have both T-independent and T-dependent components, which is largely composed of IgM and IgG2a/c plasma cells respectively (47). Given that optimal responses to both LPS and porins require TLR4, it was hypothesized that TLR4 would influence the magnitude of the EF antibody response. In order to test this, spleen sections from N.I. or STm infected mice were stained for IgM^+ and $IgG2a^+$ plasmablasts/plasma cells (**Figure 3.2.**). Both IgM^+ and $IgG2a^+$ plasma cells were produced in response to infection with STm in BALB/c and $TLR4^{-/-}$ mice. There was no visible difference in the number of IgM^+ plasma cells at day 7 in the infected BALB/c and $TLR4^{-/-}$ mice. In addition, there was no difference detected in the density of IgM and IgG2a cells by histology in the spleen (**Figure 3.3.A**). In contrast, the number of $IgG2a^+$ cells produced following infection was significantly lower in the absence of TLR4 (**Figure 3.3.B**).

Outer membrane proteins (OMPs) from STm contain a range of different antigens such as porins, flagellin and LPS. As a consequence they can be used to assess the overall quality of the antibody response against STm. Anti-OMPs titres were measured 7 days post-infection in the presence and absence of TLR4. There was a clear induction of anti-OMPs IgM, IgG, IgG2a and IgG2b in both mouse strains (**Figure 3.4.**). No difference could be detected in the anti-OMPs response in the presence or absence of TLR4, despite the impaired IgG2a class switching observed by histology. One possible explanation for this is that a few antibody secreting cells (ASCs) could be producing lots

of antibody against the pathogen and therefore compensate for the impaired class switching one week post-infection.

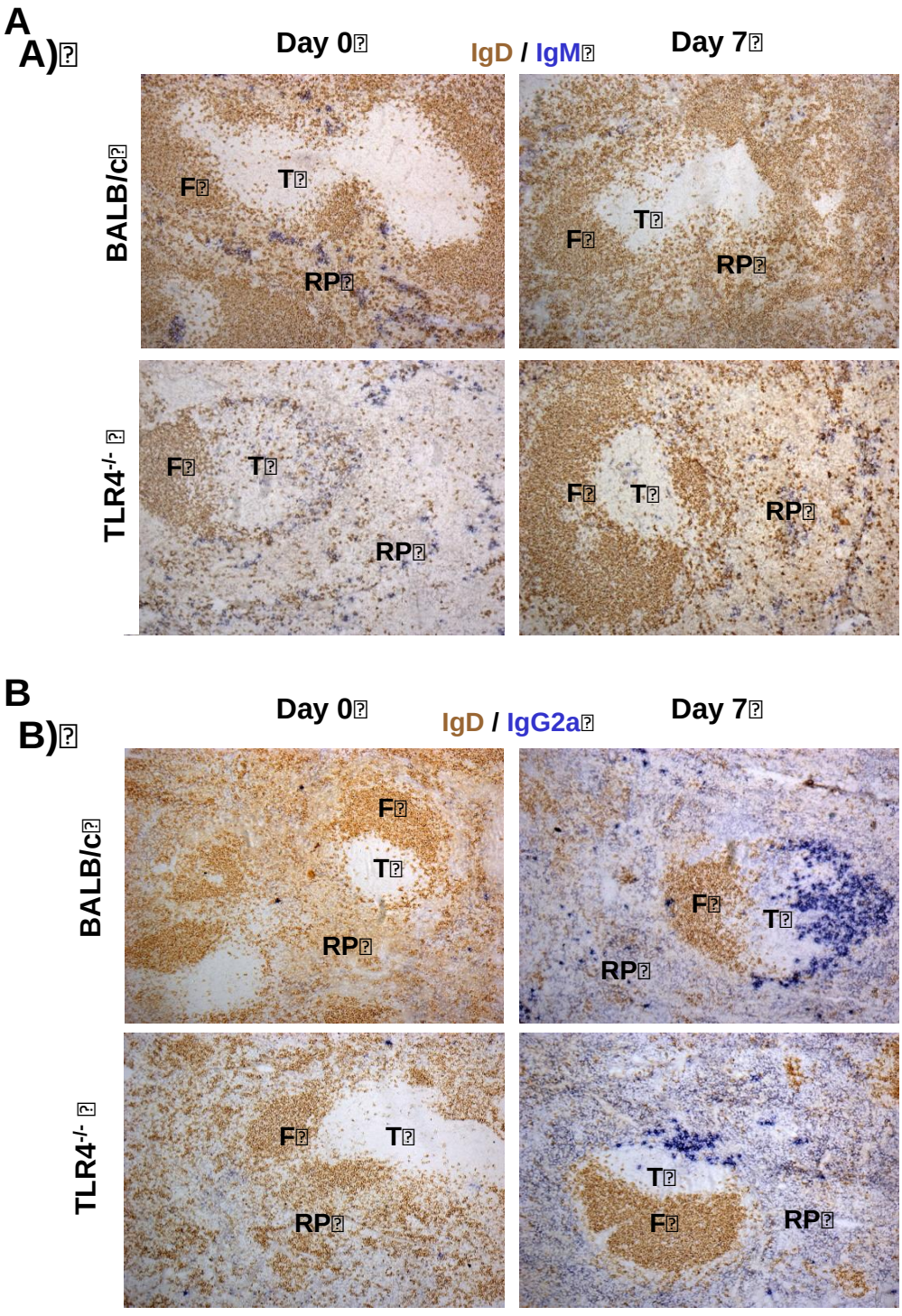

Figure 3.2:Impaired extrafollicular IgG2a class switching in TLR4$^{-/-}$ mice.
BALB/c and TLR4$^{-/-}$ mice were infected i.p. with 5 x 10^5 SL3261 and sacrificed by cervical dislocation 7 days post-infection. Spleen sections were stained for IgD (brown) and either **A**) IgM (blue) or **B**) IgG2a (blue) plasma cells. Images were taken at a x20 magnification and are representative of two independent experiments with 4 mice per group per time point per experiment. **F- Follicle, RP-Red Pulp & T-T cell zone.**

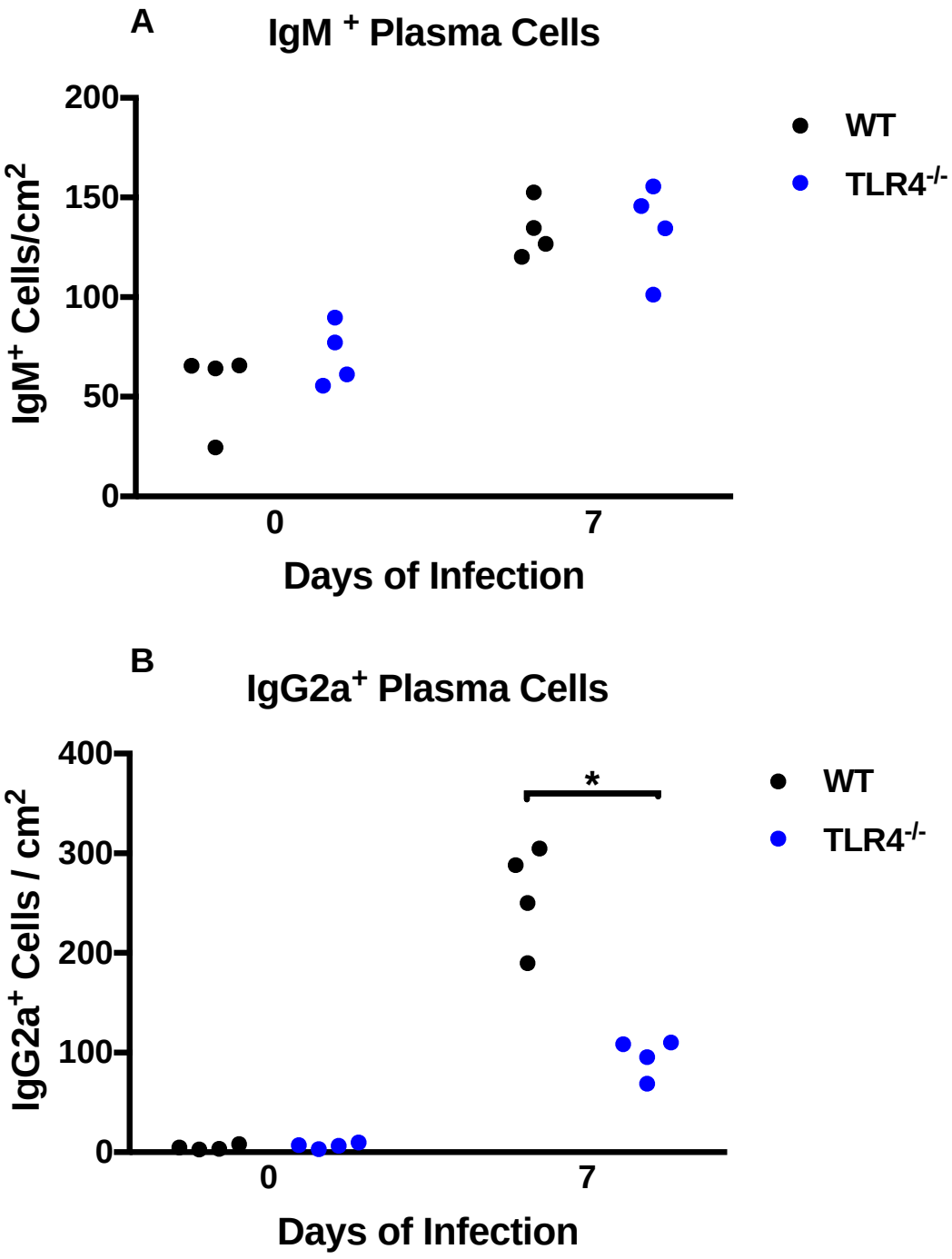

Figure 3.3:IgM and IgG2a plasma cell counts by histology
BALB/c and TLR4$^{-/-}$ mice were infected i.p. with 5 x 10^5 SL3261 and sacrificed by cervical dislocation 7 days post-infection. Spleen sections were stained for IgD (brown) and either IgM or IgG2a plasma cells. The average number of **A**) IgM and **B**) IgG2a plasma cells per spleen is shown. Data is from a single experiment with 4 mice per group at each time point.

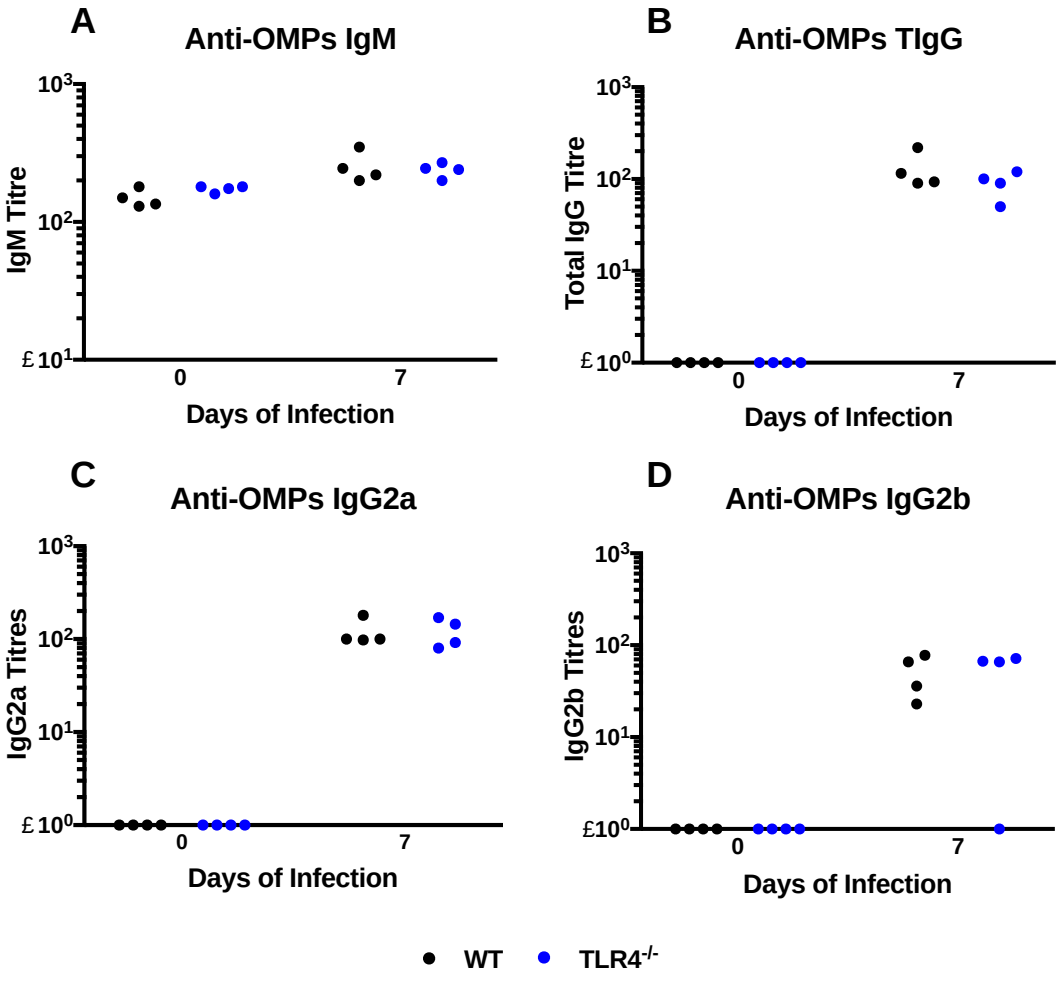

Figure 3.4:Anti-OMPs response 7 days post-infection with STm
BALB/c and TLR4$^{-/-}$ mice were infected with 5 x 10^5 SL3261 and sacrificed 7 days post-infection. *Salmonella*-specific **A**) IgM, **B**) IgG, **C**) IgG2a and **D**) IgG2b antibody titres were measured against 5 µg of outer membrane proteins (OMPs) by ELISA. Data is representative of two independent experiments with each experiment containing 4 mice per group per time point.

3.2.3. TLR4 is not required for activation of $CD4^+$ T Cells and differentiation to Th1 cells

Activated $CD4^+$ T cells are known to drive class switching in T-dependent antibody response through the provision of CD40L to cognate B cells (212). T cells and CD40L has previously been shown to be required for class switching in response to infection with STm (47, 67). Given that $TLR4^{-/-}$ mice have impaired IgG2a class switching it suggested that there could be a problem with T cell activation or their differentiation into Th1 cells. Using flow cytometry, splenocytes were stained/gated for $CD3^+CD4^+$ T cells and then activated $CD4^+$ T cells on the basis of the lower expression of CD62L (**Figure 3.5.A**). To accurately place these gates the N.I. mice and WT infected mice were used to set the gates at each time point. There was an increase in the number of $CD4^+$ T cells (**Figure 3.5.B**) and activated $CD4^+$ T cells 7 days post-infection in WT mice and $TLR4^{-/-}$ mice (**Figure 3.5.C**). As the infection progressed, the number of activated $CD4^+$ T cells increased to peak levels around two weeks post-infection and remained constant until day 35. In the absence of TLR4 there were higher levels of $CD4^+$ T cells and activated $CD4^+$ T cells at all time points, except day 35.

Th1 cells are major producers of IFN-γ, which is known to promote $IgG2a^+$ class switching (103, 126, 135). Given the reduction in $IgG2a^+$ cells observed by histology it was predicted that there could be a decrease in the number of Th1 cells in the spleen. To assess this the number of Th1 cells in the spleen was measured by flow cytometry one week after infection with SL3261. In order to gate for Th1 cells we first gated out B cells and selected T cells through a $CD3^+B220^-$ gate. T helper cells were then selected

by gating for $CD3^+CD4^+$ cells. Since Th1 cells come from activated/effector T cells these were selected on the basis of high expression of CD44. Finally, Th1 cells were identified on the basis of the expression of both IFN-γ & T-bet (**Figure 3.6.A-B**). BALB/c and $TLR4^{-/-}$ mice had a marginally, but not significantly, lower number of IFN-γ^+T-bet^+CD4^+ T cells (**Figure 3.6.C**). Although TLR4 does not influence Th1 differentiation, it does appear to be required for optimal production of polyfunctional TNF-α^+ IFN-γ^+ T cells (**Figure 3.7.A-B**), as the number (**Figure 3.7.C**) of these cells was significantly lower in $TLR4^{-/-}$ mice.

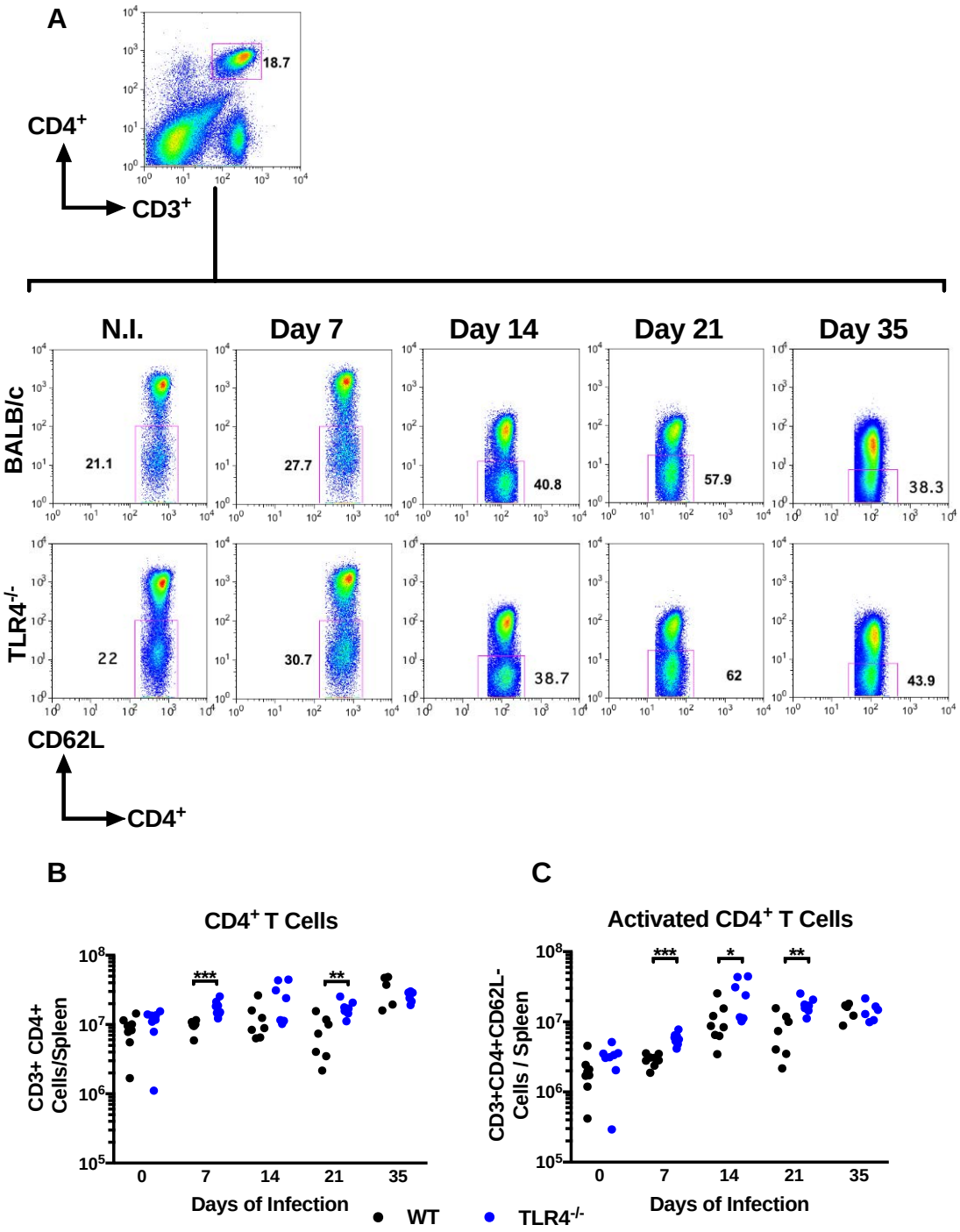

Figure 3.5:The role of TLR4 in T cell activation throughout infection with STm
BALB/c and TLR4$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 7, 14, 21 & 35 days. **A**) Gating strategy (see text for details) and representative dot plots of the percentage of activated CD4$^+$ T Cells (CD3$^+$CD4$^+$CD62L$^-$) and the total number of **B**) CD3$^+$CD4$^+$ T cells and **C**) activated/effector CD4$^+$ T cells in the spleen. Data is representative or pooled from two independent experiments with 3-5 mice per group per time point for each experiment. *, ** and *** is equivalent of P ≤ 0.05, 0.01 and 0.001 respectively.

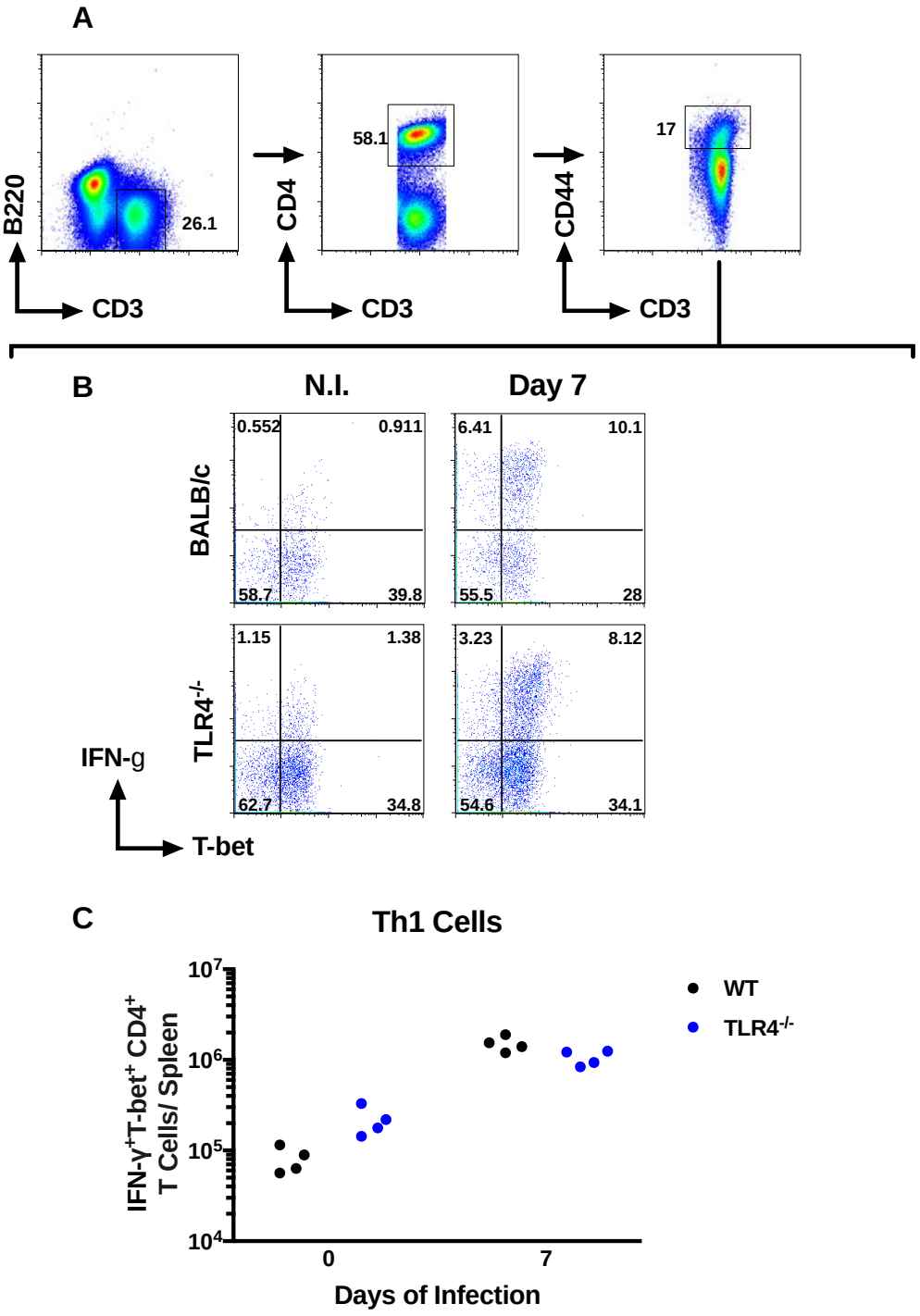

Figure 3.6: Role of TLR4 in the differentiation of Th1 Cells
BALB/c and TLR4$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 7 days. Splenocytes were cultured with or without α-CD3 and α-CD28 for 6 hours before staining for Th1 cells. **A**) Gating strategy for Th1 Cells (see text for details). **B**) Representative plots of the proportion of Th1 cells (B220$^-$CD3$^+$CD4$^+$CD44$^+$IFN-γ$^+$T-bet$^+$) and the **C**) total number of Th1 cells per spleen. Data is representative of two independent experiments with 4 mice per group per time point per experiment. *P ≤ 0.05.

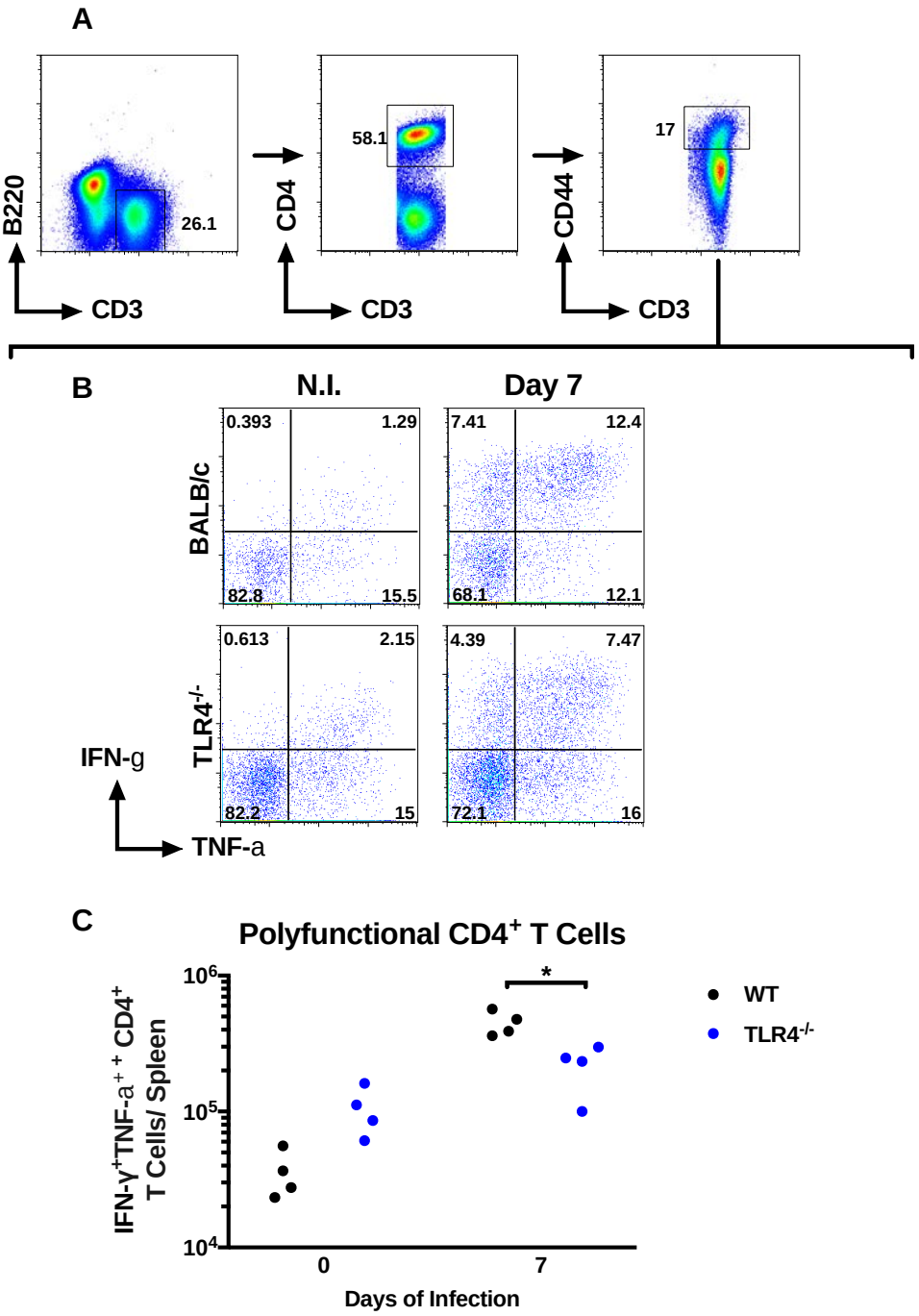

Figure 3.7:Role of TLR4 in the differentiation of polyfunctional CD4⁺ T Cells.
BALB/c and TLR4$^{-/-}$ mice were infected for 7 days with 5 x 10^5 SL3261. Splenocytes were stimulated with α-CD3 plus α-CD28 and intracellular stained for TNF and IFN- γ expressing CD4⁺ T cells. **A**) Gating strategy for Polyfunctional T Cells. B220$^-$CD3⁺CD4⁺CD44⁺ T cells were gated to select for effector T cells and then polyfunctional T cells were identified from expression of TNF & IFN-γ **B**) Representative dot plots of the percentage of TNF⁺ IFN- γ⁺ CD4⁺ effector T cells. **C**) Total number of TNF⁺IFN-γ⁺ CD4⁺CD44⁺ effector T cells (B220$^-$CD3⁺CD4⁺CD44⁺TNF⁺IFN-γ⁺). Data is representative of a single experiment with 4 mice per group.

3.2.4. Early formation of germinal centres in the absence of TLR4

Germinal centres are responsible for the production of high affinity class switched antibody, memory B cells and long lived plasma cells (43). In response to *S*Tm the appearance of this special microenvironment is restricted until bacterial numbers are low or the infection has been cleared. As a consequence germinal centres cannot be detected in WT mice until 35 days after infection (47). In support of this, the immunohistochemistry in **Figure 3.8** shows that in the WT mice there is an absence of germinal centres 7, 14 and 21 days post-infection, as determined by staining with PNA. However, germinal centres can be observed in the WT mice after 35 days of infection. In contrast, in the absence of TLR4 germinal centres are present at all time points after infection. This is further supported by the fact that these PNA positive regions were also positive for BCL-6 in the spleens of 8/8 mice tested by histology after one week of infection (data not shown). The presence of germinal centres in the $TLR4^{-/-}$ mice is also supported by flow cytometry data from the spleen which shows when gating for germinal centre (GC) B cells (**Figure 3.9.A.**) there is a greater number of $B220^{+}CD38^{-}GL\text{-}7^{+}$ GC B cells at day 14 compared to the WT (**Figure 3.9.B**). No difference in GC B cells was detected between the WT mice and $TLR4^{-/-}$ mice by flow cytometry after 21 or 35 days of infection.

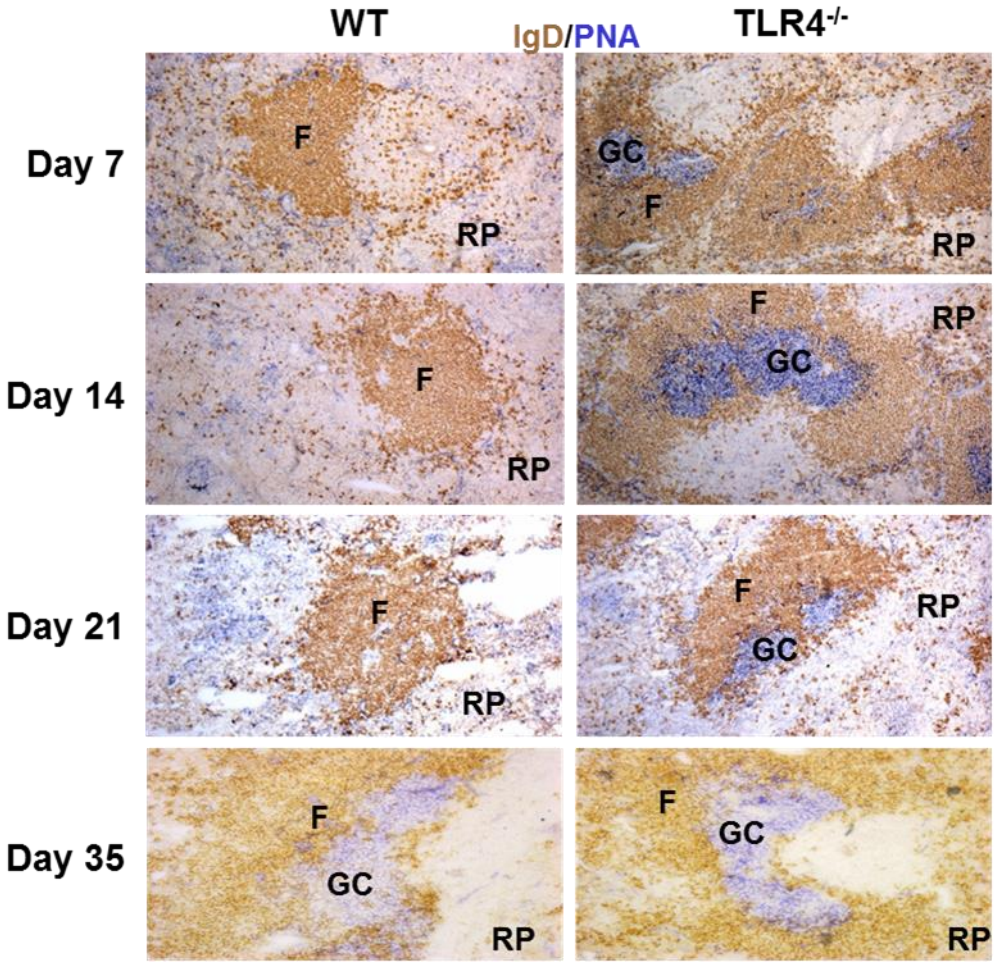

Figure 3.8: TLR4 and germinal centre formation in BALB/c mice infected with STm
BALB/c and TLR4$^{-/-}$ mice were infected with 5 x 10^5 STm for 7, 14, 21 & 35 days. 5 µm spleen sections were stained with IgD (brown) to detect B cell follicles and PNA (blue) for germinal centres. Images were taken at a 20 x magnification and are representative of two independent experiments with 3-4 mice per group per time point per experiment , except day 35 which is only from one single experiment with 5 mice per group. Staining and pictures from day 35 were done on a different day to those from day 7, 14 and 21. **F- Follicle, GC- Germinal Centre & RP-Red Pulp.**

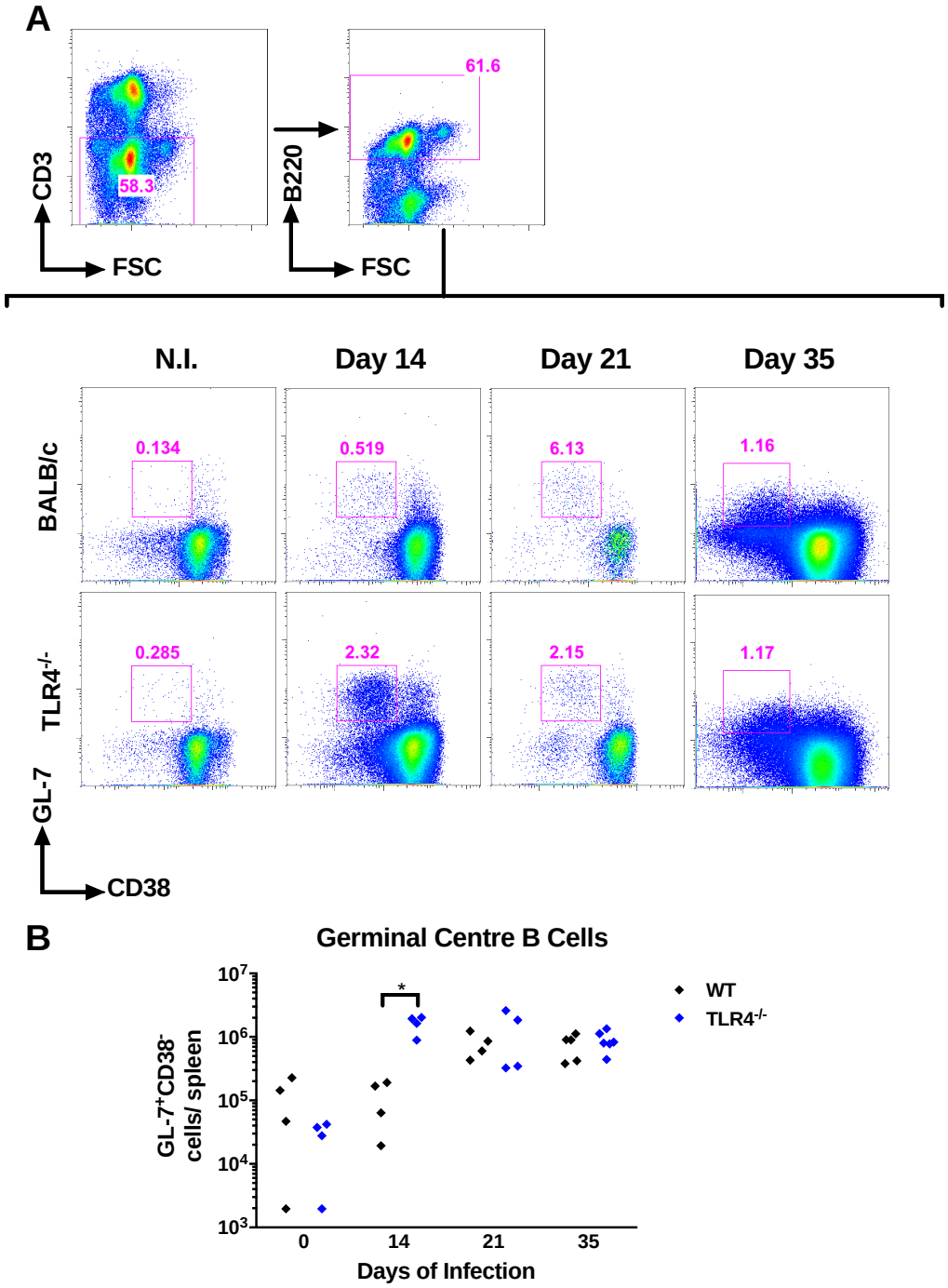

Figure 3.9: Increased number of germinal centre B cells in the absence of TLR4
BALB/c and TLR4$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 14, 21 & 35 days. **A**) Splenocytes were gated for B220 to select for B cells and then for GL-7$^+$CD38$^-$ cells to identify GC B cells. This gating strategy and representative plots of germinal centre B cells (CD3$^+$B220$^+$GL-7$^+$CD38$^-$) is shown. **B**) Total number of germinal centre B cells. Dot plots and cell numbers are representative of two independent experiments with 3-5 mice per group per time point, except day 35 which is only from one experiment. * (P≤0.05), **(P≤0.01) & *** (P≤0.001).

3.2.5. Impaired production of IgG2a$^+$ Plasma Cells in the absence of TLR4

The finding that TLR4$^{-/-}$ mice form germinal centres early throughout infection with STm suggested that they could be producing more plasma cells than WT mice, despite fewer IgG2a$^+$ cells found at day 7. To examine whether there was an accumulation of plasma cells we assessed the number of splenic CD138$^+$IgG2a$^+$ plasma cells 14 or 21 days after infection with STm. Splenocytes were stained and gated for the expression of CD138 to select for plasma cells and then IgG2a for class switched cells (**Figure 3.10.A**). It should be noted that an IgG2a intermediate population suspected to be plasmablasts could be detected in some of the N.I. and infected mice. Since we did not have markers to identify this population it was decided to gate on the IgG2a high population, as these were not present in the spleens of any of the N.I. mice. There was a significant increase in the number of CD138$^+$ cells after 14 days of infection in the spleens of TLR4$^{-/-}$ mice compared to the WT mice (**Figure 3.10.B**). However, this increase in CD138$^+$ plasma cells appears to be transient since there was no difference 21 days post-infection. Despite the increase in CD138$^+$ plasma cells there were a lower number (**Figure 3.10.C**) of IgG2a$^+$ plasma cells 14 days post-infection. This phenotype was also temporary since the representative proportion and number of IgG2a+ plasma cells was equivalent to that of the WT after 21 days of infection.

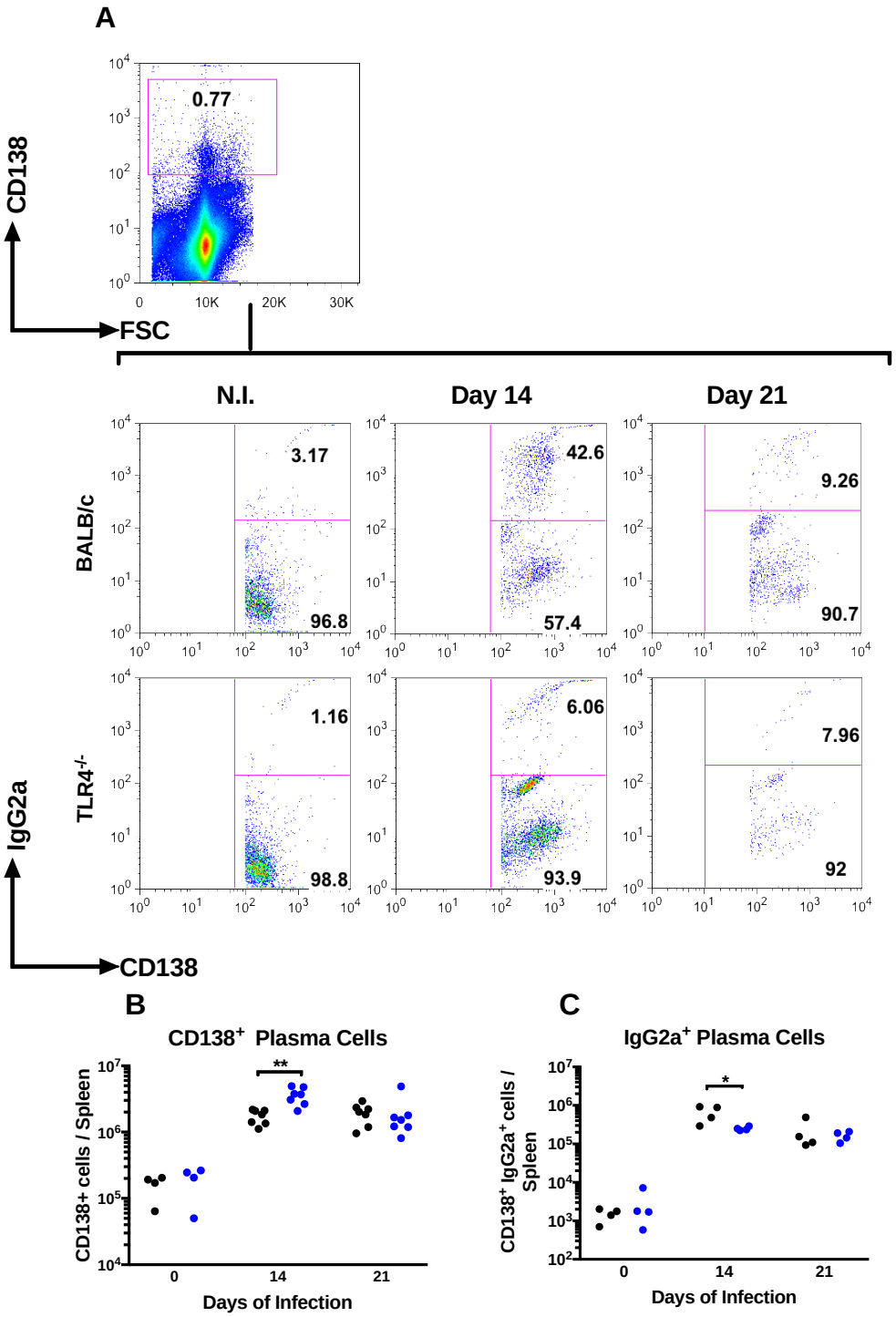

Figure 3.10:Reduced number of IgG2a$^+$ plasma cells in the absence of TLR4
BALB/c and TLR4$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 14 and 21 days. **A**) Representative dot plots of the percentage of CD138$^+$IgG2a$^+$ plasma cells in the spleen. **B**) Total number of CD138$^+$ plasma cells and **C**) CD138$^+$ IgG2a$^+$ plasma cells in the spleen. Data is pooled (B), or representative (C) of two independent experiments with 3-4 mice per group per experiment. *(P≤0.05), **(P≤0.01) & *** (P≤0.001).

3.2.6. TLR4 influences the kinetics and magnitude of the antigen-specific serum antibody response

Although no difference in the anti-OMPs response was observed one week after infection we looked at later time-points. Given the early appearance of germinal centres in the TLR4 deficient mice we hypothesized that the magnitude of the anti-OMPs response would be much greater over the course of the infection. Anti-OMPs IgM was induced following infection and did not differ significantly between the WT mice or $TLR4^{-/-}$ mice at any of the time-points (**Figure 3.11.A)**. In contrast, the anti-OMPs IgG (**Figure 3.11.B**.) response was significantly lower at day 14 and 21 in the absence of TLR4. Lower anti-OMPs IgG2a, IgG2b and IgG3 titres (**Figure 3.11.C, D & F**) were observed 14 days post-infection. The IgG2b and IgG3 anti-OMPs levels appeared to reach similar levels to the WT, 21 days post-infection. However, this was the not case for IgG2a which did not reach WT levels until 35 days after infection. The greatest level of anti-OMPs titres for all classes/subclasses of antibodies was observed 35 days post-infection. In addition, no differences were observed in the anti-OMPs titres at this time-point for any of the classes/subclasses, except IgG1. $TLR4^{-/-}$ mice had a higher anti-OMPs IgG1 titre 35 days post-infection (**Figure 3.11.E**). TLR4 can influence both the kinetics and magnitude of the antibody response to STm.

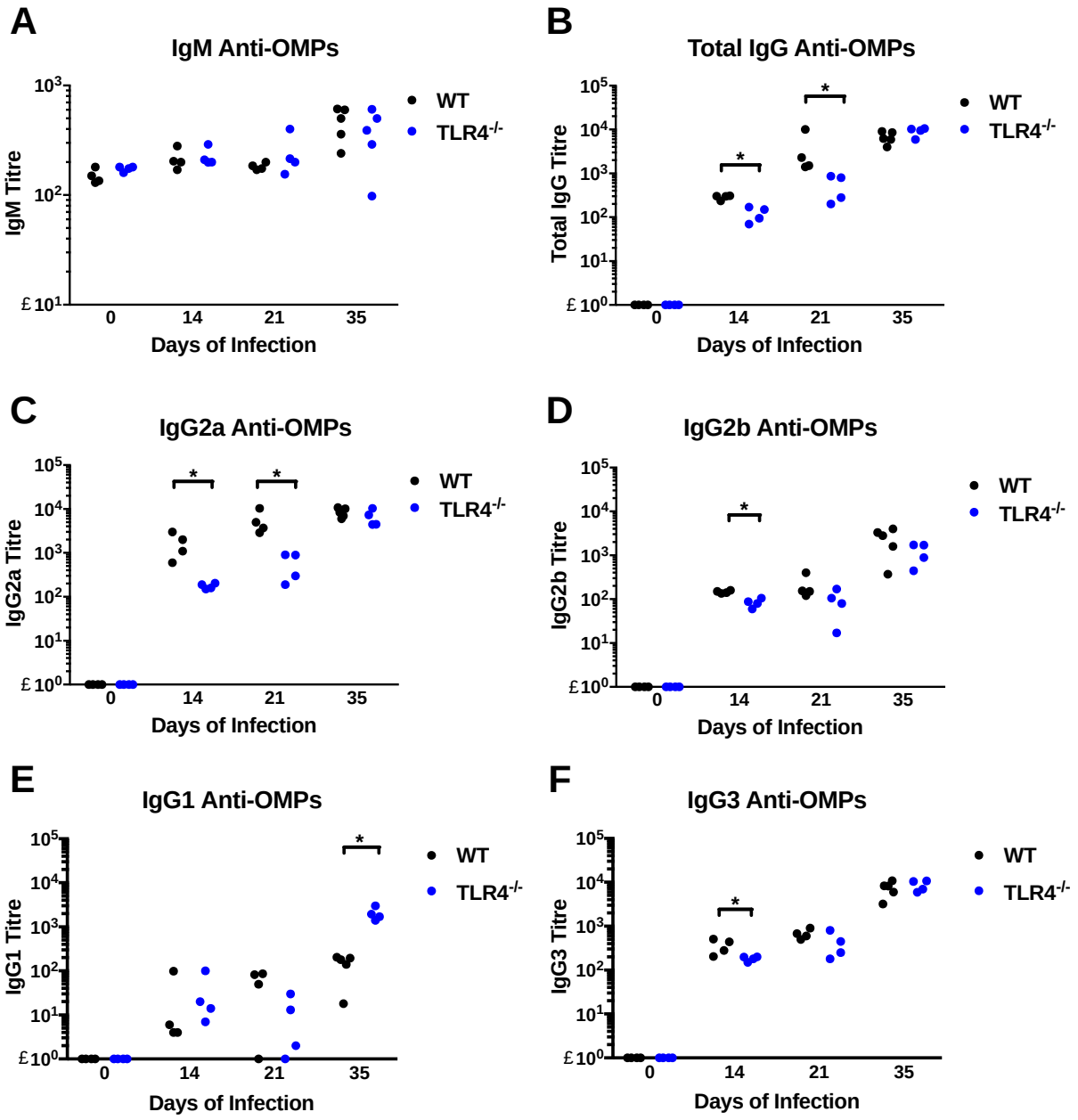

Figure 3.11: Loss of TLR4 in BALB/c mice leads to a decreased anti-OMPs response.
BALB/c and TLR4$^{-/-}$ mice were infected with 5 x 10^5 SL3261 and sacrificed 14,21 & 35 days later. *Salmonella* specific **A**) IgM , **B**) IgG , **C**) IgG2a/c , **D**) IgG2b, **E**) IgG1 and **F**) IgG3 antibody titres against 5 µg of outer membrane proteins was measured by ELISA. Data is representative of two independent experiments, except at day 35, with 3-5 mice per group per time point per experiment. *(P≤0.05), **(P≤0.01) & ***(P≤0.001).

3.2.7. *Salmonella* infection induces the production of self-reactive antibodies

Despite the presence of germinal centres, the TLR4$^{-/-}$ mice had lower OMP specific antibody titres than the WT mice. This suggested that the germinal centres are either non-functional or producing antibody to other targets such as self. To test this hypothesis a rough screening assay was devised using an ELISA to detect serum antibodies to self-antigens (**Section 2.12**).

Contrary to our hypothesis this test suggested there was an induction of antibodies to liver, heart and kidney cell homogenate in the WT mice both 14 and 21 days post-infection (**Figure 3.12**). There was no self-reactive antibody detected in the non-infected mice, surprisingly, nor was there in the infected TLR4$^{-/-}$ mice. In order to confirm the results from this assay we used an immunofluorescence based method to detect anti-self-antibodies. In this clinically approved assay, rat stomach, liver and kidney sections from RAG knockouts are blocked with goat sera, incubated with mouse sera and anti-self-antibodies detected with a secondary antibody that binds IgM, IgG and IgA (213). Self-reactive antibodies were detected to the stomach, liver and kidney in WT mice 21 days post-infection (**Figure 3.13.A**). This response appeared to be TLR4 dependent as no self-reactive antibodies were detected 21 days post-infection in TLR4$^{-/-}$ mice. The most consistent tissue staining observed was in the stomach (**Figure 3.13.B**.). It should be noted that the detection of self-reactive antibodies in WT mice was unexpected and forms the basis of the other results chapters presented in this thesis.

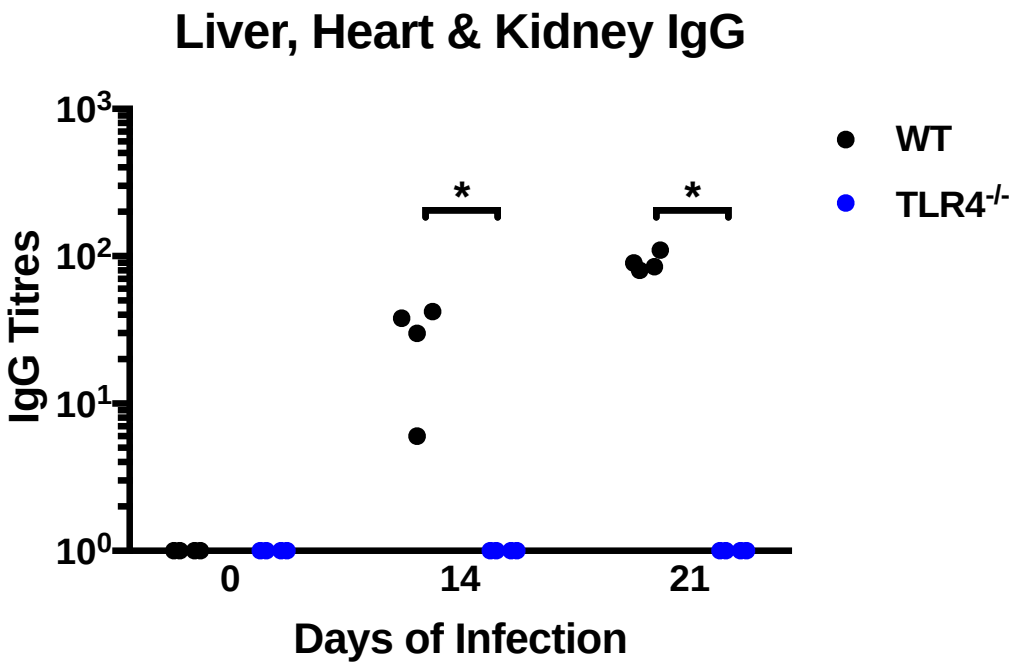

Figure 3.12: Rough screening ELISA for the detection of self-reactive antibodies
BALB/c and TLR4$^{-/-}$ mice were infected with 5 x 10^5 SL3261 and sacrificed 14 & 21 days later. 96 well plates were coated with liver, heart and kidney homogenate. Antibodies to liver, heart and kidney homogenate from infected and non-infected mice were detected by ELISA. Data is representative of two independent experiments in which each experiment had 3-4 mice per group per time point. *(P≤0.05), **(P≤0.01) & ***(P≤0.001).

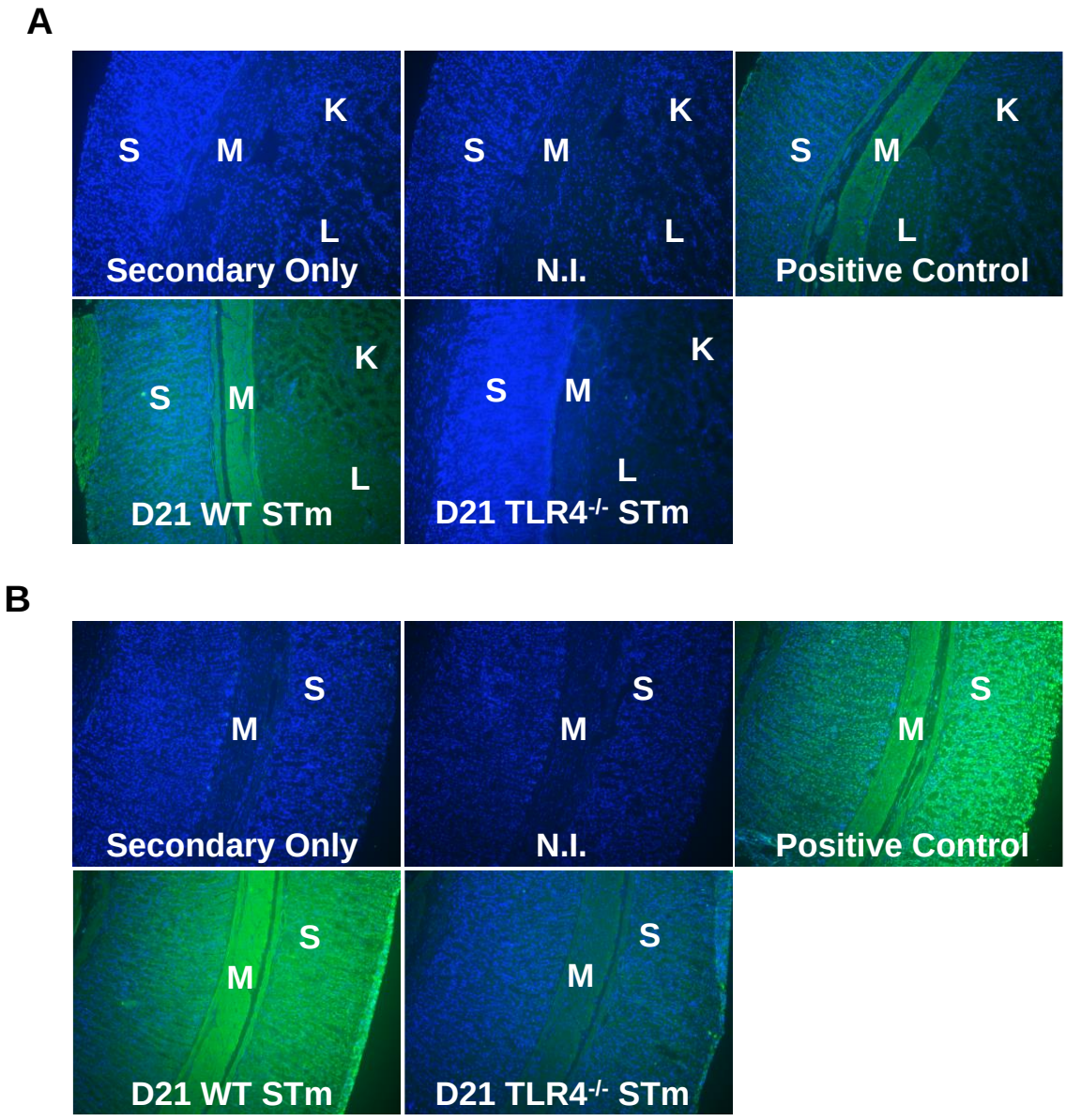

Figure 3.13: Staining for Stomach, Liver & Kidney Self-reacting Antibodies
BALB/c and TLR4$^{-/-}$ mice were infected with 5 x 10^5 SL3261 and sacrificed 21 days later. RAG$^{-/-}$ rat stomach, liver and kidney slides were blocked with goat serum and stained with mouse sera at a 1/20 dilution from infected and non-infected mice. A secondary antibody that binds IgM, IgA and IgG was used to detect self-reactive antibodies. **A**) Stomach, liver and kidney self-reactive antibodies. **B**) Stomach self-reactive antibodies. Data is representative of two independent experiments in which each experiment had 3-4 mice per group per time point. **K** – Kidney , **L –** Liver, **M** – Muscle & **S** – Stomach.

3.2.8. TLR4$^{-/-}$ mice on a C57BL/6 background do not control the initial growth of *Salmonella* Typhimurium

During this research project it was reported that germinal centres are present 10 days after infection with STm in the spleens of MyD88$^{-/-}$ mice on a BALB/c genetic background. However, these germinal centres were not observed in MyD88$^{-/-}$ mice on a C57BL/6J background (214). Since these experiments did not rule out a role for TRIF signaling nor did they exclude the possibility of germinal centres forming earlier than 35 days post-infection we decided to investigate this further.

C57BL/6J and TLR4$^{-/-}$ mice on a C57BL/6J background were infected intravenously (*i.v.*) with 5 x 10^5 SL3261 and spleens were harvested both 7 and 21 days post-infection. The effects of *S*Tm on the bacterial burden and splenic mass were first assessed. Confirming published results, TLR4$^{-/-}$ mice had a higher bacterial burden both 7 and 21 days after infection suggesting that they could not control the initial growth of the pathogen (**Figure 3.14.A**). In contrast to the BALB/c TLR4$^{-/-}$ mice (**Figure 3.1**), there was no difference in the spleen size 7 days post-infection. In contrast, the spleen size was significantly larger 21 days post-infection in the absence of TLR4 compared to the C57BL/6J WT (**Figure 3.14.B.**).

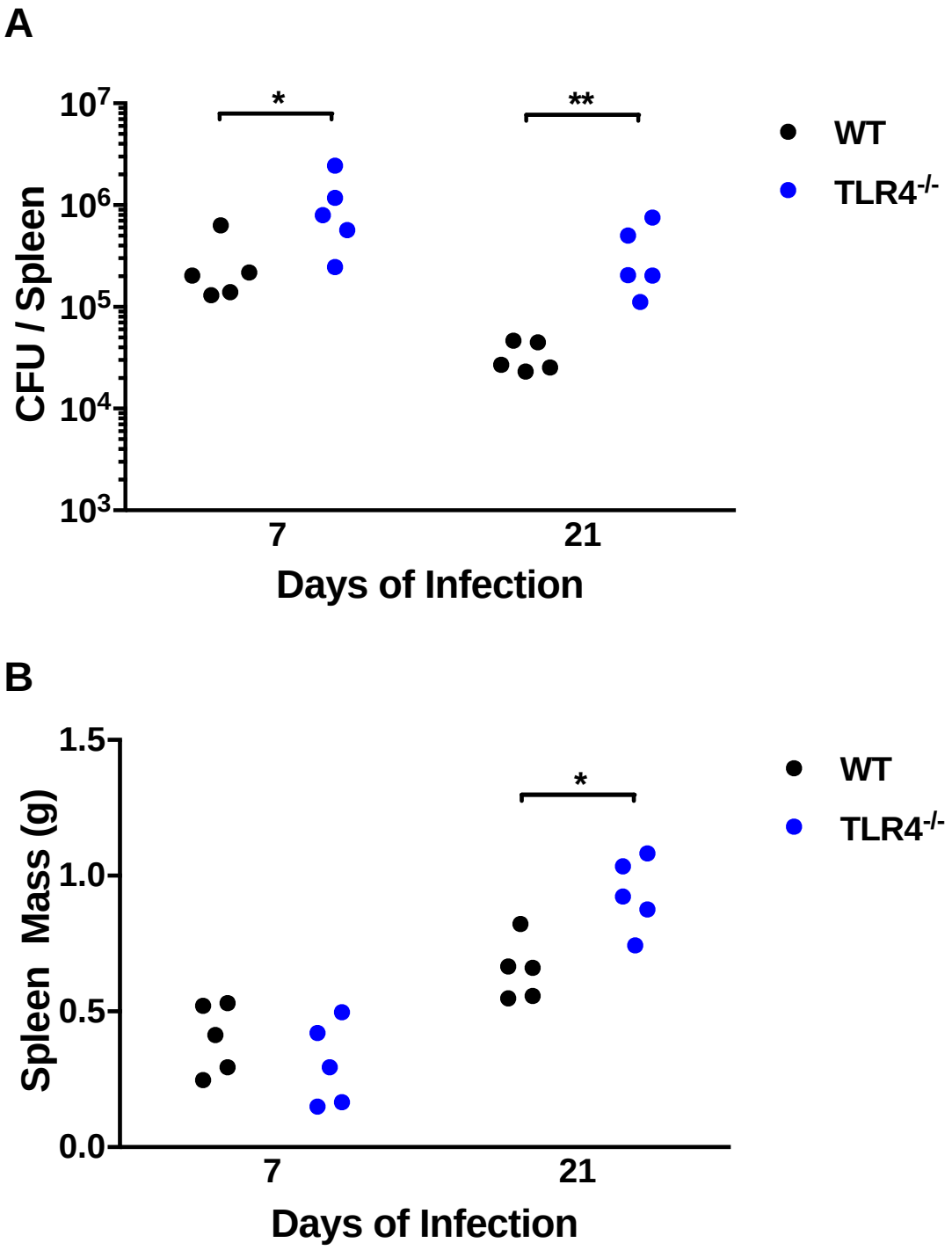

Figure 3.14:Bacterial burden and spleen mass in TLR4$^{-/-}$ mice with a C57BL/6 background. C57BL/6 and TLR4$^{-/-}$ mice were infected i.v. with 5 x 10^5 SL3261 and sacrificed by cervical dislocation at 7 and 21 days post-infection. **A**) Splenic bacterial counts and **B**) spleen size after infection. Each point represents one mouse from a single experiment with 4-5 mice per group. * (P≤0.05), ** (P≤0.01) & ***(P≤0.001).

3.2.9. Early germinal centre formation is not observed in TLR4$^{-/-}$ mice on a C57BL/6J background

Germinal centre formation in TLR4$^{-/-}$ mice with a C57BL/6J genetic background was assessed by flow cytometry at 7 and 21 days post-infection with *S*Tm. Splenic GC B cells were identified as TCR-β^{-}B220^{+}GL7^{+}FAS^{+} cells due to problems with the CD38 and GL-7 staining. TCR-β^{-}B220^{+} cells were gated to remove αβ T cells and to select for B cells. GC B cells were then identified from the TCR-β^{-}B220^{+} population on the basis of the expression of GL-7 and FAS (**Figure 3.15.A-B**). Given the absence of a N.I. control the gates were placed using the WT infected mice as a guide as these were known to be negative for germinal centres (47). No difference could be detected in the number of TCR-β^{-}B220^{+}GL7^{+}FAS^{+} GC B cells (**Figure 3.15.C**) at either time point, in the presence or absence of TLR4. Although there was a small decrease in the number of GC B cells detected in WT mice, 21 days post-infection compared to 7 days. The absence of early germinal centre formation in WT mice and TLR4$^{-/-}$ mice was confirmed through PNA^{+} staining of spleen sections (data not shown).

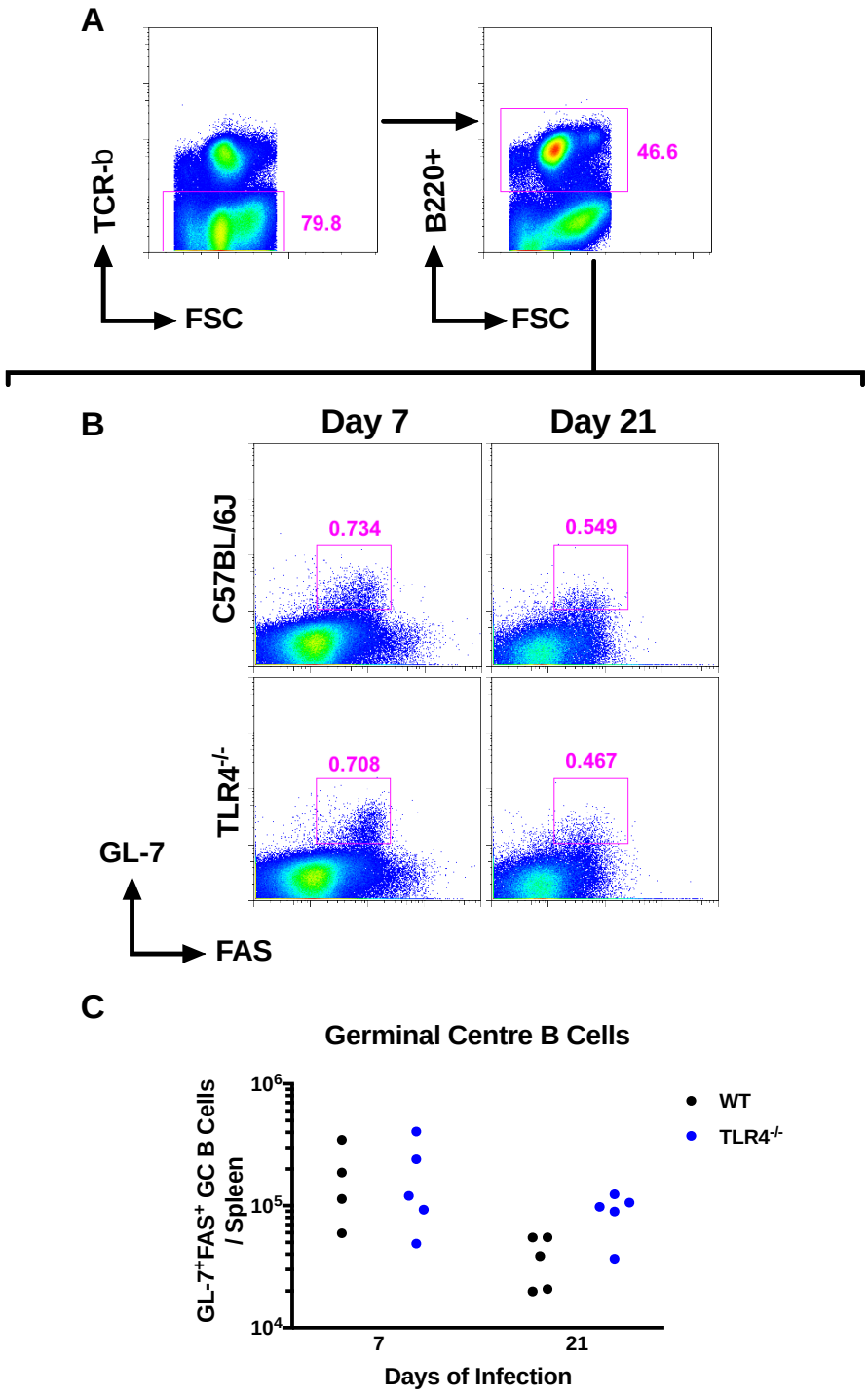

Figure 3.15:Loss of TLR4 does not lead to early germinal centre formation in C57BL/6J mice. C57BL/6J and TLR4$^{-/-}$ mice were infected *i.p.* with 5 x 10^5 SL3261 for 7 and 21 days. **A**) Gating strategy – TCR-β$^-$ cells were selected to remove αβ T cells and B cells were then identified on the expression of B220. GC B cells were then gated for on the basis of the expression of GL-7 and Fas, **B**) Representative plots of germinal centre B cells and **C**) number of TCR-β$^-$B220$^+$GL-7$^+$FAS$^+$ germinal centre B cells in the spleen. Data is representative of a single experiment with 4-5 mice per group.

3.2.10. Impaired IgG2a/c class switching is not observed in the absence of TLR4 on a C57BL/6J genetic background.

Since BALB/c TLR4$^{-/-}$ mice have an impaired ability to produce class switched IgG2a plasma cells in response to infection, we decided to test whether or not a similar phenotype could be observed in TLR4$^{-/-}$ mice on a C57BL/6J genetic background. Staining for CD138^{+} plasma cells and intracellular IgG2a/c by flow cytometry (**Figure 3.16.A**) showed a similar number of plasma cells (**Figure 3.16.B**) and IgG2a plasma cells at day 7 and day 21 (**Figure 3.16.C**). There was no difference detected between the WT mice and TLR4$^{-/-}$ mice at either of these time-points. TLR4 is not required for the optimum production of IgG2a/c plasma cells on a C57BL/6J background.

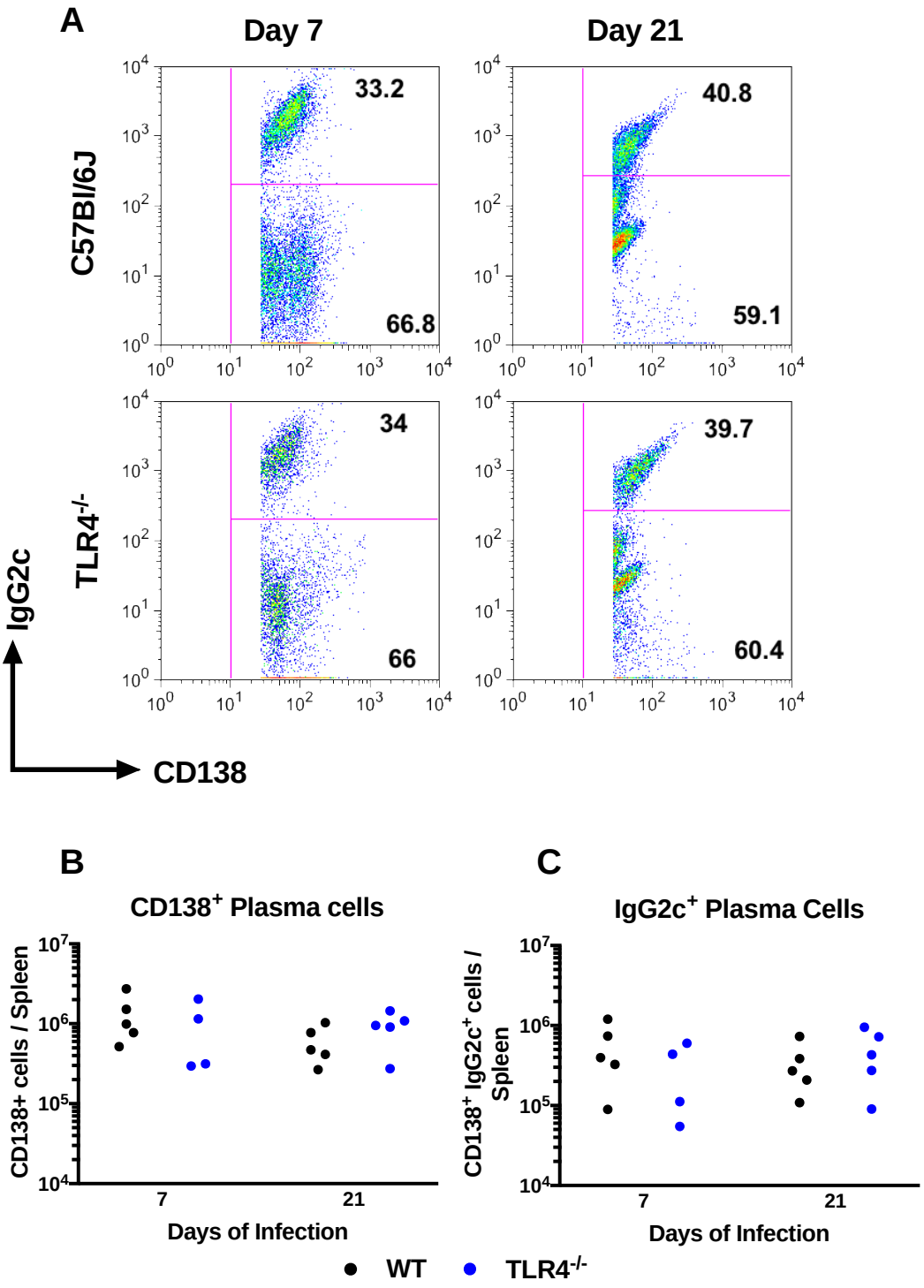

Figure 3.16:TLR4 is not required for optimal IgG2a/c class switching in C57BL/6J mice. C57BL/6J and TLR4$^{-/-}$ mice were infected *i.v.* with 5 x 10^5 SL3261 for 7 and 21 days. **A**) Representative plots of the percentage of CD138$^+$IgG2a/c$^+$ cells in the spleen. Splenocytes were gated for CD138 to select plasma cells and then intracellular IgG2c positive cells. **B**) Total number of CD138$^+$ and **C**) CD138$^+$IgG2a$^+$ plasma cells in the spleen. Data is representative of a single experiment with 4-5 mice per group per time point.

3.2.11. TLR4 does not influence the kinetics or magnitude of the anti-OMPs response in C57BL/6J TLR4$^{-/-}$ mice.

TLR4$^{-/-}$ mice from a BALB/c genetic background, have lower antibody titres against OMPs compared to the WT mice after 2-3 weeks post-infection. To test whether TLR4 has a similar influence on the antibody response on a different genetic background, C57BL/6J and TLR4$^{-/-}$ mice were infected with STm for 7 and 21 days. The antibody response to STm was assessed by measuring the anti-OMPs titres by ELISA (**Figure 3.17**). The IgM antibody response appears to have peaked by day 7 as there was no further increase observed in the antibody titres 21 days post-infection (**Figure 3.17A**). In contrast, the magnitude of the IgG response and all subclasses, except IgG1, appeared to increase at day 21 compared to day 7 (**Figure 3.17B-F**). No difference between the WT or TLR4 knockout could be detected in the OMPs antibody titres of IgM, IgG or any of the IgG subclasses both 7 and 21 days post-infection.

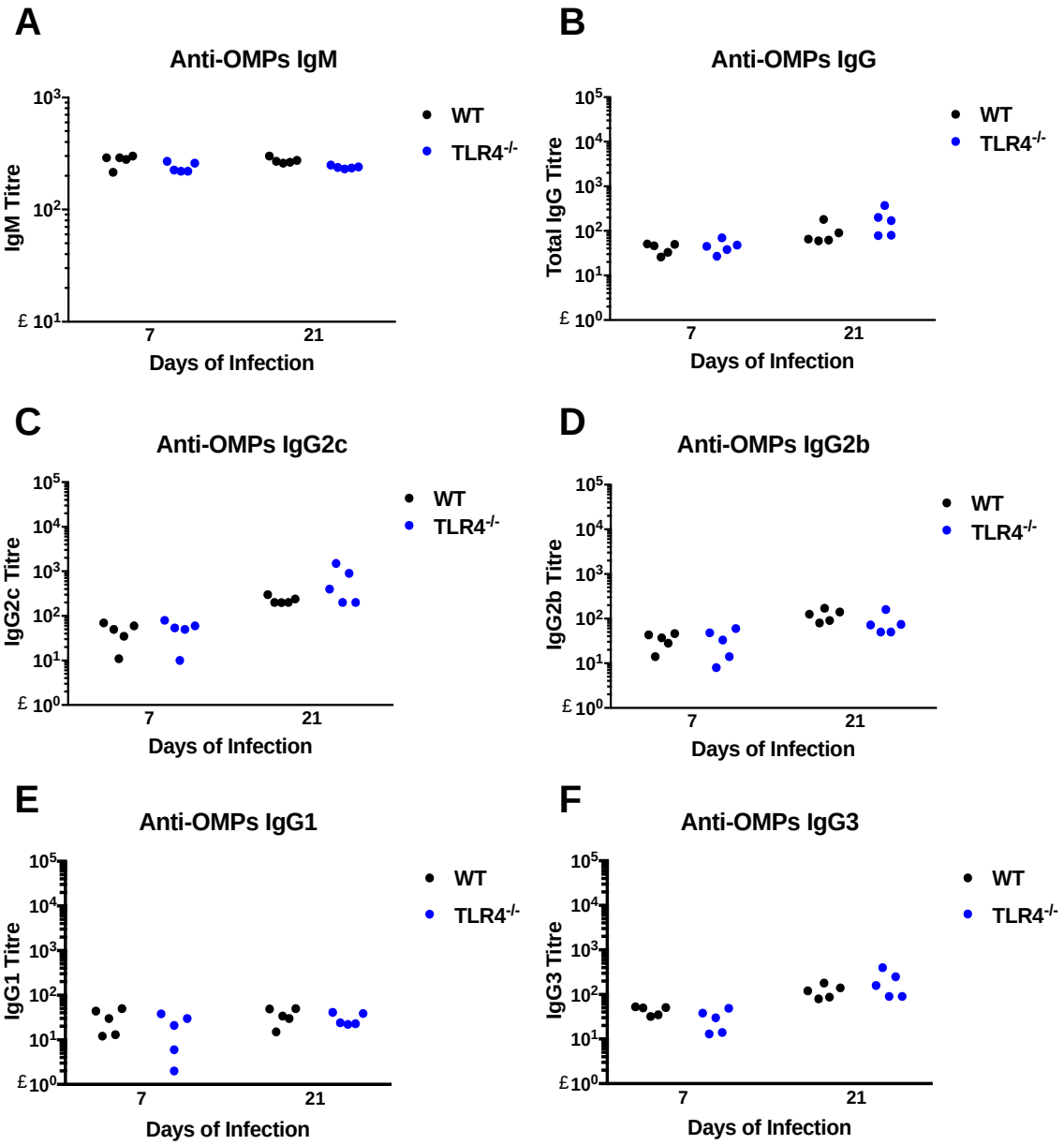

Figure 3.17:TLR4 does not influence the magnitude of the antibody response to STm in C57BL/6J mice.
C57BL/6J and TLR4$^{-/-}$ mice were infected i.v. with 5 x 10^5 SL3261 for 7 and 21 days. *Salmonella*-specific **A**) IgM , **B**) IgG, **C**)IgG2a/c, **D**) IgG2b, **E**) IgG1 and **F**) IgG3 antibody titres were measured against 5 µg of OMPs by ELISA. Data is representative of a single experiment with 5 mice per group per time point.

3.2.12. IFN-γ alone does not prevent the formation of germinal centres in C57BL/6J mice

Previous reports have suggested that BALB/c mice are intrinsically more able to polarize towards a Th2 cytokine response, compared to C57BL/6J mice, which are more associated with Th1 responses (215, 216). IFN-γ has been shown to inhibit the differentiation of PD-1^{hi} GC TfH cells and prevent the formation of germinal centres in malaria models (217). Given that IFN-γ is classically associated with Th1 responses we hypothesized that this cytokine could be responsible for the difference in the phenotypes observed in the BALB/c and C57BL/6J TLR4$^{-/-}$ mice. In order to test this hypothesis, C57BL/6J and IFN-γ$^{-/-}$ mice from a C57BL/6J genetic background were infected for 7 days with STm. The spleens were then harvested and examined by flow cytometry for GC B cells. No difference could be detected in the number of GL-7^{+}FAS^{+} GC B cells between the WT mice and IFN-γ$^{-/-}$ mice (**Figure 3.19**). Therefore, IFN-γ alone is not responsible for preventing germinal centre formation in C57BL/6J mice following infection with STm.

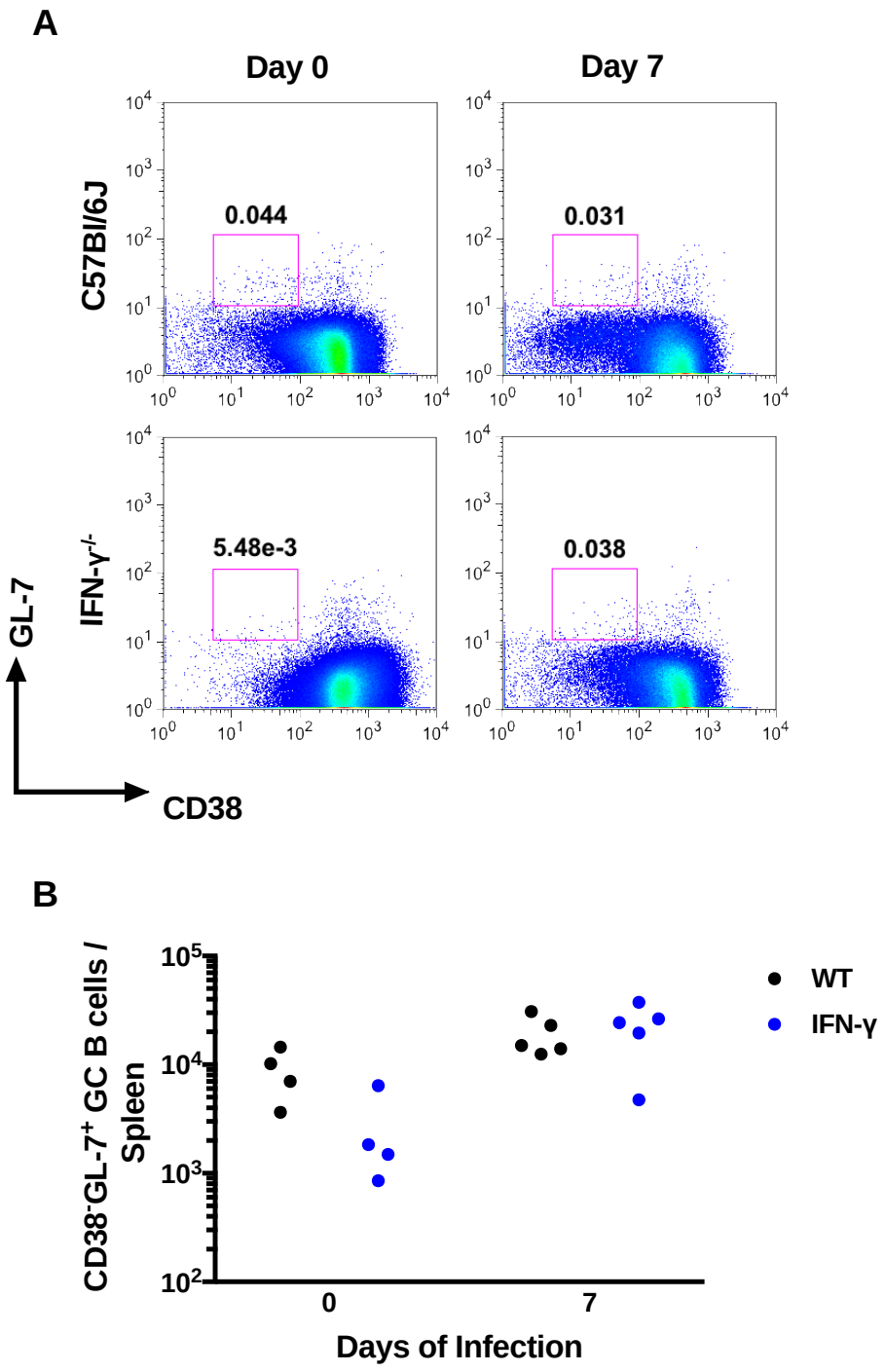

Figure 3.18:IFN-γ deficient C57BL/6J mice do not form early germinal centres.
C57BL/6J and IFN-$\gamma^{-/-}$ mice were infected i.p. with 5 x 10^5 SL3261 for 7 days. Spleens were harvested and GC B cells measured by flow cytometry ($B220^+GL\text{-}7^+CD38^-$). **A**) Representative plots of the percentage of GC B cells. Day 0 mice were used as a reference in order to set the gates for the day 7 mice **B**) Total number of GC B Cells ($CD3^-B220^+GL\text{-}7^+CD38^+$). Data is representative of two independent experiments with each experiment containing 4-5 mice per group per time point.

3.3. DISCUSSION

The original aim of this chapter was to investigate the role of TLR4 in the antibody response to STm. Throughout the course of this research project it became clear that the mouse genetic background could potentially influence the role of TLRs in the adaptive response to infection with STm. Given the differences in germinal centre formation reported in MyD88 knockouts from a BALB/c and C57BL/6J background we decided to explore whether the phenotypes we were observing in TLR4$^{-/-;}$ mice is conserved across different mouse genetic backgrounds.

TLR4 is expressed on a range of different innate and adaptive immune cells. Innate cells such as macrophages, neutrophils and natural killers cells have been shown to be important for controlling the growth of STm (218, 219). TLR4 and MyD88 signaling are important for restricting the initial growth of virulent STm through the action of pro-inflammatory cytokines such as IFN-γ, TNF-α and reactive oxygen species generated by iNOS (116). Consistent with these findings, our experiments have shown that TLR4$^{-/-}$ mice have a higher bacterial burden 7-21 days post-infection regardless of the mouse genetic background. The function of TLR4 in the innate response is conserved.

Despite the consistently higher bacterial burden in the absence of TLR4, the rate of splenic expansion, but not the total spleen size (between 1-1.5g), differed depending on the genetic background. For example, the spleen size of C57BL/6J TLR4$^{-/-}$ mice was similar to the WT one week post-infection but remained larger after 21 days. In contrast, BALB/c TLR4$^{-/-}$ mice had spleens approximately half the size of the WT mice, 14 days

post-infection. One possible explanation is that TLR4 plays a role in the recruitment of immune cells in BALB/c mice. If this is the case, alternative receptor-signaling pathways may be able to replace the TLR4 derived signals over a longer period of time. Splenomegaly in response to STm is largely due to a massive induction of erythropoiesis (220). At the peak of a STm infection around 80 % of the spleen is composed of $Ter119^+$ cells with the majority of these being immature $CD71^+$ $Ter119^+$ reticulocytes. The accumulation of these cells is related to the production of EPO in a MyD88/TRIF dependent manner and shunting from the bone marrow (220-222) .As a consequence the reduced splenomegaly at day 14 may be more likely to reflect the levels of EPO, since other TLRs would be able to compensate for the loss of TLR4 at a later time point such as day 21.

$CD4^+$ T cells, in particular, Th1 cells are required for clearance of STm from day 18 onwards(223). Given that the bacterial burden in the spleen of both the BALB/c and TLR4 knockout was roughly equivalent 35 days post-infection it suggests that $CD4^+$ T cell activation is not impaired. In support of this, the proportion of activated/effector $CD4^+$ T cells formed in response to STm was similar to the WT at all time points measured post-infection. However, when looking at the total number of activated/effector $CD4^+$ T cells in the spleen, the numbers were actually higher in the absence of TLR4 at all time-points, except day 0 and day 35. The greater levels of activated $CD4^+$ T cells may be due to the higher bacterial burden resulting from an impaired innate immune response. This suggests that TLR4 is not required for maximal activation of $CD4^+$ T cells. Additional evidence supporting this is the fact that no difference in the proportion or

number of Th1 cells could be detected in the presence or absence of TLR4. The fact TLR4 is not required for T cell activation is conserved since $CD4^+$ T cell activation was equal or greater than the WT in the $TLR4^{-/-}$ C57BL6/J mice (Data not shown). In addition, it has been reported that that TLR4 is not required by C57BL/6J mice to clear infection with STm (190). This is also consistent with work done using SL7207OVA. Intravenous immunisation with this strain has shown that mice deficient in either or both $TLR2^{-/-}$ and $TLR4^{-/-}$ can generate $CD4^+$ IFN-γ^+ and $CD8^+$ T cells at levels equivalent or greater than WT mice (206). Although TLR4 does not appear to influence the activation of $CD4^+$ T cells nor the differentiation of Th1 cells, we did observe a reduced proportion and number of polyfunctional Th1 cells on BALB/c background (**Figure 3.7**). The exact contribution of these cells throughout primary infection is difficult to determine from our data and further experiments are needed to confirm this observation. It should be noted that B7-H1 deficient mice have reduced polyfunctional Th1 cells on a C57BL/6J background and this might play a role in the differentiation of these cells in BALB/c mice (224).

The EFR helps restrict the extracellular spread of *S*Tm through the rapid production of low/moderate affinity antibody at the T cell zone-red pulp border of the spleen. Previous reports have demonstrated that systemic *S*Tm infection induces a T-independent EFR with T-dependent class switching that persists until the infection is cleared at day 35 (47, 67). Characterisation of the antibody response in C57BL/6J mice has shown that the EFR is largely composed of IgM and IgG2a/c plasma cells with some switching to IgG2b. BALB/c mice had a large induction of IgM and IgG2a plasma cells (**Figure 3.2**)

with some IgG2b (data not shown) 7 days post-infection. BALB/c mice lacking TLR4 appear to have a normal number of IgM plasma cells when compared to the WT. This data suggests that TLR4 does not influence the T-independent response. Further evidence supporting this is the fact that no difference in the anti-OMPs IgM response could be detected at any of the time points (**Figure 3.4 & 3.11.A.**). Production of IgM to OMPs has previously been shown to be associated with B1b cells (67) and no difference was detected in the proportion or number of these cells in the presence or absence of TLR4 (data not shown). This was surprising since TLR4$^{-/-}$ mice have previously been reported to make lower IgM responses to ST porins (174). In addition, TLR4 has typically been shown to drive T-independent responses to LPS and porins in isolation, which are both found in the outer membrane of STm. One possible explanation for these observations are that other TLRs could compensate for the lack of TLR4 as B1 cells are reported express a range of PRRs (225). Alternatively, STm could be acting as a TI-1 or TI-2 antigen depending on the type of B cell it binds to (59).

In contrast to IgM, class switching to IgG2a was impaired in the absence of TLR4 on a BALB/c background after 7 days (**Figure 3.2. & 3.3.**) of infection and this appeared to persist until it reach WT levels at day 21 (**Figure 3.10**.). Impaired IgG2a class switching appears to be independent of Th1 cells since the differentiation of these cells was equal to that of the WT. IFN-γ is known to be required for IgG2a class switching. The observation that TLR4$^{-/-}$ BALB/c mice can produce some IgG2a plasma cells suggests that IFN-γ production could be lower or that B cells have an impaired ability to respond to this cytokine, possibly through altered expression of IFN-γR. T-bet expression on B

cells, not T cells, has been shown through mixed bone marrow chimaeras to be essential for IgG2a class switching in response to primary infection with STm (186). In addition, T-bet deletion on B cells has been shown to prevent IgG2a class switching in response to infection with lymphocytic choriomeningitis virus (226). T-bet induction on B cells is reported to require stimulation of the BCR, IFN-γR and engagement of TLR7, TLR9 or CD40 (227). TLR4 might directly or indirectly alter T-bet expression on B cells in BALB/c mice thus influencing IgG2a class switching. However, TLR4 does not influence IgG2a class switching in C57BL/6J mice (**Figure 3.14**). This suggests that other mechanisms or TLRs could compensate for the loss of TLR4 and that this receptor is not crucial for the humoral response.

Germinal centre formation is absent in WT mice until the infection is cleared around 35 days later (**Figure 3.8 & 3.9**). In contrast, BALB/c mice lacking TLR4 are able to form germinal centres 7 days after infection and these appear to persist at-least until 21 days post-infection. Germinal centres can also be detected 35 days post-infection but whether these are the same ones observed at day 21 is unknown. Antibiotic treatment of STm infected mice has previously been shown to allow germinal centres to form 20 days after infection due to a reduced bacterial burden to $1/10^{th}$ (< 5000 CFU) that of the original inoculant (47). Suggesting that a live pathogen actively suppresses germinal centre formation. Additional evidence supporting this is the fact SPI-2 expression is required to prevent the induction of GC B cells in *Salmonella*-OVA models (146). One possible explanation for impaired germinal centre formation is to do with TfH differentiation. The appearance of PD-1^{lo} and PD-1^{Hi} TfH cells during STm infection coincides with the

extrafollicular and germinal centre response respectively (228). In addition, data from the Cunningham lab has shown that the presence of germinal centres and PD-1^{Hi} TfH population induced in response to flagellin are not present when mice are co-immunised with flagellin and STm (104). Collectively, this data suggests that throughout infection with STm there is an impaired differentiation of pre-TfH to mature TfH cells. Preliminary data supporting this is the fact a greater proportion of PD-1^{Hi} TfH cells can be detected 14 days after infection in the TLR4 BALB/c knockouts (data not shown). This population appears to be transient as it disappears around 21 days post-infection suggesting that the germinal centres could be collapsing, possibly due to changes in splenic architecture. However, further experiments would be required to confirm these findings. Loss of TLR4 could also enables early formation of germinal centres in BALB/c mice due to its potential role in altering the structure of the spleen. For example, TLR4 stimulation induced by STm but not by other bacteria such as *E.coli* and *L.monocytogenes* causes disruption in the architecture of the popliteal lymph node (229). This disruption is proposed to be dependent upon reduced CXCL13 plus CCL21 expression, which is required for maintenance of the T and B cell zones. It is therefore possible that the absence of TLR4 in BALB/c mice prevents such a disruption from occurring in the spleen, which in turn enables an immune response to develop normally. However, neither of these explanations alone fully explains why C57BL/6J mice do not form germinal centres suggesting that inflammatory cytokines are important. IFN-γ is known to suppress TfH differentiation but the loss of this cytokine on a C57BL/6J background does not lead to germinal centre formation 7 days post-infection. This suggests that the differences in Th1 and Th2 polarisation reported in C57BL/6J and BALB/c mice is not

responsible for inhibiting germinal centre formation. It thus possible that a combination of TLRs and cytokines are interfering with the germinal centre response.

Given the early appearance of germinal centres in TLR4$^{-/-}$ mice (BALB/c background) it was expected that there would be higher titres of OMP specific antibodies. ELISA data from BALB/c mice shows that anti-OMP IgM titres were not affected by presence or absence of TLR4. However, the levels of anti-OMPs IgG, IgG2a, IgG2b and IgG3 are much lower in the TLR4$^{-/-}$ mice 14 days post-infection. Although the titres start to increase 21 days post infection with no difference between the WT and TLR4 knockout being detected in the levels of IgG2b and IgG3. This suggests that the early germinal centres are either functionally inactive or that they are producing antibody to some unknown target. The levels of IgM and all IgG isotypes increase by approximately 1-2 logs after 35 days post-infection. This time point coincides with a low bacterial burden (0-2000 CFU/spleen) and the appearance of germinal centres in both the WT and TLR4 knockout. Given that no difference in all antibody isotypes, except IgG1, could be detected in the presence or absence of TLR4 it suggests that functional germinal centres are formed at day 35. It is therefore highly probable that the anti-OMPs antibodies being detected after 35 days is high affinity antibody. In the context of STm infection it is unlikely that TLR4 expression on FDCs is essential to promote SHM in the germinal centres as shown through different *in vivo* and *in silico* models (176). Although the anti-OMPs response was impaired in TLR4 derived BALB/c mice no reduction in IgM or IgG isotypes could be detected in the presence or absence of TLR4 in C57BL/6J mice at either day 7 or 21. The role of TLR4 in the antibody response to STm differs

depending on the genetic background. Although the expression of TLRs on B cells is most likely required for optimal antibody responses in C57BL/6J mice. Evidence supporting this is that MyD88$^{-/-}$ B cells have lower *Salmonella* specific titres of IgM and all IgG subclasses, except IgG1 and IgG3 (209). Similar observations have been found in response to VLPs. Intrinsic differences in the B cells from the two different genetic backgrounds could be responsible. B cell responses to different TLR adjuvants comparing C57BL/6J vs BALB/c mice have shown no difference in TLR expression but a greater tendency of BALB/c B cells to produce immunoglobulin's in response to adjuvants (230). Although differences in the strain of *Salmonella* and route of administration could also influence how the immune response develops.

Attempts to identify potential antibody targets for TLR4$^{-/-}$ mice revealed that WT mice produce self-reactive antibodies to rat stomach, liver and kidney sections 21 days post-infection. The production of these antibodies was dependent on TLR4 in BALB/c, but not C57BL/6J mice (data not shown). This suggests that the self-reacting antibodies are derived from the EFR and TLR4 is important for diversifying the antibody response. It should be noted that in C57BL/6J mice the self-reactive antibody staining was quite weak and therefore it is difficult to conclude whether there really is no difference in titres in the presence or absence of TLR4. To confirm these findings additional experiments would have to be done and the mice immunized i,p. rather than i.v.. It is interesting to note that the self-reactive antibodies accumulate at day 21 when the EFR dominates the anti-Salmonella response. TLRs in combination with other inflammatory cytokines could potentially influence the development of extrafollicular derived self-reactive antibodies in

the spleen, as this has been identified as source of autoantibodies in rheumatoid arthritis (231) .

The role of TLR4 in the innate control of STm is conserved across BALB/c and C57LBL/6J mice. However, the influence on the humoral response varies with the genetic background. In BALB/c mice, TLR4 prevents germinal centre formation, promotes IgG2a class switching, and enhances the magnitude, kinetics and diversity of the antibody response. In contrast, early germinal centre formation, impaired IgG2a class switching and reduced anti-OMPs titres are not evident in C57BL/6J TLR4$^{-/-}$ mice. Differences in the genetics of the host can therefore significantly alter the role of TLR4 in the adaptive response to primary infection with STm.

4. CHAPTER 4 - KINETICS OF THE PATHOGEN-SPECIFIC AND SELF-REACTIVE ANTIBODY RESPONSE

4.1. INTRODUCTION

In Chapter 3, we identified the early formation of germinal centres after infection with STm in the absence of TLR4 in BALB/c mice. Assessment of the pathogen-specific antibody response by ELISA suggested that these germinal centres were not productive or were generating antibodies against other targets, such as self. However, the induction of self-reactive antibodies was detected after infection in WT mice, but not in TLR4$^{-/-}$ mice (**Figure 4.1**). The data suggested that infection with STm could be stimulating both a pathogen-specific and self-reactive antibody response and that this could represent a potential link to the induction of autoimmune disease.

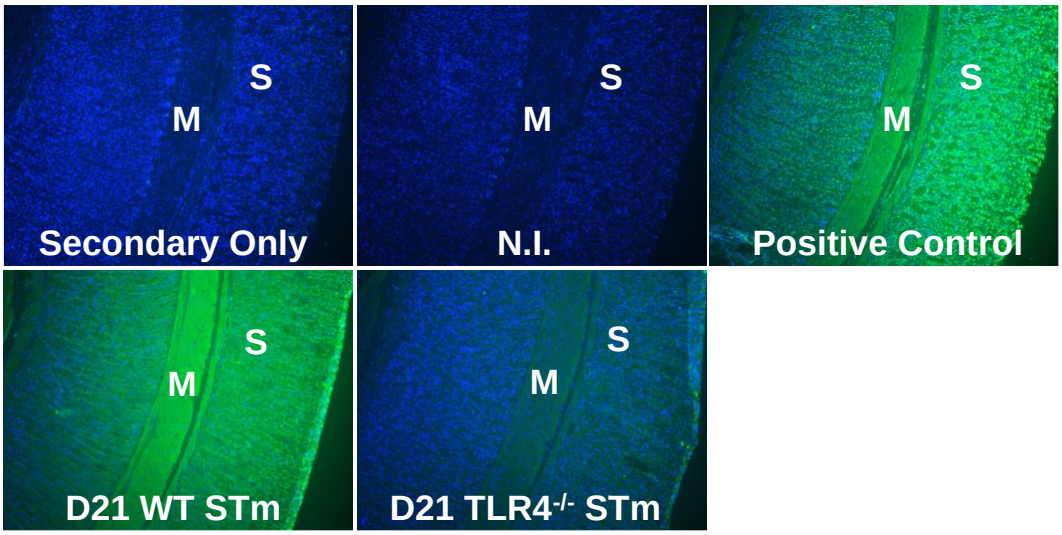

Figure 4.1:Detection of Stomach, Liver & Kidney Self-Reactive Antibodies
BALB/c and TLR4$^{-/-}$ mice were infected with 5 x 10^5 SL3261 and sacrificed 21 days later. RAG$^{-/-}$ rat stomach, liver and kidney slides were blocked with goat serum and stained with mouse sera from infected and non-infected mice. Stomach and muscle self-reactive antibodies (green) were then detected using a F(ab')2 secondary that detects IgA, IgG and IgM antibodies. Cells nuclei (blue) were counterstained with DAPI. This figure is representative of two independent experiments with 3-4 mice per infection time-point per experiment. Please note that this figure and figure legend is reproduced from **Figure 3.13**.

Autoimmune diseases are known to occur when normal or dysregulated immune responses develop and attack host tissues and can be affected by multiple factors including age, genetics and environment (161). Infections with bacteria, parasites or viruses are one of the key environmental factors known to facilitate a breakdown in peripheral immune tolerance (232). Pathogens can break immune tolerance through several mechanisms such as molecular mimicry (233, 234), bystander activation, epitope spreading (235) and the release of cryptic epitopes (232). An example of molecular mimicry is the immune responses against the cell wall of Group A Streptococcus. Antibodies raised against M protein can cross-react with heart muscle, leading to rheumatic heart disease (236-238). The other mechanisms mentioned have been observed throughout various infections plus mouse models of diabetes and multiple sclerosis (239-241).

B cells are implicated in many different autoimmune diseases through secretion of autoantibodies, release of pro-inflammatory cytokines and presentation of autoantigens to self-reactive T cells. B cell responses have been shown to be a major contributor to the pathogenesis of SLE and rheumatoid arthritis (242). In SLE, excess IFN-γ can drive spontaneous formation of germinal centres and the production of high affinity class switched autoantibodies (243, 244). In addition, triggering of the BCR, IFNγR and TLRs on B cells has been shown to induce T-bet expression (245). Deletion of T-bet from B cells in some SLE mouse models results in a reduced number of spontaneous germinal centres, delayed appearance of autoantibodies and prolonged survival (246). The importance of B cells is demonstrated by the effectiveness of rituximab in the treatment

of rheumatoid arthritis, SLE, idiopathic thrombocytopenic purpura and pemphigus vulgaris (247). Experimental models of these diseases are often investigated under steady state conditions. This does not help us understand how autoimmune responses are regulated by infection. This is important since abnormal B cell responses are known to occur during infection with pathogens such as B.*burgdorferi*, a known cause of lyme arthritis (248-251). In addition, extrafollicular antibody responses have been shown to be a source of autoantibodies in MRL.*Fas*^{lpr} mice (252, 253). Pathogens that induce atypical antibodies could therefore be a source of self-reactive antibodies and could potentially trigger or increase a person's likelihood of developing autoimmunity.

Gram-negative bacteria have been linked to the development of autoimmune diseases (254). Infection with STm can cause reactive arthritis (ReA), particularly in people with the HLA-B27 allele (255). In addition, there are some reports linking STm infection with a range of other conditions such as autoimmune haemolytic anaermia, cold agglutinin disease, acute transverse myelitis, ulcerative colitis and Guillain-Barré Syndrome (256-260). However, the exact role of STm in influencing an individual's susceptibility to developing autoimmune diseases other than ReA is unknown. Data from mouse models is conflicting as some have reported that a vaccine strain of STm can induce hypergammaglobulinaemia (261). On the other hand studies in NOD mice have shown that infection with STm can delay the onset of type 1 diabetes (262, 263). Therefore, STm infection is important to study for autoimmune disease as this model allows us to examine the responses over a longer term as the infection resolves.

In this chapter I investigate the kinetics of the pathogen-specific and self-reactive antibody response induced after systemic infection with STm to understand the induction and maintenance of these responses both during and after the infection has resolved.

4.2. RESULTS

4.2.1. Bacterial burden and splenomegaly resolve after one month of infection

In order to contextualize the pathogen-specific and self-reactive response, initially the rate of clearance was assessed in WT mice. C57BL/6J mice were infected *i.p.* with 5 x 10^5 SL3261 for 7, 21, 35 & 110 days. As shown previously, the bacterial burden in the spleen peaked by 7 days of infection with 400,000 – 1,000,000 CFU per spleen (**Figure 4.2 A**). The bacterial burden decreased there after so that by day 21 it was approximately 10 fold lower and a 100 fold lower at day 35 and 110. No bacteria can be detected in the spleen 110 days after infection. Spleen size increased between 3-6 fold after one week of infection, compared to N.I. mice (**Figure 4.2 B**). The peak of splenomegaly is observed 21 days post-infection with spleen sizes in a range of 0.99-1.17g. After 35 days of infection the spleen shrinks to a size similar to that of mice infected for one week. After the infection is cleared at 110 days the spleen is nearly the same size as the N.I. mice but it does not return to the starting weight.

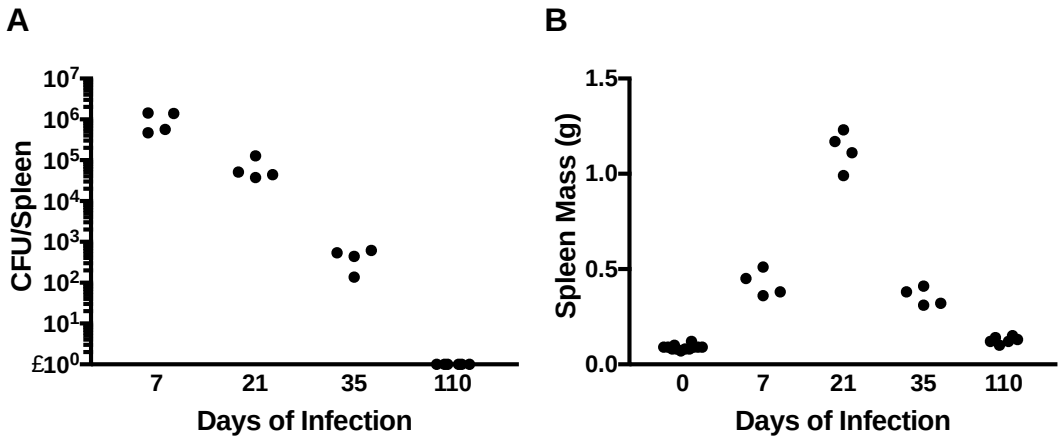

Figure 4.2:Bacterial burden and spleen mass after infection with STm
C57BL/6J mice were infected i.p. with 5 x 10^5 SL3261 for 7, 21, 35 & 110 days. Mice were culled by cervical dislocation. Spleens were isolated and the **A**) Total number of CFU per spleen and **B**) spleen mass was measured. Data is representative of two independent experiments with 4-6 mice per time point per experiment.

4.2.2. The Kinetics of the Anti-OMPs response following infection with STm

To assess the kinetics of the pathogen-specific B cell response after infection with STm, the titres of antibodies produced against OMPs were measured by ELISA. There is a significant increase in anti-OMPs IgM after one week of infection (**Figure 4.3 A**). Similar levels of IgM titres are observed both 21 and 35 days post-infection. However, the anti-OMPs IgM titres decrease after 110 days to reach background levels equivalent to those detected in the N.I. mice. In contrast, the titres of IgG (**Figure 4.3 B**) and all IgG subclasses (**Figure 4.3. C,D & F**), except IgG1 (**Figure 4.3 E**), significantly increased by 7 days post-infection and continued to do until day 35 where it plateaued until 110 days. The largest increase in the titres of all IgG classes is observed 35 days post-infection, a time when germinal centres are known to be present (47). Unlike the majority of IgG subclasses, anti-OMPs IgG1 cannot be observed 7 or 21 days after infection. There appears to be an induction of anti-OMPs IgG1 five weeks after infection in approximately 50% (4/8) of the mice. IgG1 could also be detected at similar levels after 110 days of infection. Therefore, the pathogen-specific antibody response peaks after 35 days of infection.

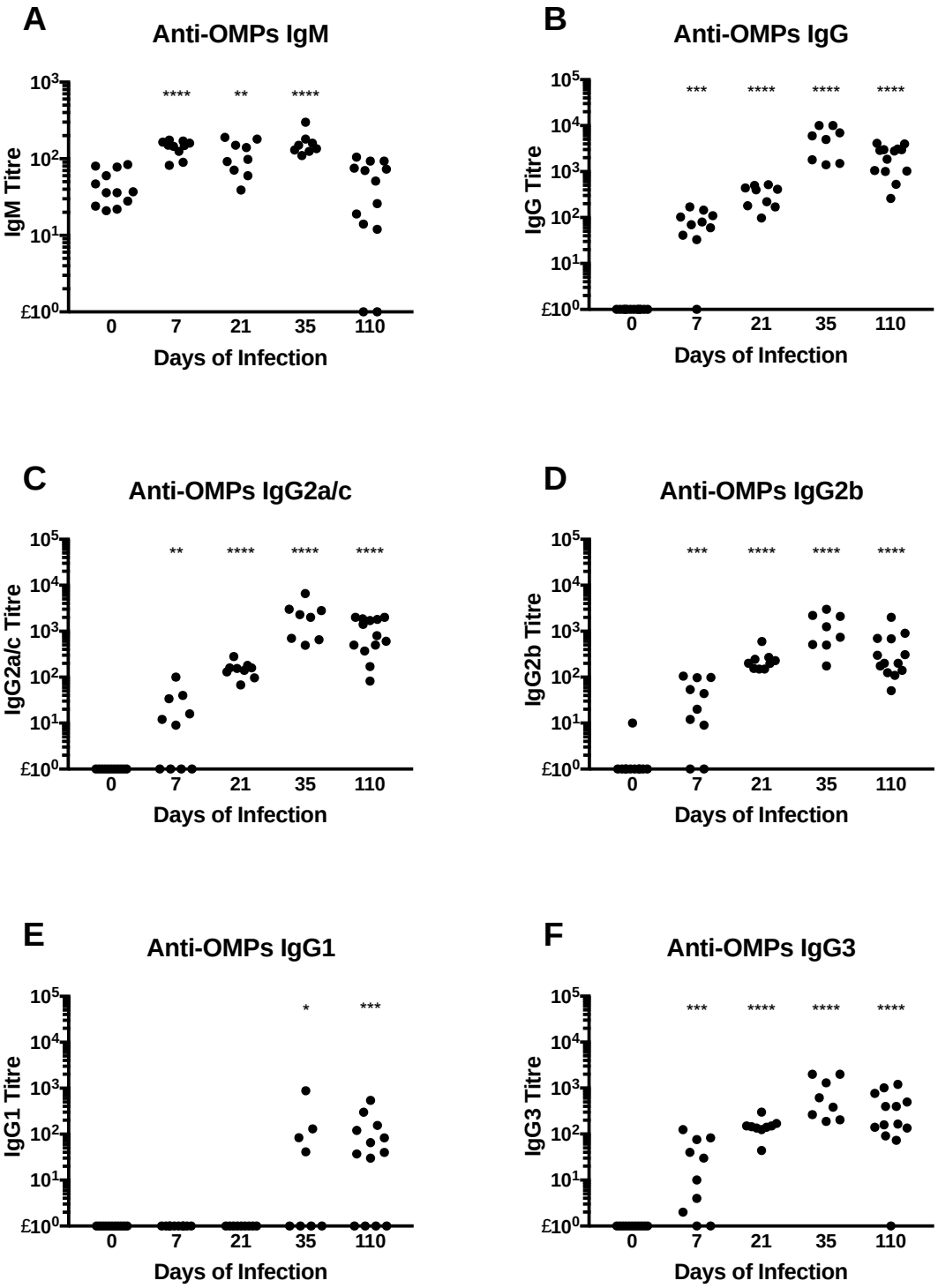

Figure 4.3:Anti-OMPs response following infection with STm
C57BL/6J mice were infected i.p. with 5 x 10^5 SL3261 for 7, 21, 35 & 110 days. *Salmonella* specific **A**) IgM, **B**) IgG, **C**) IgG2a/c **D**) IgG2b **E**) IgG1 and **F**) IgG3 antibody titres were assessed by ELISA against 96 wells plates coated with 5 µg of OMPs from STm. Data is pooled from two independent experiments with 4-6 mice per time point per experiment. *(P≤0.05), ** (P≤0.01), ***(P≤0.001) & **** (P≤0.0001).

4.2.3. Kinetics of the self-reactive antibody response following infection with STm

After establishing that the pathogen-specific antibody response peaks after five weeks of infection the self-reactive antibody response was measured to determine whether this follows similar kinetics. To achieve this, sera were screened by an ELISA method that was previously mentioned in Chapter 3 (**Figure 3.12**). ELISA plates coated with liver, heart and kidney (LHK) homogenate were incubated with mouse sera and self-reactive antibodies were detected using a secondary antibody to IgM or IgG. Modest titres of LHK binding IgM and IgG antibodies were detectable three weeks after infection with STm **(Figure 4.4**). The titres of IgM and IgG LHK binding antibodies declined to levels near background by day 110.

To confirm these findings Nova Lite rat stomach, liver and kidney slides were stained with DAPI (stains cell nuclei), incubated with sera and self-reactive antibodies were detected using a secondary antibody that can bind IgA, IgG and IgM. $LTBR^{-/-}$ mice are known to have impaired negative selection (264, 265). Sera from these mice was kindly provided by Dr Manuela Carvalho-Gaspar (University of Birmingham) and used as a positive control for this assay following confirmation against 15 week old *Roquin*$^{san/san}$ mice, which are known to give rise to autoantibodies and tissue pathology resembling SLE (266).

The positive control shows the presence of self-reactive antibodies against the rat stomach and the underlying muscle (**Figure 4.5**.). In contrast, no staining can be detected to any of the tissues in N.I. mice. There is a small increase in the anti-rat

smooth muscle and liver antibodies in the N.I. mice that were maintained alongside the infected mice for 110 days, the levels detected are similar to WT mice that have been infected for 7 days. Following infection with STm, self-reactive antibodies can be detected 7 days post-infection to the stomach, muscle and liver in **8/8** mice. After 21 days of infection the intensity of the self-reactive antibody staining against the stomach, muscle and liver increased compared to mice infected for one week. In addition, self-reactive antibodies can also be detected against the kidney. In total, **8/9** mice were positive for self-reactive antibodies against the stomach, muscle and liver after three weeks of infection. In contrast, the intensity of the staining appeared to decrease with time both 35 and 110 days after infection. Despite this, self-reactive antibodies could be detected in **7/8** mice and **10/12** mice after 35 and 110 days, respectively. The most persistently intense staining observed across two experiments was in the stomach and the underlying smooth muscle.

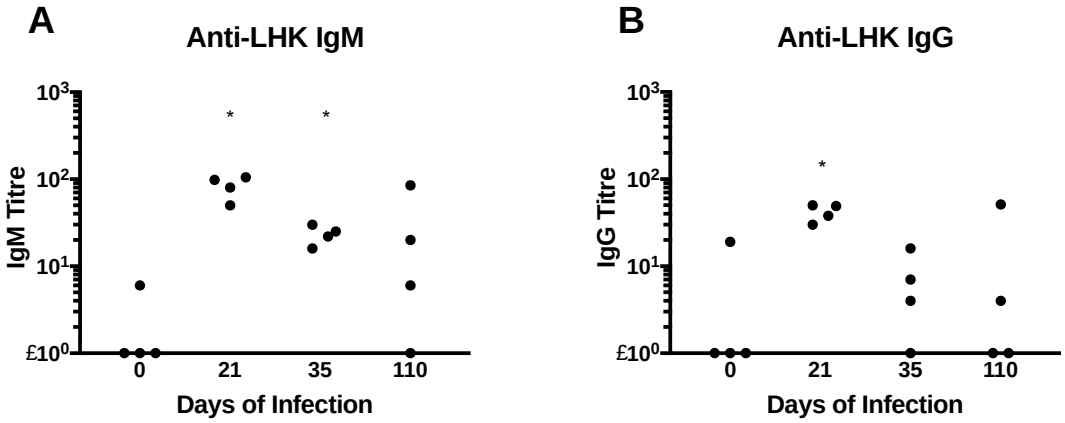

Figure 4.4:Screening for self-reactive antibodies by ELISA after infection with STm.
C57BL/6J mice were infected i.p. with 5 x 10^5 SL3261 and sera was collected 7, 21, 35 & 110 days later. Self-reactive **A**) IgM and **B**) IgG antibody titres were screened at a 1/20 dilution against 96 well plates coated with mouse heart, liver and kidney homogenate. Data is from a single experiment with 4 mice per group. *(P≤0.05).

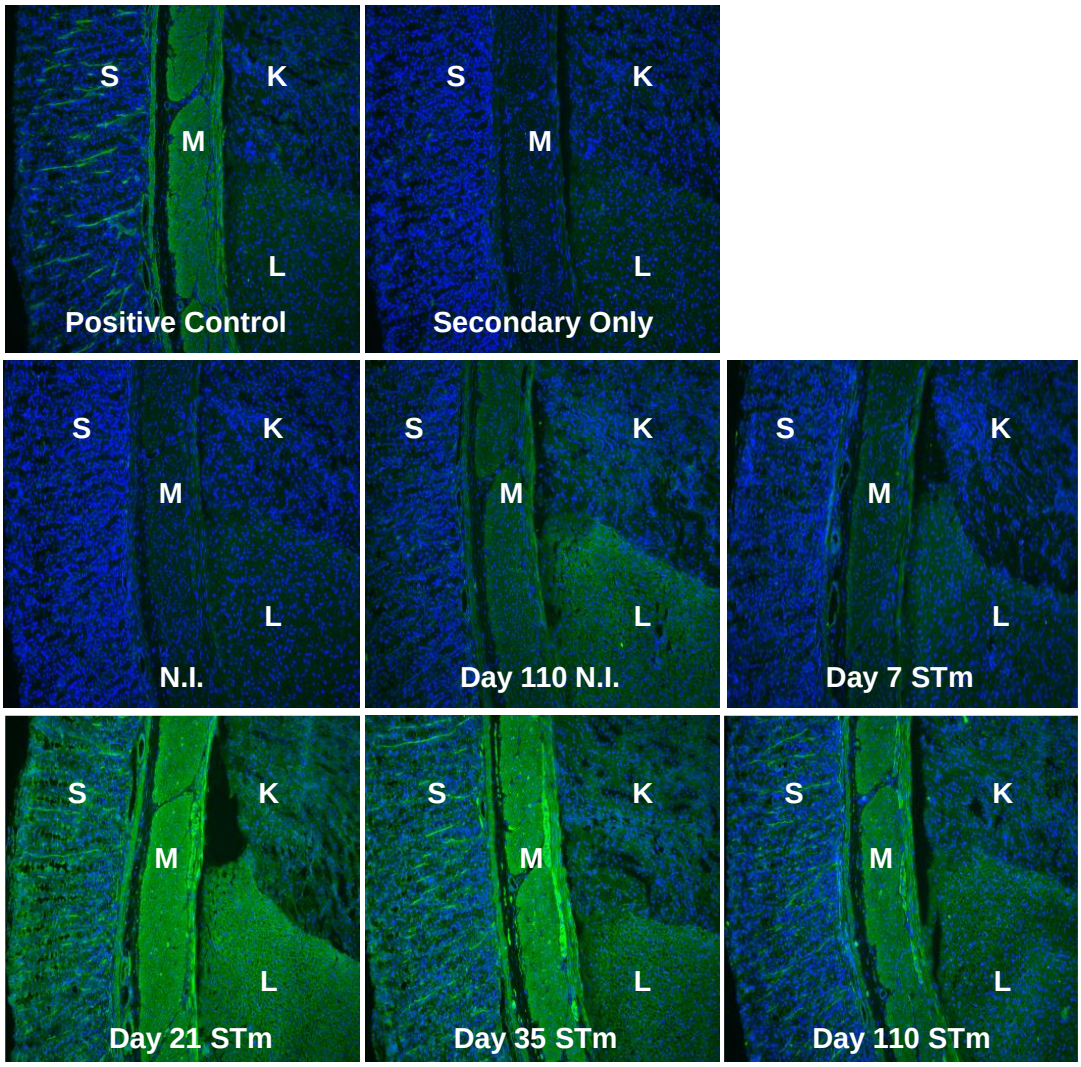

Figure 4.5:Kinetics of the rat stomach, liver & kidney antibody response after infection with STm. C57BL/6J mice were infected i.p. with 5 x 10^5 SL3261 and sacrificed 7, 21, 35 & 110 days post-infection. Nova lite rat stomach, liver & kidney slides were incubated with mouse sera at a 1/20 dilution and self-reacting antibodies were detected using a F(ab/)2 IgG-FITC secondary. Cell nuclei were stained with DAPI and images taken at a 20x magnification. **S**-Stomach, **M**-Muscle, **L**-Liver & **K**-Kidney. Data is representative of two independent experiments with 4-6 mice per group per experiment. Please note that the images from day 21 and 35 infected mice are part of the IL-6 experiment presented in Chapter 5 and that the same positive control, N.I. control and secondary only control is presented.

4.2.4. Characterisation of the self-reactive antibody isotypes induced in response to STm

Given the most intense staining for self-reactive antibodies were observed in mice that had been infected for three weeks, we hypothesized that the class of self-reactive antibody being produced was IgG. To test this rat stomach, liver and kidney slides were stained for IgM, IgG or IgA antibodies three weeks post-infection.

Self-reactive IgM staining can be detected to both the stomach and muscle in the N.I. mice (**Figure 4.6.A.**). Some background staining for IgM antibodies could be detected in the N.I. control. The intensity of the self-reactive IgM staining increases after three weeks of infection with STm and a total of 7/8 mice were positive across two independent experiments. This suggests that self-reactive IgM antibodies are induced by infection. However, the pattern of the IgM staining appears to be vague as no distinct pattern can be observed. The secondary antibody for IgM is specific as no IgM self-reactive antibodies can be detected in the secondary only control, nor in the sera of B cell deficient mice lacking the immunoglobulin heavy chain ($Ig\mu^{-/-}$).

IgG staining could be detected against the muscle, stomach and cell nuclei in the LTBR positive control (**Figure 4.6.B**). No staining could be detected in the N.I. mice. In contrast, there was clear IgG antibody staining observed to the stomach and muscle after 21 days of infection. This was detected in **8/8** mice from two independent experiments. In addition, the streaked staining pattern observed in the stomach is consistent with that seen when using the F(ab')2 secondary antibody that can detect

IgM, IgG and IgA antibodies. This suggests that the F(ab')2 secondary antibody is largely picking up antibodies of the IgG class in the stomach. However, we cannot rule out that self-reactive IgA antibodies are also being produced. Attempts to stain for self-reactive IgA antibodies were negative and since an appropriate positive control could not be acquired we cannot be certain that this is a technical failure or a genuine result. Therefore, IgM and IgG self-reactive antibodies are induced three weeks after infection with STm.

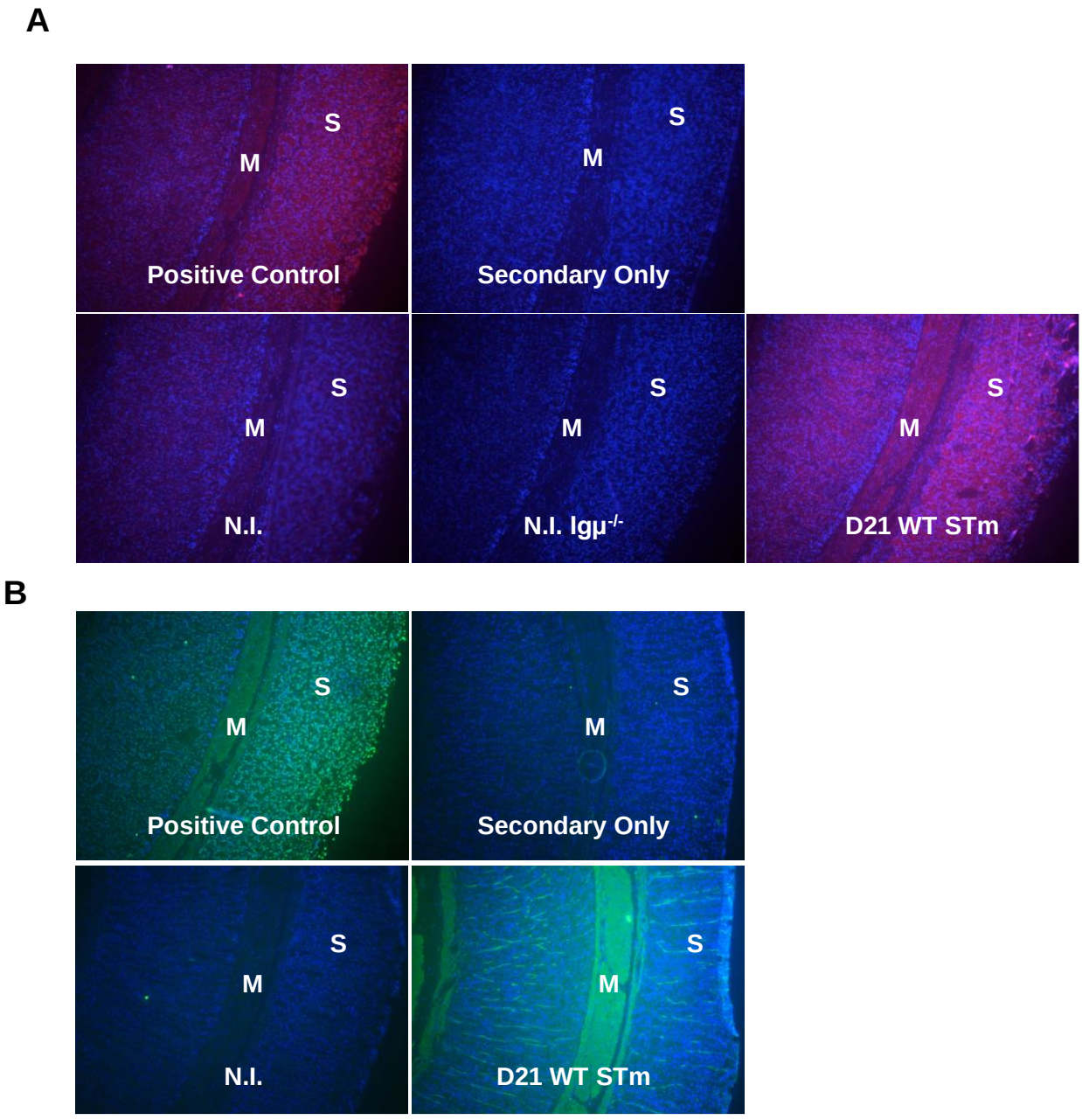

Figure 4.6:IgM and IgG staining for self-reactive antibodies after infection with STm.
C57BL/6J mice were infected i.p. with 5 x 10^5 SL3261 and sacrificed 21 days post-infection. Nova lite rat stomach, liver & kidney slides were incubated with mouse sera at a 1/20 dilution and self-reacting antibodies were detected using a secondary for either **A**) IgM (TRITC Conjugate) or **B**) IgG (FITC Conjugate). Cell nuclei were stained with DAPI and viewed using a fluorescent microscope. **S**-Stomach and **M**-Muscle. Data is representative of two independent experiments with 4-6 mice per group per experiment.

4.2.5. Identification of cellular targets of IgG antibodies induced during infection with STm

Following the detection of IgG antibodies against the stomach and underlying muscle we tested what cellular components the antibodies induced by infection are binding. In order to investigate this we stained human epithelial substrate (HEp-2) slides with sera from mice that had been infected with 5 x 10^5 SL3261 for 7, 21, 35 and 110 days (**Figure 4.7.**). As a positive control we used 15 week old Roquin$^{san/san}$ mice and LTBR mice. The antibodies from LTBR mice stain nuclei a clear bright apple green, while the N.I. and secondary IgG antibody controls show no IgG self-reactive antibodies. N.I. mice that have aged 110 days have some background antibodies directed against nuclear and cytoplasmic components. After three weeks of infection with STm there is a clear induction of antibodies that can bind both the nuclear and cytoplasmic components. These self-reactive antibodies were detected in 4/4 mice at this time-point. Similar findings were observed after 35 days and 110 days of infection with 4/4 and 4/6 mice being positive. However, the individual staining pattern was highly variable between mice at each time-point. It was therefore difficult to identify the specific cellular components that these antibodies are targeting, as there were no consistent patterns. This data confirms that self-reactive IgG antibodies are induced after infection with STm to both nuclear and cytoplasmic components.

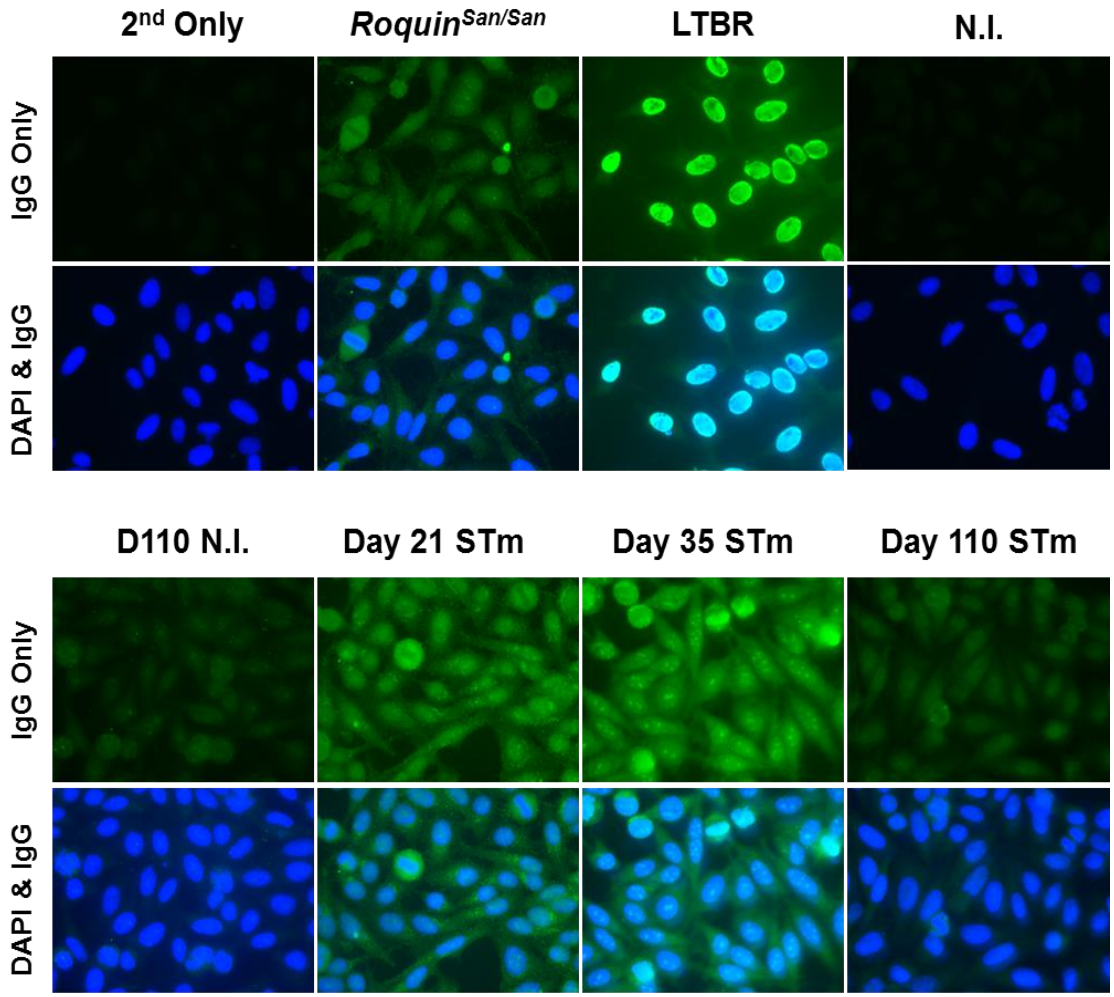

Figure 4.7:HEp-2 staining for self-reactive antibodies after infection with STm.
C57BL/6J mice were infected *i.p.* with 5 x 10^5 SL3261 and sera were collected 21, 35 & 110 days later. HEp-2 slides were incubated with mouse sera at a 1/20 dilution from N.I. and infected mice. Self-reactive antibodies were detected with a secondary for IgG and cell nuclei were counterstained with DAPI. Self-reactive IgG antibodies are shown in green and DAPI is shown in blue. Data is from a single experiment and is representative of 4-6 mice per time point. (4/4) mice were positive for anti-nuclear and cytoplasmic staining after three and five weeks of infection. After 110 days of infection 4/6 mice were positive for anti-nuclear and cytoplasmic staining.

4.2.6. Identification of self-reactive IgG subclasses following infection with STm.

The peak of the self-reactive antibody response was detected in mice that had been infected for three weeks, a time when T cells are known to be important for controlling and resolving infection (135, 267). In addition, our two different immunofluorescent staining's for self-reactive antibodies suggested that the F(ab')2 secondary antibody was largely picking up IgG antibodies at this time-point. As a consequence it was hypothesized that IgG2a/c would be the dominant IgG subclass given that this is the main isotype induced against the pathogen (47). To test this we screened for liver, heart and kidney antibodies by ELISA after 21, 35 and 110 days of infection (**Figure 4.8**). There was a clear induction of LHK reactive IgG2c and IgG2b after three weeks of infection. Some IgG3 could also be detected at this time-point. The appearance of IgG2a/c and IgG3 LHK reactive antibodies was transient as they could not be detected 35 and 110 days post-infection. In contrast, the IgG2b anti-LHK antibodies titres persisted at similar levels until day 35 and then declined by day 110. No IgG1 LHK reactive antibodies could be detected at any time-point.

Since the peak of the self-reactive response detected by screening ELISA was three weeks post-infection we tested for IgG2a/c, IgG2b and IgG3 self-reactive antibodies at this time-point, by immunofluorescence. IgG2a/c antibodies were detected against the stomach and smooth muscle after 21 days of infection in **8/8** mice (**Figure 4.9 A**). In contrast, only some weak muscle staining could be detected in the IgG2b (**Figure 4.9 B**) and IgG3 (**Figure 4.9 C**) subclasses. In addition, only **6/8** mice were positive for anti-

muscle IgG2b antibodies and **4/4** for IgG3. Therefore, IgG2a/c is the dominant subclass of IgG self-reactive antibodies induced after infection with STm.

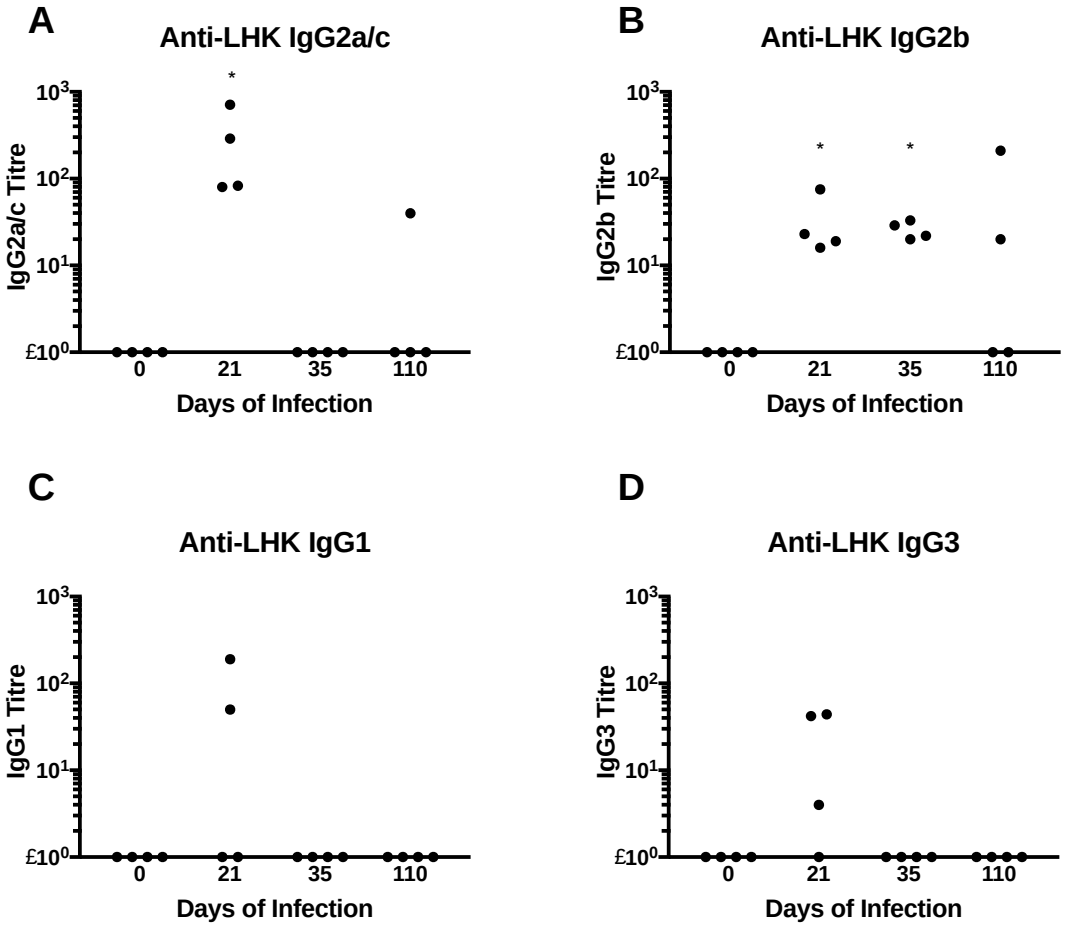

Figure 4.8:Screening for LHK IgG subclasses after infection with STm
C57BL/6J mice were infected i.p. with 5 x 10^5 SL3261 and sera was collected 7, 21, 35 & 110 days later. Self-reactive **A**) IgG2a/c **B**) IgG2b **C**) IgG1 and **D**) IgG3 antibody titres were screened against 96 well plates coated with mouse heart, liver and kidney homogenate. Data is from a single experiment with 4 mice per time point. *(P≤0.05).

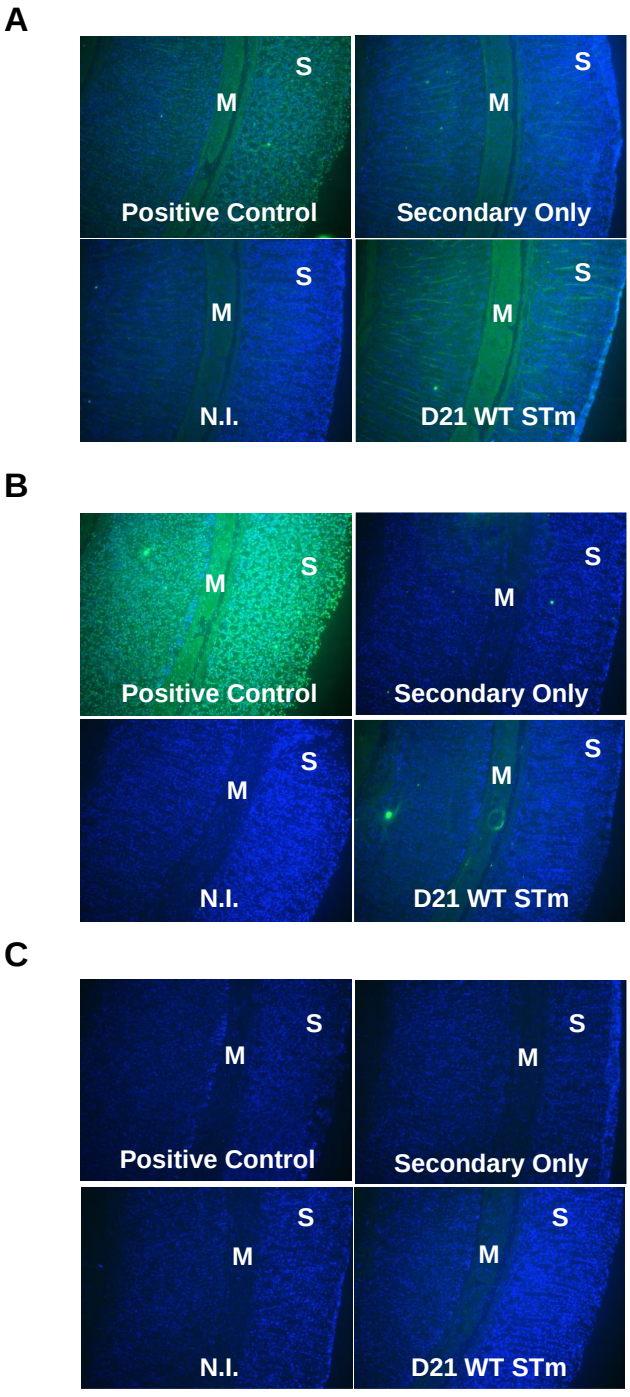

Figure 4.9:IgG2a/c, IgG2b and IgG3 staining for self-reactive antibodies after infection with STm. C57BL/6J mice were infected i.p. with 5 x 10^5 SL3261 and sacrificed 21 days post-infection. Nova lite rat stomach, liver & kidney slides were incubated with mouse sera at a 1/20 dilution and self-reacting antibodies were detected using a secondary for either **A**) **IgG2a/c**, **B**) **IgG2b** or **C**) **IgG3**. Cell nuclei were stained with DAPI and sections were viewed using a fluorescent microscope. **S**-Stomach & **M**-Muscle. Data is representative of two independent experiments.

4.2.7. Role of IFN-γ and T-bet in the generation of self-reactive antibodies.

The detection of IgG2a/c self-reactive antibodies directed against rat stomach and muscle suggested that this may be an isotype-specific response induced after infection. As a consequence we hypothesized that if we infected mice that have impaired IgG2a/c class switching then there would be no self-reactive antibodies. IFN-γ and T-bet are required for IgG2a/c class switching in mice (268). In addition, the aberrant production of IFN-γ can promote the development of systemic autoimmune diseases such as SLE (244, 269). It was therefore predicted that Th1 responses and IFN-γ would be required for the development of the self-reactive antibody response. Past experiences from our laboratory have shown that IFN-$\gamma^{-/-}$ mice succumb to infection with SL3261 after 10 days. In order to compare the production of self-reactive antibodies in WT mice and IFN-$\gamma^{-/-}$ mice, these were infected with SL3261 for one week. In Chapter 4, self-reactive antibodies could not be detected in WT mice after one week of infection at a 1/20 dilution, as a consequence a 1/10 dilution of sera was used. Stomach and muscle antibodies were detected one week after infection in 4/4 WT mice and 3/4 IFN-$\gamma^{-/-}$ mice (**Figure 4.10 A**). There was no observable difference detected in the intensity of the staining.

To test if IFN-γ might be having more of an influence later on in the infection we screened sera from bone marrow chimeras in which either the stromal or haematopoietic compartment was IFN-$\gamma^{-/-}$. These chimeras were generated by Dr Ruth Coughlan and Dr Ewan Ross. In brief, WT and IFN-$\gamma^{-/-}$ mice were irradiated and then re-constituted with bone marrow from either IFN-$\gamma^{-/-}$ mice or WT mice. Anti-LHK IgM (**Figure 4.10 B**) and

IgG (**Figure 4.10 C**) antibodies could be detected three weeks after infection, but no difference could be found in the absence of IFN-γ from either the stromal (WT→IFN-$\gamma^{-/-}$) or haematopoietic (IFN- $\gamma^{-/-}$→WT) compartment. T-$bet^{-/-}$ mice cannot produce IgG2a/c antibodies after infection with STm (186). To help confirm the findings from IFN-$\gamma^{-/-}$ mice, sera were screened from WT mice and T-$bet^{-/-}$ mice that had been infected for 18 or 35 days. Self-reactive IgM and IgG antibodies were induced in WT mice after 18 days of infection and these persisted until day 35. No difference between the WT mice and T-$bet^{-/-}$ mice could be detected at either time-point before or after infection (**Figure 4.11 A & B**). These findings were confirmed through rat stomach, liver and kidney slide staining. No difference could be detected in the stomach or muscle staining between the infected WT mice and T-$bet^{-/-}$ mice both 18 and 35 days post-infection (**Figure 4.11 C**). For example, 4/4 mice WT mice and 4/4 T-$bet^{-/-}$ mice were positive for self-reactive antibodies against the stomach and muscle after 18 days of infection. However, after 35 days of infection 4/4 WT mice were positive but only 2/4 T-$bet^{-/-}$ mice had self-reactive antibodies after infection despite the higher numbers of bacteria in T-bet knockouts. Therefore, the induction of self-reactive antibodies is neither dependent upon Th1 cells nor IgG2a/c. This implies that other antibody isotypes can also contribute to the self-reactive response and that Th1 cells might be required to maintain the anti-self-antibody response.

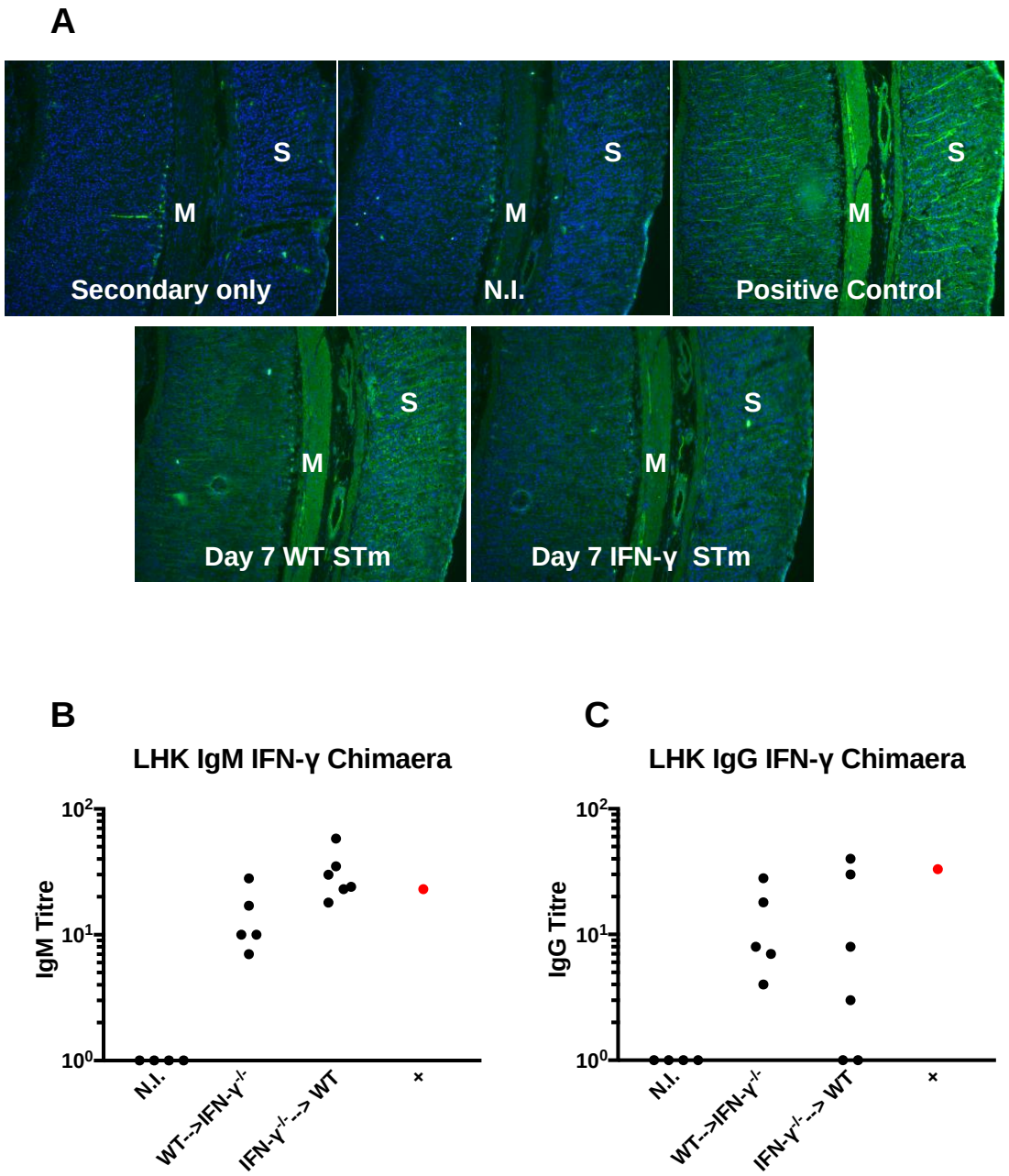

Figure 4.10:Testing for self-reactive antibodies in IFN-γ$^{-/-}$ mice after infection with STm
C57BL/6J and IFN-γ $^{-/-}$ mice were infected for 7 days with STm. **A**) Rat Stomach, Liver and Kidney slides were stained with sera from infected WT and IFN-γ$^{-/-}$ mice for self-reactive antibodies using a F(ab$'$)2 IgG-FITC secondary. Cell nuclei were stained with DAPI (blue). **S**- Stomach & **M** – Muscle. Ruth Coughlan had previously generated mixed bone marrow chimaeras where IFN-γ$^{-/-}$ mice had been irradiated plus reconstituted with C57BL/6J bone marrow (WT→ IFN-γ$^{-/-}$) or vice versa (IFN-γ$^{-/-}$→WT) and infected them with STm for 21 days. Sera were screened by ELISA for **B**) IgM and **C**) IgG antibodies to mouse liver, heart and kidney homogenate. The + is a positive control from San Roque mice which possess autoantibodies against rat stomach and muscle. Data is representative of two independent experiments with 5-6 mice per group (except the positive control) per experiment.

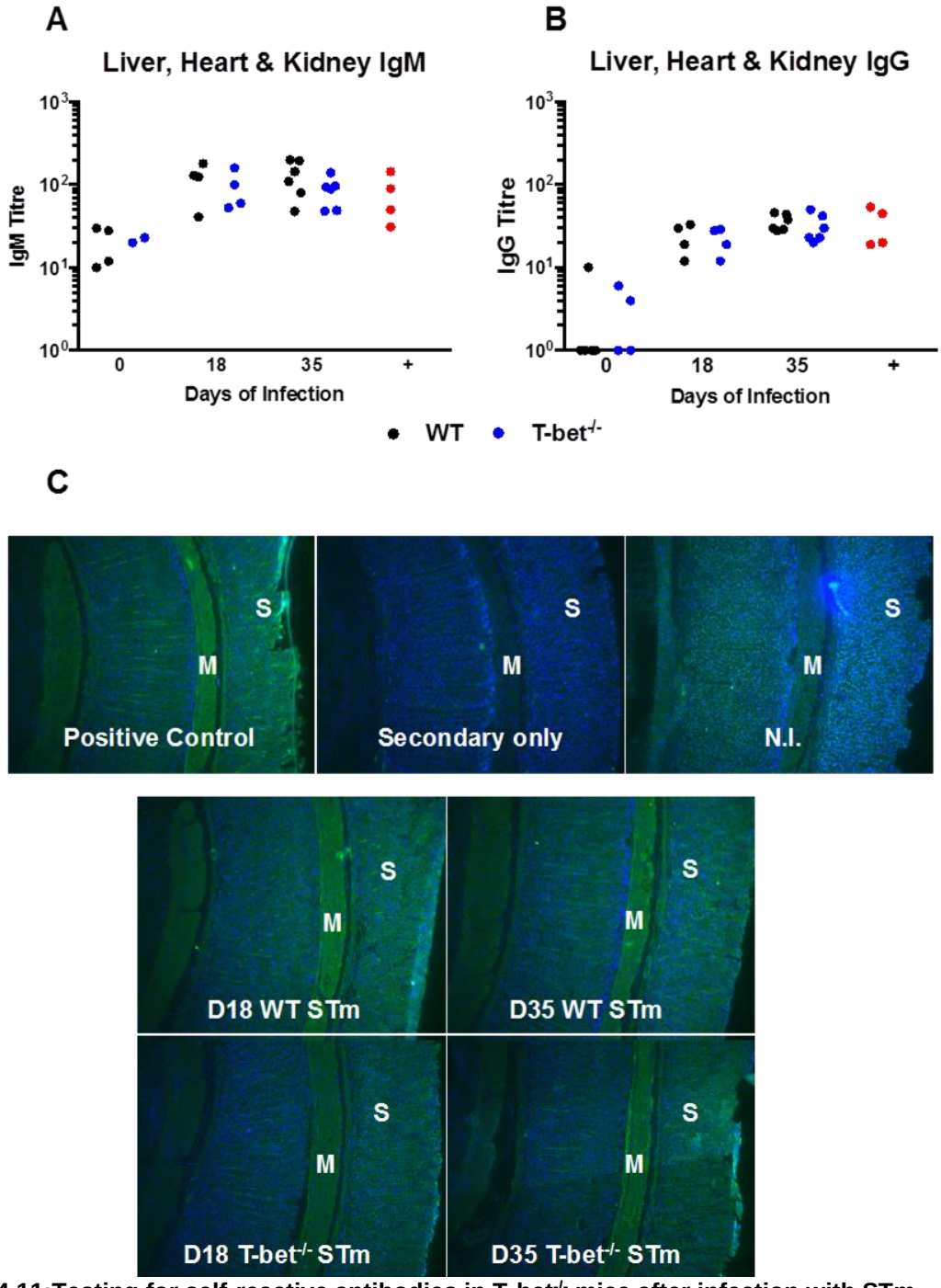

Figure 4.11:Testing for self-reactive antibodies in T-bet$^{-/-}$ mice after infection with STm.
C57BL/6J and T-bet$^{-/-}$ mice were infected i.p. with STm for 18 and 35 days. Sera were screened by ELISA for **A**) IgM and **B**) IgG antibodies to mouse liver, heart and kidney homogenate. The + sign represents a group of San Roque mice that were provided by Dr Fabrina Gaspal and confirmed to have autoantibodies and autoimmune disease. **C**) Nova lite rat stomach sections were incubated with mouse sera and tissue specific antibodies (green) detected with a F(ab$'$)2 IgG-FITC secondary. DAPI staining for cell nuclei is shown in blue. Immunofluorescence data is representative of two independent experiments with 4 mice per group per experiment, whilst ELISA data is only from a single experiment.

4.3. DISCUSSION

In the previous chapter the production of self-reactive antibodies against the rat stomach, liver and kidney were detected after infection with STm. This was shown to be TLR4-dependent in BALB/c mice, but was inconclusive in C57BL/6J mice due to the strength of the staining. The self-reactive response may not be a product of polyclonal B cell stimulation by LPS. Evidence supporting this is that the total number of switched and non-switched extrafollicular plasmablasts detected by ELISPOT after infection with STm was not affected by the absence of TLR2, TLR4 or MyD88 (214). To investigate the self-reactive antibody response we first infected C57BL/6J mice for 7, 21, 35 and 110 days. The highest bacterial burden was observed one week after infection and thereafter-bacterial numbers declined in the spleen (**Figure 4.2 A**) and the majority of mouse livers (data not shown) by 110 days. It should be noted that the infection was not consistently cleared from the kidneys as in one experiment 5/6 mice had between 20-170 CFU/kidney after 110 days (data not shown). This could potentially influence the persistence of the antibody response to self-antigens.

Salmonella-specific antibodies were measured by looking at the titres of anti-OMPs IgM and IgG. IgM was rapidly induced 7 days after infection and remained at similar levels until day 35. In addition, the IgM response appears to decline to background levels by day 110. Given that no increase in anti-OMPs IgM was observed at day 35 it suggests that limited pathogen-specific IgM antibodies are coming from the germinal centre reaction. This could also explain why the IgM titres return to background levels after 110 days of infection, as the germinal centre is typically the main source of long lived plasma

cells. This is further supported by B cell ELISPOT data in which no difference between the N.I. and infected mice was detected in the number of LPS or porin-specific IgM^+ plasma cells in the spleen or bone marrow after 110 days (data not shown). In contrast, the anti-OMPs titres of IgG and all subclasses, except IgG1, increased from day 7 onwards up until day 35. The largest increase in IgG titres was observed 5 weeks after infection. These observations are consistent with previous work from our laboratory, which have shown that this coincides with the appearance of germinal centres in the spleen (47). The longevity of this response is most likely due to a combination of persisting germinal centres giving rise to antibody secreting cells and long lived plasma cells in the bone marrow that continue to secrete antibodies against the pathogen.

After one week of infection with STm, self-reactive antibodies were detected against the stomach, liver and muscularis/muscle in the time course experiment. The intensity of the staining peaked after three weeks of infection and self-reactive antibodies could be detected against the stomach, muscularis/muscle, liver and kidney. In contrast, the titres and intensity of self-reactive antibodies declined after five weeks of infection but persisted until day 110. Since the pathogen-specific antibodies were increasing five weeks after infection, at a time when the self-reactive response is declining, it suggests that there is a discourse in the kinetics of these two responses.

The peak of the self-reactive response was consistently observed three weeks after infection, a time when the EFR against the pathogen is known to dominate. Since the EFR to STm is known to produce low affinity pathogen-specific antibodies this self-

reactive antibody response could be a product of molecular mimicry and inflammation as the stomach-specific antibody response decreases as the bacterial burden declines in the spleen. This low affinity antibody response would also explain the variable cytoplasmic and nuclear staining observed against HEp-2 slides. Nevertheless, LPS could be responsible for the induction of nuclear antibodies, as this has been shown to induce dsDNA antibodies in NZB x NZW F_1 mice (270). However, the total amount of LPS in an infection with 5 x 10^5 SL3261 is much lower. Self-reactive antibodies persist until 110 days post-infection and the exact source of these at later time-points is not known as they could be derived from the EFR, the GC reaction or both. One interesting observation was the identification of stomach antibodies as these appeared similar to the staining observed with gastric parietal cell antibodies, which are associated with pernicious anaemia and atrophic gastritis (271). It would therefore be interesting to test for intrinsic factor specific antibodies as these could impair vitamin B12 absorption (272). The stomach antibodies could also be targeting the H^+/K^+ ATPase proton pump. Antibodies against this pump have been associated with H.*pylori* infection and autoimmune gastritis (273, 274). Alternatively, antibodies from Crohn's disease patients have been shown to bind flagellin from STm. In addition, these same antibodies can also bind TLR5 and Pals 1-associated tight junctions, which hold epithelial cells together. Antibodies against these two molecules can induce the activation of monocytes and increase the permeability of the intestine (275). This suggests that the anti-stomach antibodies induced after infection with STm could be anti-flagellin antibodies that can cross-react with stomach epithelial cells. To take these findings forward, WT mice and $TLR5^{-/-}$ mice would need to be infected for three weeks with

SL3261 and a mutant strain that does not express flagellin in order to compare the stomach-specific antibody response. The production of anti-stomach antibodies could be clinically relevant as they might promote a leaky gut or enhance escape of the pathogen into the gastrointestinal tract. Anti-smooth muscle antibodies were also detected after infection with STm and are associated with conditions such as autoimmune/chronic hepatitis and primary billary cirrhosis (PBC) (276). The appearance and persistence of these antibodies may be a product of liver damaged induced by the infection. The clinical significance of such antibodies is currently unknown but is interesting since STm and *S*.Minnesota have been linked to the pathogenesis of PBC (277, 278). It should be noted though that anti-smooth muscle antibodies are normally only found in 20-40% of PBC patients whereas anti-mitochondrial antibodies are detected in approximately 90% of patients (279). To confirm if the antibodies can drive tissue damage we would have to carry out *in vitro* experiments to test the ability of these antibodies to induce complement-mediated killing and antibody-dependent cell-mediated cytotoxicity against cells. In addition, we could look at the stomach and muscle before and after infection through histology in WT mice and BLIMP-$1^{/-}$ mice, as these cannot produce plasma cells. Alternatively, a serum transfer experiment could be done by injecting mice with sera from N.I. and STm infected mice. Tissue pathology could then be detected by histology.

Self-reactive IgM antibodies were also induced against the stomach, muscle and liver after three weeks of infection with STm. However, the background levels of self-reactive IgM were variable in N.I. mice, which suggested that we are detecting natural antibodies

(69). It appeared that the IgM self-reactive antibodies stained similar tissues to our F(ab')2 antibodies but the staining was more intense and the pattern less discernable. In contrast, IgG self-reactive antibodies appeared to stain tissues with a similar pattern to that of the $F(Ab^2)$ antibody. The data suggests that IgG is the main class of self-reactive antibodies that we are detecting after infection using our F(ab')2 antibody. However, we cannot rule out whether or not we are detecting some IgM or that self-reactive IgA antibodies are being induced by infection. It would be interesting to see if an oral infection alters the levels and class of self-reactive antibodies especially since there would be inflammation at the mucosa of the gastrointestinal tract to allow bacterial entry from the gut.

The detection of anti-self IgG antibodies against the stomach implied that this would be a subclass-specific immune response since IgG2a/c is the main isotype produced against the pathogen. This was initially supported by staining that showed the stomach and muscle antibody response appeared to be dominated by IgG2c response as very little IgG3 or IgG2b could be detected. Rat stomach, liver and kidney slides were also stained for IgG1 self-reactive antibodies but none could be detected in a preliminary staining (Data not shown). The dominance of the IgG2c antibody response suggested that the main antibody isotype raised against the pathogen also binds self. This implied that IFN-γ and T-bet were driving both the pathogen-specific and self-reactive immune response. However, IFN-$\gamma^{-/-}$ mice had similar levels of stomach and muscle-specific antibodies to the WT mice after 7 days of infection. Similar observations were measured by anti-LHK ELISA after the infection of IFN-γ bone marrow chimaeras for three weeks.

Although these findings need to be confirmed by tissue sections as the majority of antibodies detected by the screening ELISA are most likely not anti-self-antibodies and therefore the assay could be more sensitive but less specific. Despite this, no difference could be detected in the self-reactive antibody response between the WT mice and T-bet$^{-/-}$ mice after 18 or 35 days of infection. The stomach and muscle-specific antibody response is therefore not restricted by a particular IgG subclass, nor is it dependent upon Th1 responses. The induction of self-reactive antibodies could be due to molecular mimicry. Alternatively, given the redundancy of pro-inflammatory cytokines a range of other molecules could drive the response in the absence of IFN-γ or T-bet. The resulting inflammation could cause tissue damage leading to the release of self-antigens, bystander activation, epitope spreading and the expression of cryptic epitopes. In addition, new epitopes could be generated as dsDNA could potentially complex with STm through curli on the bacterial membrane (280, 281). This pattern would explain why the response peaks when tissues are highly inflamed and declines with the clearance of the bacteria. However, it cannot explain why self-reactive antibodies persist especially since it could be that antibodies derived from the EFR do not survive until 110 days post-infection. The reason for this is that the EFR is short lived in the absence of persistent antigen/infection and that the half-life of IgG antibodies ranges from 4-8 days depending on the subclass (282). It is therefore possible that some self-reactive plasma cells are undergoing positive selection in the germinal centre, which would also imply that self-reactive T cells are being induced in response to STm.

Overexpression of IFN-γ has been shown to drive systemic autoimmune disease and induce the production of anti-dsDNA and anti-histone antibodies (283). Many of these observations have been made in transgenic models of systemic autoimmune disease under steady state conditions. Infections with bacteria like STm could therefore be inducing autoreactive responses through different immune pathways. Especially since other strains of STm has been shown to drive the development of autoreactive TfH cells which in turns leads to hypergammaglobulinaemia and the deposition of immune complexes in the kidney (261). Therefore, it would be interesting to understand what molecules regulate the pathogen-specific and self-reactive antibody responses.

In conclusion, there is a clear discourse in the kinetics of the pathogen-specific and self-reactive antibody response. Self-reactive IgM and IgG antibodies are induced against the stomach, muscle and liver. The dominant subclass of self-reactive IgG induced is IgG2a/c. However, the response is not dependent upon a particular IgG subclass and does not appear to be dependent upon two of the major drivers of systemic autoimmune disease, IFN-γ and T-bet.

5. CHAPTER 5 – REGULATION OF THE PATHOGEN-SPECIFIC AND SELF-REACTIVE ANTIBODY RESPONSE

5.1. INTRODUCTION

In Chapter 4, we identified that infection with STm induces an IgM and IgG self-reactive antibody response in WT mice. The induction of this response was independent of IFN-γ and T-bet, which suggested that it is not Th1 dependent. This led to the hypothesis that other immunoregulatory checkpoints might be important in controlling this response. As a consequence we decided to examine the response to STm in a range of mice that have defects in molecules associated with the induction of adaptive immunity after infection with STm.

To generate antibody responses the cross talk between APCs like DC and T cells and B cells and T cells is required. CD80 and CD86 are co-stimulatory molecules that are essential for the activation of T cells and are expressed by APCs. They are normally upregulated following their exposure to antigen and activation through PRRs. CD80 and CD86 provide co-stimulation to T cells through engagement of CD28 at the immune synapse. CD28 is constitutively expressed on naïve and resting T cells and binding to this releases IL-2 that can drive the initial growth of activated T cells (284, 285). The importance of this interaction is evident in CD80/86$^{-/-}$ and CD28$^{-/-}$ mice, as these cannot develop effective T cell responses during a primary immune response. In addition, CD28 stimulation leads to sequential expression of ICOS on T cells (286). CD28 and ICOS are reported to operate synergistically to drive the differentiation of TfH cells which

depending on their maturation state can induce extrafollicular or germinal centre antibody responses. Evidence supporting this is observed in response to SRBCs and STm infection in which $CXCR5^{+}$ $PD\text{-}1^{low}$ or $PD\text{-}1^{High}$ TfH cells have been shown to appear with the EF or GC response respectively (228).

Pre-TfH cells can migrate to T-B border and interact with cognate B cells. B cells provide additional stimulation to T cells through provision of various co-stimulatory (CD80, CD86, ICOSL and OX40L) and co-inhibitory molecules (PD-L1 and PD-L2) (285). ICOSL is the only known ligand for ICOS. The expression of ICOSL is not required for germinal centre formation but is essential for the maintenance of these structures. $ICOS^{-/-}$ mice have lower number of TfH cells, germinal centres and exhibit impaired antibody responses (287, 288). The role of ICOS in antibody production appears to be restricted to TD responses. Evidence of this is that $ICOSL^{-/-}$ mice have normal IgM and IgG3 immune responses following immunization with the TI antigen, NP-LPS (286, 289). The exact role in the EFR is less clear. Dysregulation of ICOS signaling can also contribute to the development of autoimmunity as impaired negative feedback contributes to the accumulation of TfH cells and a lupus-like autoimmune phenotype in *Roquin*$^{San/San}$ mice (290).

IL-6 is a key cytokine that is required for optimal immunity to a range of different pathogens (291). This cytokine is important for both the innate and adaptive immune response. Depending upon the context it can promote inflammation or facilitate resolution (292). In the adaptive immune response, IL-6 is known to promote the growth

of B cells and support the differentiation, survival and maintenance of antibody-secreting cells (291, 293-296). In addition, IL-6 in conjunction with IL-21 can directly affect T cells by promoting the differentiation of TfH cells. For example, IL-21$^{-/-}$ mice have normal antibody responses in STm infections (297). However, previous findings from our laboratory have shown that loss of IL-6 delays the formation of germinal centres after infection with STm (186). IL-6 can therefore influence B cell responses at various stages of the adaptive response by acting directly on them or through T cells. Excess IL-6 is associated with a range of different autoimmune conditions in which it can promote aberrant TfH and Th17 responses (298).

In this chapter we attempted to address how the antibody response to the pathogen and host is regulated. The goal was to identify whether these two antibody responses are co-regulated or independently regulated. As a result we characterized the antibody response after infection with STm in a range of different genetic knockout mice.

5.2. RESULTS

5.2.1. IL-6 is not required for the control of systemic infection with STm

IL-6 is a pleiotropic cytokine that plays a key role in both the innate and adaptive response to a range of different pathogens (291). To investigate the role of this cytokine in the immune response to STm, we infected WT mice and IL-6$^{-/-}$ mice for 21 and 35 days. IL-6$^{-/-}$ mice had a similar number of bacteria in the spleen 21 days post-infection, but a significantly higher number after 35 days (**Figure 5.1.A**). In contrast, no difference in the bacterial burden could be detected at either time point in the liver (**Figure 5.1.B**). IL-6$^{-/-}$ mice had similar spleen (**Figure 5.1.C**) and liver (**Figure 5.1.D**) masses to the WT mice throughout the course of the infection. IL-6 is not required for the control of systemic infection with STm nor does it influence the expansion of the spleen or liver.

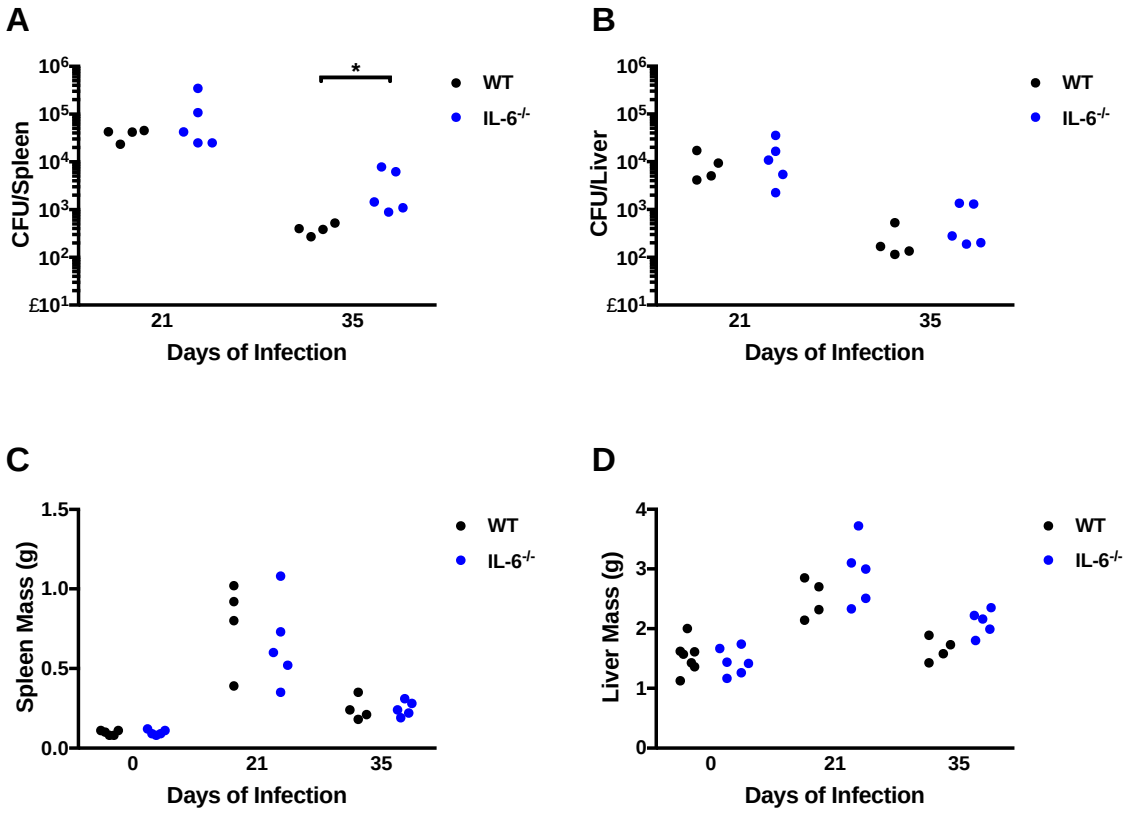

Figure 5.1:Bacterial burden in the liver and spleen of IL-6$^{-/-}$ mice after infection with STm
C57BL/6J and IL-6$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 21 and 35 days. Bacterial burden was assessed in the **A**) Spleen and **B**) Liver. The mass of the **C**) Spleen and **D**) Liver was measured. Data is representative of a single experiment with 4-5 mice per group per time point. *(P≤0.05).

5.2.2. IL-6 is not required for activation of CD4+ T Cells

IL-6 has previously been reported to influence the proliferation, commitment and survival of T cells (291). We hypothesized that IL-6$^{-/-}$ mice would have a reduced number of activated CD4^{+} T cells in the spleen. WT mice and IL-6$^{-/-}$ mice were infected with SL3261 for three to five weeks, and CD4^{+} T cells in the spleen were assessed by flow cytometry. Splenocytes were first gated for CD3^{+}CD4^{+} cells (as shown in previous chapters) and then activated CD4^{+} T cells (CD3^{+}CD4^{+}CD44^{+}) (**Figure 5.2.A**). No difference could be detected in the total number of activated CD4^{+} T cells in WT mice or IL-6$^{-/-}$ mice at any of the time-points. The maximum number of activated CD4^{+} T cells was generated 21 days post-infection and remains at similar levels at day 35 in both strains of mice (**Figure 5.2.B**). Therefore, IL-6 is not required for optimal CD4^{+} T cell activation.

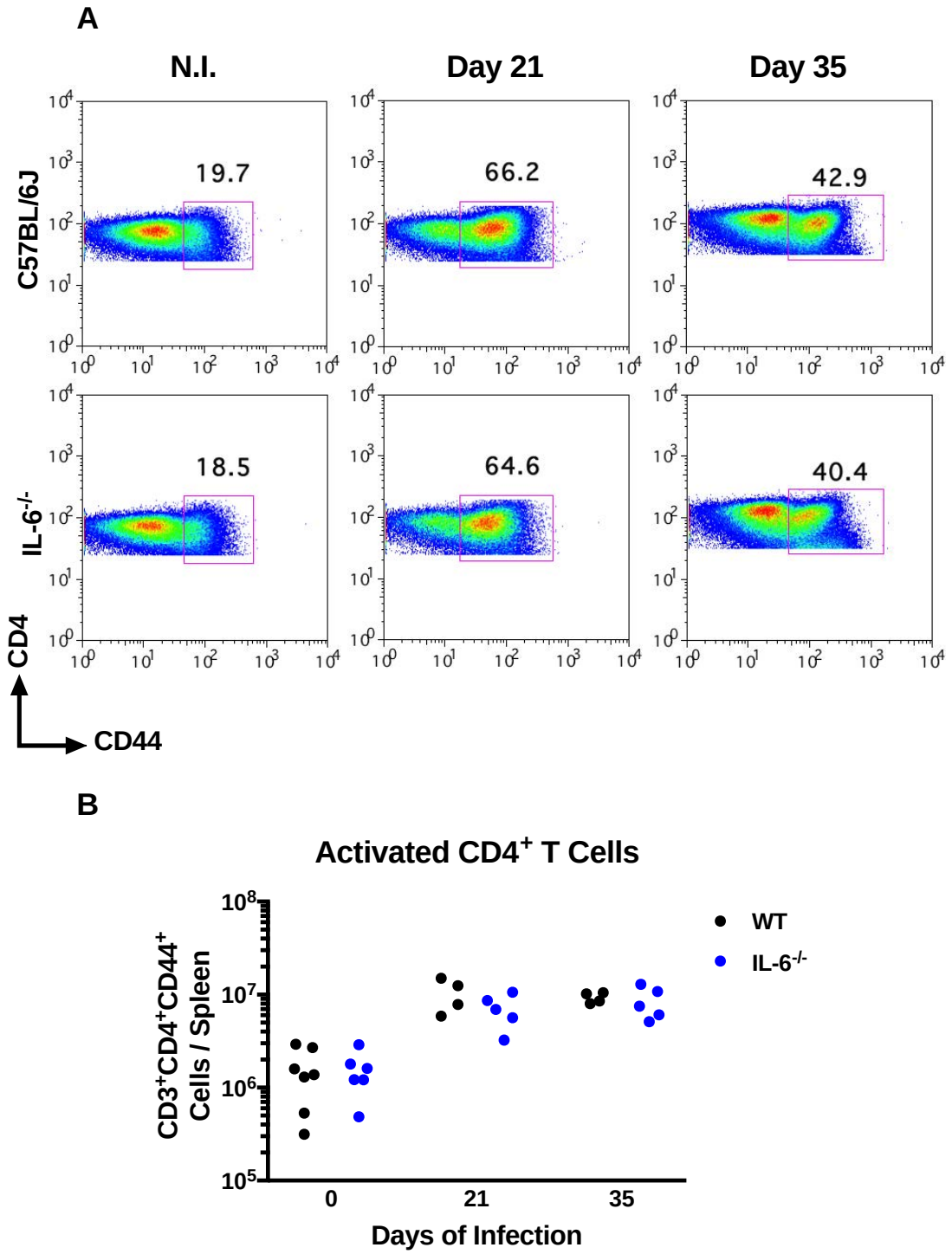

Figure 5.2:Role of IL-6 in the activation of CD4⁺ T Cells in response to infection with STm
C57BL/6J and IL-6$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 21 and 35 days. **A**) Representative plots of the relative frequency of activated/effector CD4⁺ T Cells. $CD3^+CD4^+$ cells were gated as shown in previous chapters. **B**) Number of activated/effector CD4⁺ T Cells ($CD3^+CD4^+CD44^+$) in the spleen was assessed by flow cytometry. Data is representative of a single experiment with 4-6 mice per group.

5.2.3. IL-6 is required for optimal plasma cell responses to STm

Previous reports from our laboratory have shown that plasma cells and pathogen-specific antibodies are derived from the EFR at day 18-21, while at later time-points, germinal centres become the main source (47). In addition, we have observed reduced antibody responses with similar kinetics to the WT mice in both IL-6$^{-/-}$ mice and IL-6$^{-/-}$ bone marrow chimaeras (186). It was therefore hypothesized that IL-6$^{-/-}$ mice would have a reduced antibody response to STm. Production of IgM^{+} and IgG2a/c^{+} plasma cells (CD138^{+}) was assessed by flow cytometry in the spleens of mice that had been infected for 21 and 35 days. Splenocytes were first gated for CD138 to select for plasma cells and then intracellular IgM (**Figure 5.3.A**), IgG2a/c (**Figure 5.3.B**) or IgG2b (data not shown).

There were an increased number of CD138^{+} plasma cells detected in the spleens of WT mice and IL-6$^{-/-}$ mice after 21 days of infection, compare to N.I. controls (**Figure 5.4.A**). A similar number of plasma cells were also measured after 35 days of infection, but no difference in the number of CD138^{+} plasma cells could be detected between either the WT mice or IL-6$^{-/-}$ mice at any of the time-points. A modest induction of IgM^{+} plasma cells was observed 21 days post-infection but this declined to background levels after 35 days (**Figure 5.4.B**). In contrast, there was a much more marked increase in the number of IgG2a/c plasma cells detected 21 days post-infection in the WT mice (**Figure 5.4.C**). At day 21 the total numbers are significantly larger in WT than IL-6$^{-/-}$ mice, although both strains had 10-100 fold greater numbers of IgG2a/c plasma cells than the N.I controls. This difference in IgG2a/c plasma cells between WT mice and IL-6$^{-/-}$ mice was not

detected at day 35. A similar pattern was observed for IgG2b^{+} plasma cells (**Figure 5.4.D**). Therefore, IL-6 is required for optimal production of class switched plasma cells at day 21, but not 35 days post-infection.

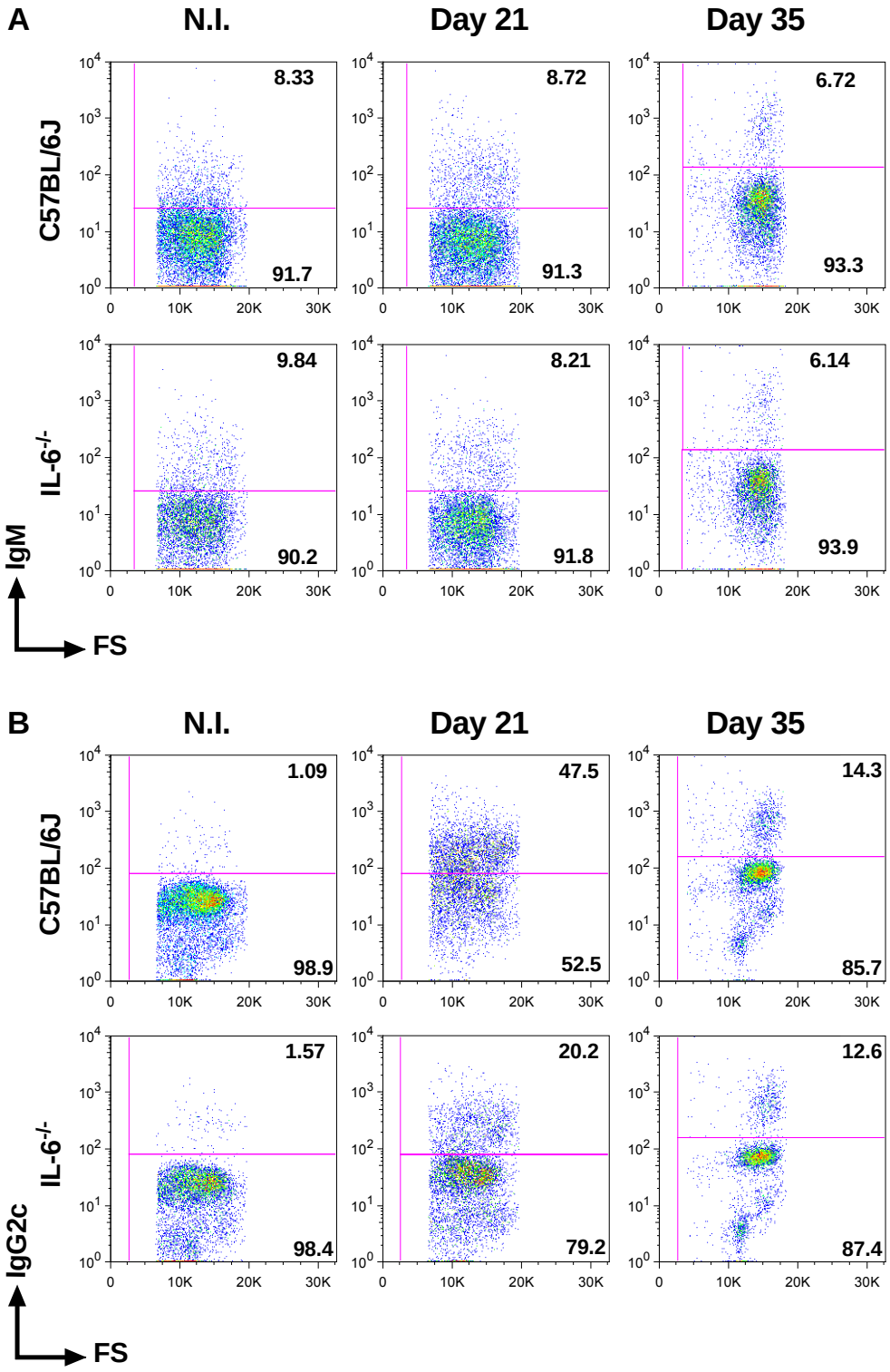

Figure 5.3:Gating of IgM and IgG2c Plasma Cells in IL-6$^{-/-}$ mice after infection with STm.
C57BL/6J and IL-6$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 21 and 35 days. Representative dot plots of the relative frequency of **A**) CD138$^+$ IgM$^+$ and **B**) CD138$^+$ IgG2c$^+$ plasma cells in the spleen were assessed by flow cytometry. Data is representative of a single experiment with 4-6 mice per group.

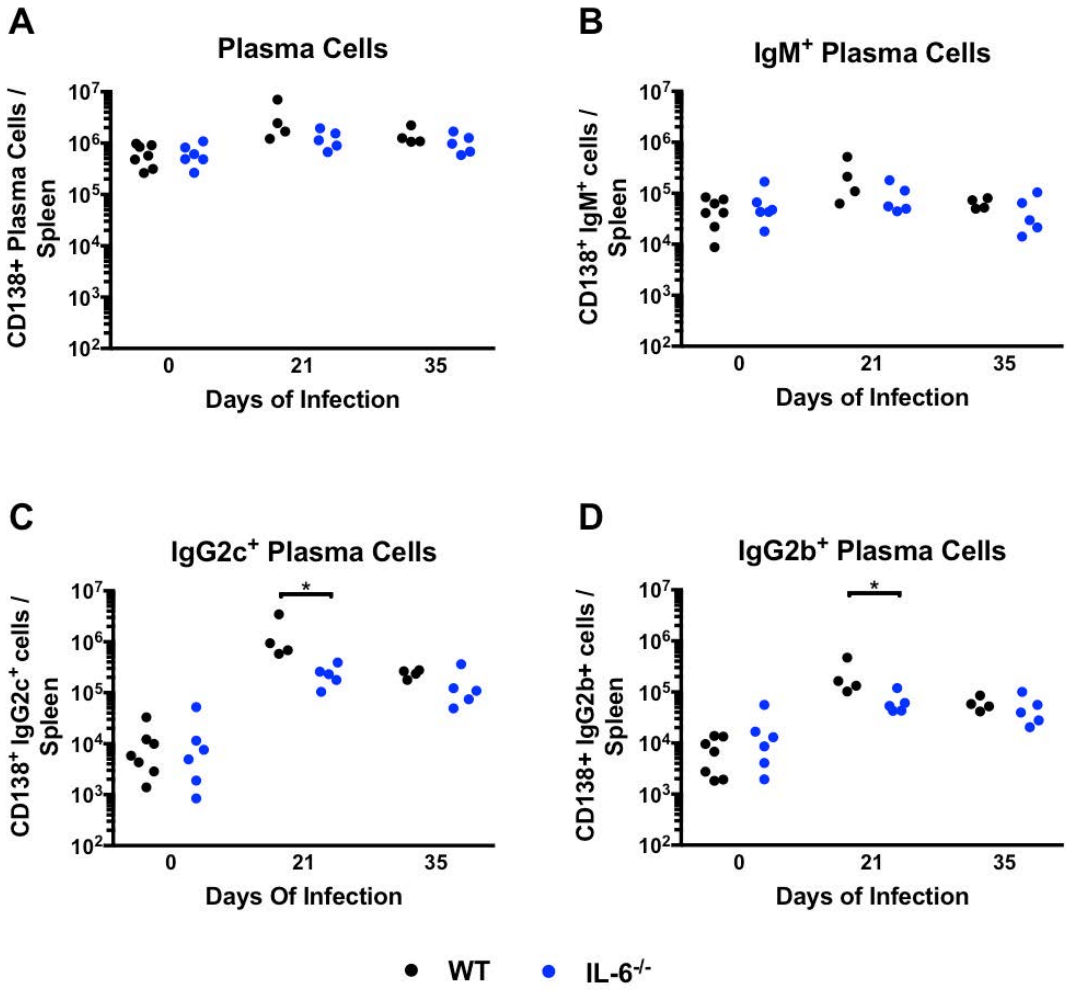

Figure 5.4:Total number non-switched and switched plasma cells in the absence of IL-6
C57BL/6J and IL-6$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 21 and 35 days. All cells were gated for CD138 to identify plasma cells and then either IgM, IgG2a/c or IgG2b. Total number of **A**) CD138$^+$, **B**) IgM$^+$, **C**) IgG2a/c$^+$ and **D**) IgG2b$^+$ plasma cells per spleen. Data is representative of a single experiment with 4-6 mice per group per time point. *(P≤0.05).

5.2.4. Impaired extrafollicular antibody responses in the absence of IL-6

The levels of serum antibodies to STm were measured by ELISA. IL-6$^{-/-}$ mice had lower levels of anti-OMPs IgM compared to WT mice after five weeks of infection (**Figure 5.5.A**). For Anti-OMPs IgG and IgG isotypes, with the exception of IgG1, there was a significantly lower response detected at 21 days post-infection in the absence of IL-6 (**Figure 5.5.B-F**). However, unlike the anti-OMPs IgM response, no difference could be detected 35 days post-infection in the titres of anti-OMPs IgG or the IgG subclasses in IL-6$^{-/-}$ mice. No anti-OMPs IgG1 antibodies could be detected in either the WT mice or the IL-6$^{-/-}$ mice (**Figure 5.5.E**). The data suggests that IL-6 is required for optimal extrafollicular antibody responses.

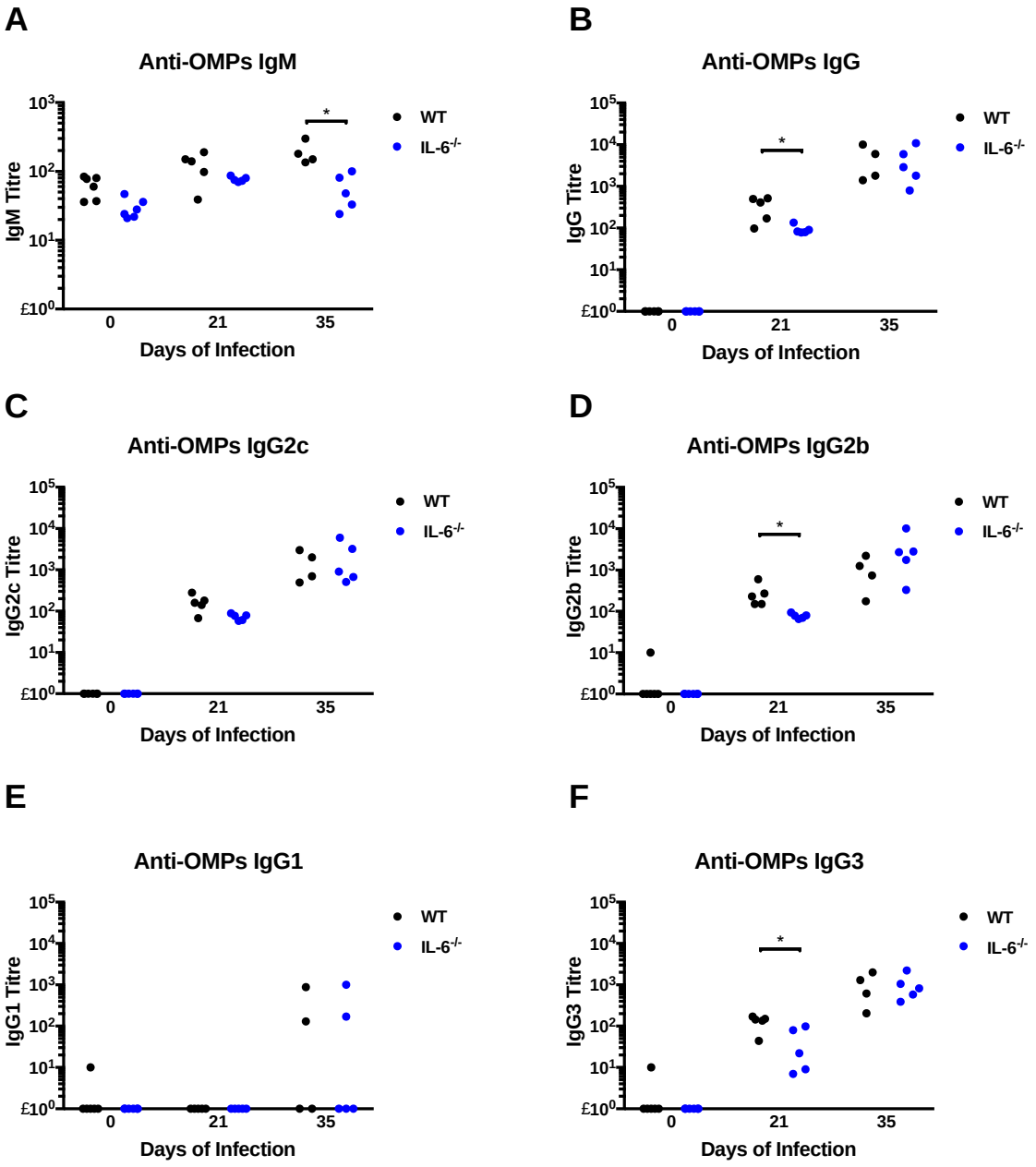

Figure 5.5:Role of IL-6 in the anti-OMPs response after infection with STm.
C57BL/6J and IL-$6^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 21 and 35 days. Mouse **A**) IgM, **B**) IgG, **C**) IgG2c, **D**) IgG2b, **E**) IgG1 and **F**) IgG3 antibodies against *Salmonella* Typhimurium outer membrane proteins were assessed by ELISA. Data is representative of a single experiment with 4-6 mice per group per time point. *($P \leq 0.05$)

5.2.5. The self-reactive antibody response is not IL-6 dependent

In the previous chapter we identified that the peak of the self-reactive antibody response was observed after three weeks of infection with STm. Since IL-6 was required for optimal pathogen-specific antibody responses at this time-point, we hypothesized that loss of IL-6 could impeded the production of self-reactive antibodies. In order to test this, mice were infected with 5 x 10^5 SL3261 for 21 and 35 days. Sera were collected and rat stomach, liver and kidney slides were stained for self-reactive antibodies. Self-reactive antibodies against the stomach and underlying muscle could be detected in **4/5** WT mice and **4/4** IL-$6^{-/-}$ mice at all time-points (**Figure 5.6**). The intensity of the staining for self-reactive antibodies in the stomach appeared to be moderately lower in the absence of IL-6 compared to WT mice. To confirm this observation we attempted to quantify the fluorescent intensity using Image J but this was impeded by the scattered pattern of the staining.

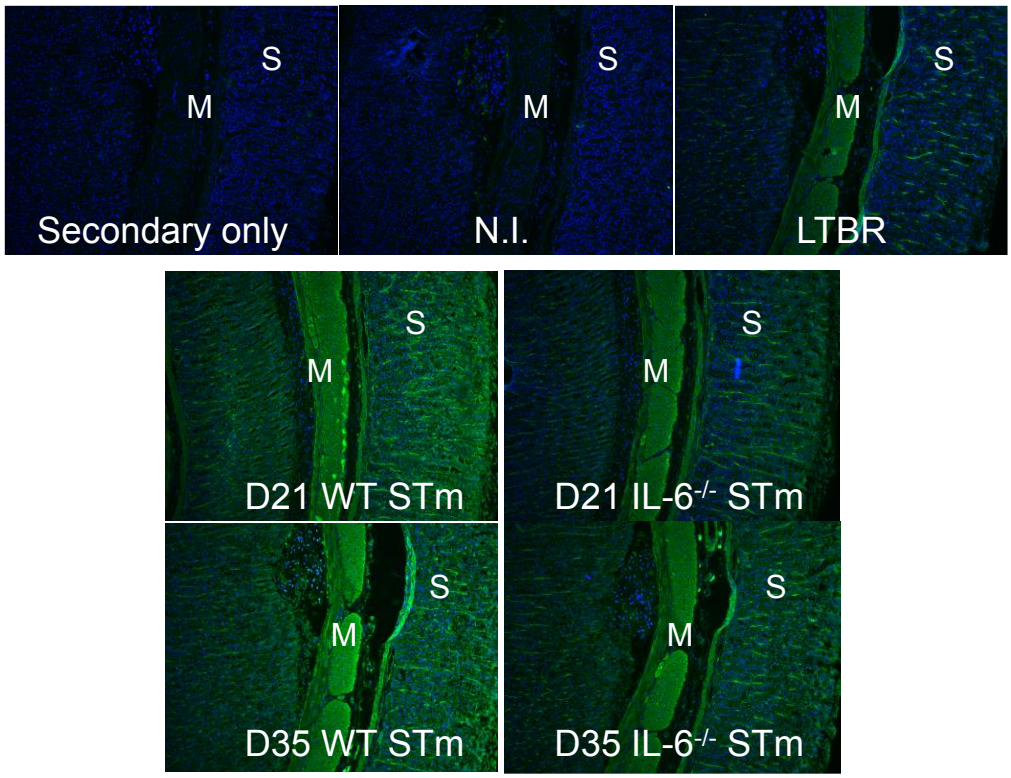

Figure 5.6:IL-6 is not required for the development of the self-reactive antibody response
C57BL/6J and IL-6$^{-/-}$ mice were infected with STm for 21 and 35 days. Self-reactive antibodies were measured by immunofluorescence against rat stomach, liver and kidney slides. Pictures of the rat stomach and underlying smooth muscles were taken at a 20 x magnification. Self-reactive antibodies are shown in green (F(ab$'$)2 IgG-FITC secondary) and cell nuceli are counterstained with DAPI in blue. Data is representative of a single experiment with 4-5 mice per group per time point. 4/4 WT mice and 4/4 IL-6$^{-/-}$ mice infected with STm were positive for stomach, muscle and liver self-reactive antibodies. Note that the images of the secondary, N.I. and Positive control are from the same experiment presented in Figure 4.5 as these were stained at the same time and was part of the time course experiment.

5.2.6. Screening ELISA suggests that IL-6 on stromal cells is important for optimal production of self-reactive antibodies.

Loss of IL-6 potentially resulted in a reduction in antibody binding to rat stomach after infection with STm. Since multiple cell types can produce IL-6 we investigated whether IL-6 from haematopoietic or stromal cells contribute to this response. As a consequence sera was screened from IL-6 bone marrow chimeras that had previously been generated and infected by Dr Ruth Coughlan, for 35 days with 5 x 10^5 SL3261 (186). In brief, bone marrow chimeras were generated through the irradiation of WT mice or IL-$6^{-/-}$ mice, which were then reconstituted with bone marrow from either IL-6 sufficient or deficient mice (**See Section 2.3**). This resulted in the construction of mice that can (WT→WT) or cannot produce IL-6 at all (IL-$6^{-/-}$→IL-$6^{-/-}$), or mice that cannot secrete IL-6 from haematopoietic (IL-$6^{-/-}$→WT) or stromal cells (WT → IL-$6^{-/-}$).

No anti-LHK antibodies could be detected in N.I. mice, but after infection there was an induction of anti-LHK IgM in IL-6 sufficient mice (WT→WT chimeras) (**Figure 5.7 A**). Similar titres of anti-LHK antibodies were observed in the absence of IL-6 from haematopoietic cells (IL-$6^{-/-}$→WT). In contrast, lower levels of anti-LHK IgM was observed in the absence of IL-6 expression in the stromal cell compartment (WT→IL-$6^{-/-}$). Complete loss of IL-6 resulted in no detectable levels of anti-LHK IgM antibodies after infection.

A similar role for IL-6 was found in the production of anti-LHK IgG antibodies, but it was more pronounced (**Figure 5.7 B**). No anti-LHK IgG antibodies could be detected in the

absence of IL-6 on either the stromal cell compartment (WT → IL-$6^{-/-}$) or in mice with a complete loss of IL-6 (IL-$6^{-/-}$→IL-$6^{-/-}$). Stromal cells are therefore a key source of IL-6 for the optimal production of anti-LHK self-reactive antibodies.

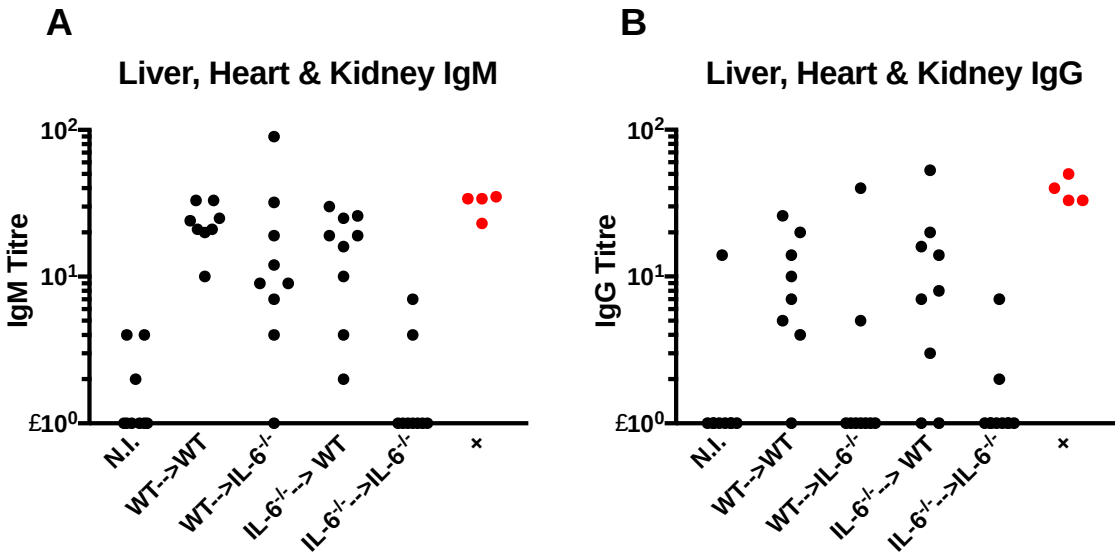

Figure 5.7:Screening for LHK antibodies in IL-6 chimaeras after infection with STm
C57BL/6J or IL-6$^{-/-}$ mice were irradiated and reconstituted with either WT (C57Bl/6J) or IL-6$^{-/-}$ bone marrow cells. IL-6 chimaeras were generated in which IL-6 production was normal (WT→WT), deficient (IL-6$^{-/-}$→IL-6$^{-/-}$), missing from the stromal (WT→IL-6$^{-/-}$) or the haematopoietic compartment (IL-6$^{-/-}$→WT). These mice were then infected for 35 days and the serum was screened by ELISA for **A**) IgM and **B**) IgG antibodies against mouse liver, heart and kidney homogenate. Sera from 4 mice infected with STm for three weeks with confirmed self-reactive antibodies were used as a positive control (+). Data is pooled from two independent experiments. Serum was kindly provided by Dr Ruth Coughlan (University of Birmingham).

5.2.7. TNF is required for control of STm in the Liver

TNF is a pro-inflammatory cytokine that exerts its biological effects through the p55 and p75 receptors. TNF can cause tissue damage in both acute and chronic models of inflammation and autoimmune disease (299, 300). Unpublished observations from our laboratory have shown that $p55^{-/-}p75^{-/-}$ mice form antibody secreting cells that do not migrate to the splenic red pulp and instead are maintained in the T-zone one week after infection with STm. As a consequence we investigated the role of these receptors in the antibody response after infection. In order to do this we first studied the role of TNF in the controlling the growth of STm by infecting WT mice and $p55^{-/-}p75^{-/-}$ mice for 21 days. Unfortunately due to a decline in the health of the animals the experiment had to be taken 17 days post-infection. The bacterial burden in the spleen was marginally higher, on average, in the absence of p55 and p75, but significantly greater in the liver (**Figure 5.8. A & B**). In addition, the splenic mass was approximately half the size of the WT mice in the absence of TNF (**Figure 5.8.C**). No difference could be observed in the mass of the liver (**Figure 5.8.D**). This confirmed published observations that TNF is required for the optimal control of the bacterial burden in the liver (301).

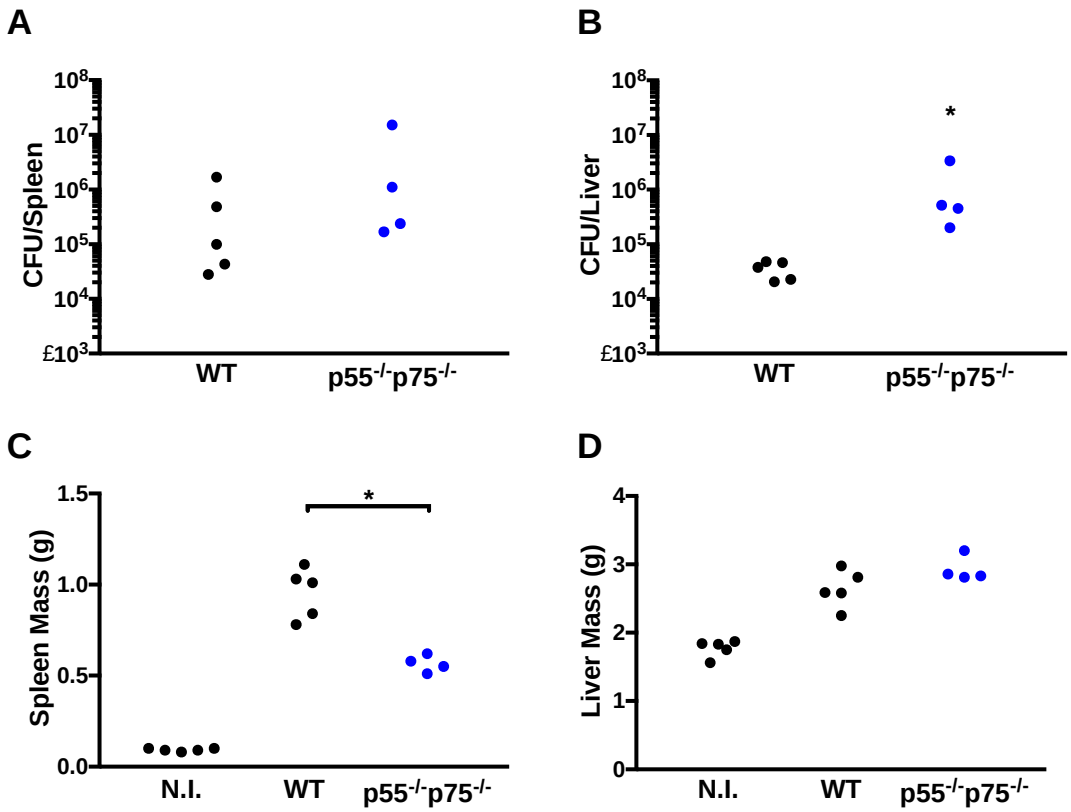

Figure 5.8:TNF is required for control of STm infection in the Liver, but not the spleen.
C57BL/6J and p55$^{-/-}$p75$^{-/-}$ mice were infected for 17 days with 5 x 10^5 SL3261. Bacterial burden was assessed in the **A**) Spleen and **B**) Liver. The mass of the **C**) Spleen and **D**) Liver was measured. Data is from a single experiment with 4-5 mice per group. *(P≤0.05).

5.2.8. TNF is not required for the generation of effector CD4$^+$ T cells

Given the higher bacterial burden in p55$^{-/-}$p75$^{-/-}$ mice, it was hypothesized that this could be due to a defect in the activation of CD4$^+$ T cells and not just a product of impaired innate immunity. To test this, WT mice and p55$^{-/-}$p75$^{-/-}$ mice were infected with SL3261 and the CD4$^+$ T cells in the spleen were assessed by flow cytometry. We first gated for CD3$^+$CD4$^+$ T cells and then activated/effector CD4$^+$ T cells (**Figure 5.9.A**) as shown in Chapter 3. After infection with STm there were a greater number of CD4$^+$ T cells in the spleen of infected WT mice compared to N.I. controls (**Figure 5.9.B**). In addition, a marginally higher number could be detected in the absence of TNF. A three to four fold increase was observed in the relative proportion of activated/effector CD4$^+$ T cells in the spleens of infected WT mice and p55$^{-/-}$p75$^{-/-}$ mice. Consistent with these observations there was a significantly higher number of activated/effector CD4$^+$ T cells in the infected p55$^{-/-}$p75$^{-/-}$ mice compared to WT mice (**Figure 5.9.C**), despite the smaller spleen sizes. TNF is not required for optimal CD4$^+$ T cell activation and a deficiency in the biological activity of this cytokine appears to lead to an increased number of effector CD4$^+$ T cells.

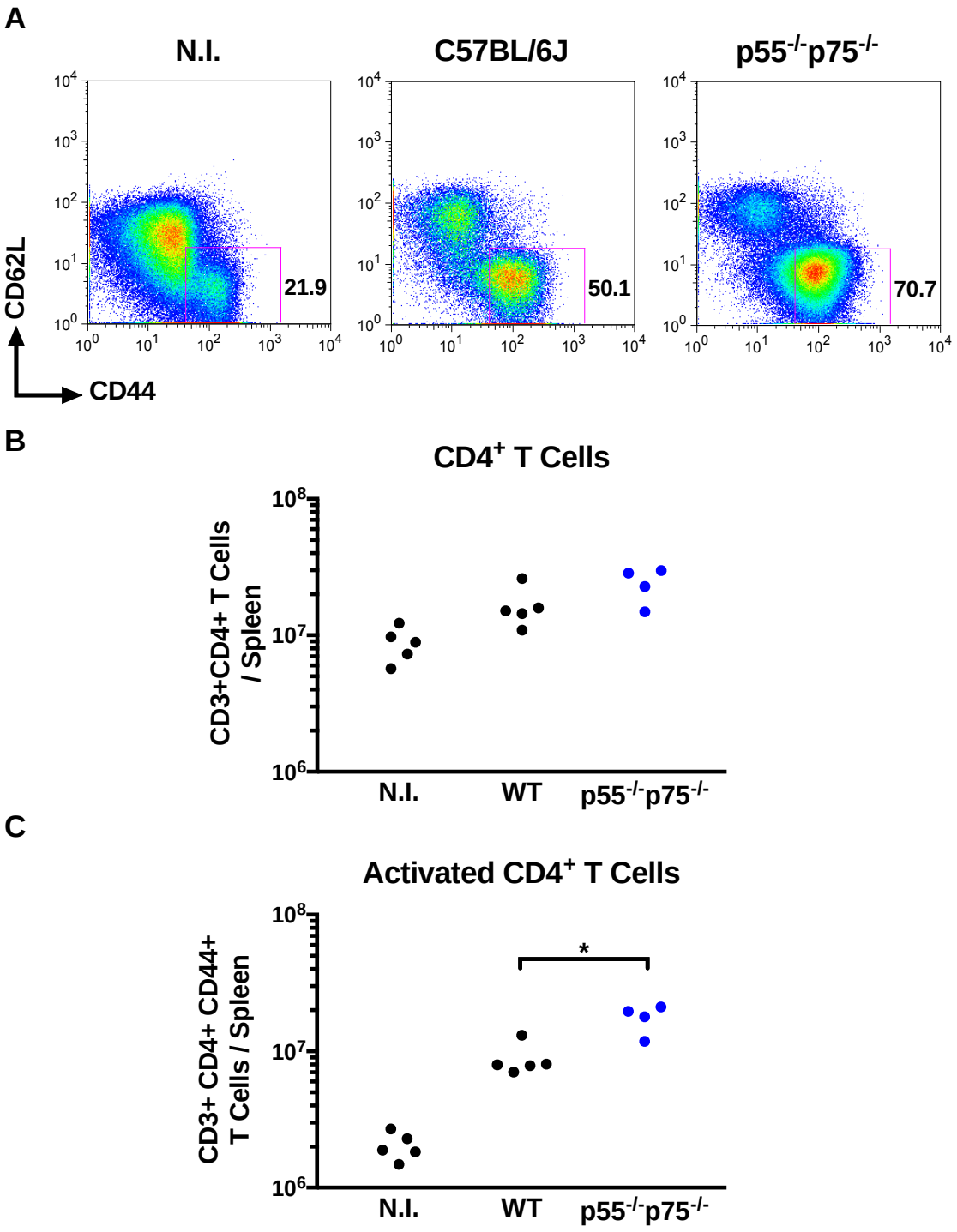

Figure 5.9:Effector CD4⁺ T cell production in the spleen of $p55^{-/-}p75^{-/-}$ mice
C57BL/6J mice and $p55^{-/-}p75^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 17 days. $CD4^+$ T cell populations in the spleen were assessed by flow cytometry. **A**) Representative dot plots of the relative frequency of activated/effector $CD4^+$ T cells ($CD3^+CD4^+CD62L^-CD44^+$). The Total number of **B**) $CD3^+CD4^+$ and **C**) activated/effector $CD4^+$ T cells per spleen. Data is representative of a single experiment with 4-5 mice per group. *(P≤0.05).

5.2.9. Plasma cells do not migrate to the T-zone in $p55^{-/-}p75^{-/-}$ mice

Unpublished observations from our laboratory have shown that $p55^{-/-}p75^{-/-}$ mice that have been infected with STm for one week produce plasma cells that fail to migrate to the red pulp. In order to identify what also happened later, spleens were sectioned and stained for T cells through the expression of CD3, and IgM or IgG2c plasma cells (**Figure 5.10**). N.I. WT mice have distinct B cell follicles and T cell zones that are fairly close together. Following infection there is a large expansion in the red pulp of both the WT mice and TNF deficient mice. IgM and IgG2c cells can be detected in the red pulp of WT infected mice. In contrast, in the absence of TNF the IgM and IgG2c cells appear to be restricted to the T zone, although some cells can also be found in the red pulp. This data suggests that in the absence of TNF that antibody-secreting cells do not migrate to the red pulp to the same degree as in WT infected mice.

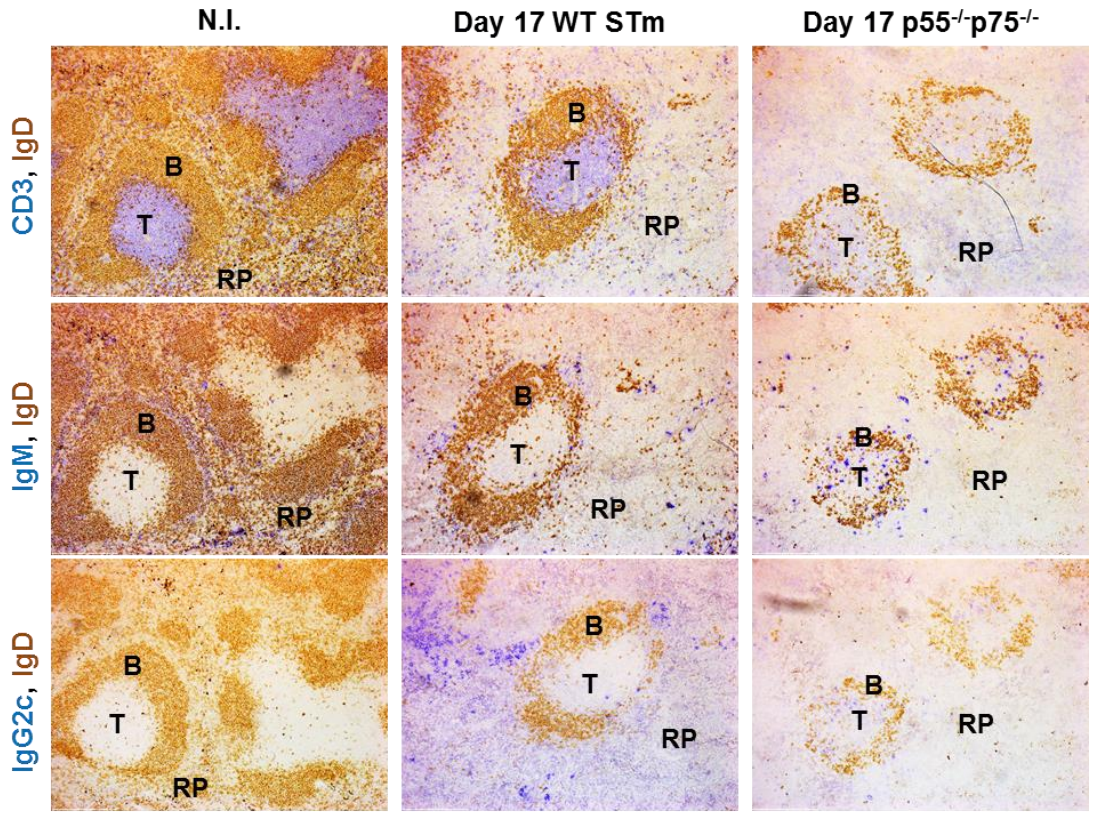

Figure 5.10:Localization of IgM and IgG2c Plasma Cells after Infection with STm in the absence or presence of TNF.
C57BL/6J mice and $p55^{-/-}p75^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 17 days. Spleens were frozen and cut into 5 µm sections. Splenic B cell follicles were stained with IgD (brown) and either CD3, IgM or IgG2c (blue) in order to identify the T zone and plasma cells respectively. Images were taken at x10 magnification and are representative of a single experiment with 4-5 mice per group. Images displayed are representative images. **B** – B cell follicles, **T** – T cell Zone & **RP** – Red Pulp.

5.2.10. TNF is not required for *Salmonella*-specific antibody responses

Given the atypical localization of splenic plasma cells in $p55^{-/-}p75^{-/-}$ mice we tested whether or not this influences the overall production of plasma cells and pathogen-specific antibodies. Splenic populations of plasma cells were assessed before and after infection by flow cytometry. Cells were first gated for $CD138^{+}$ cells and then intracellular IgM, IgG2a/c or IgG2b (**Figure 5.11.A-C**).

Despite the smaller spleens of the infected $p55^{-/-}p75^{-/-}$ mice, no difference could be detected in the total number of non-switched and switched plasma cells compared to infected WT mice (**Figure 5.12 A-D**). Therefore, the induction of plasma cells after infection is not dependent upon TNF. Furthermore, levels of STm-specific antibodies were similar between both groups of infected mice (**Figure 5.13**), except IgG2b (**Figure 5.13 D**)

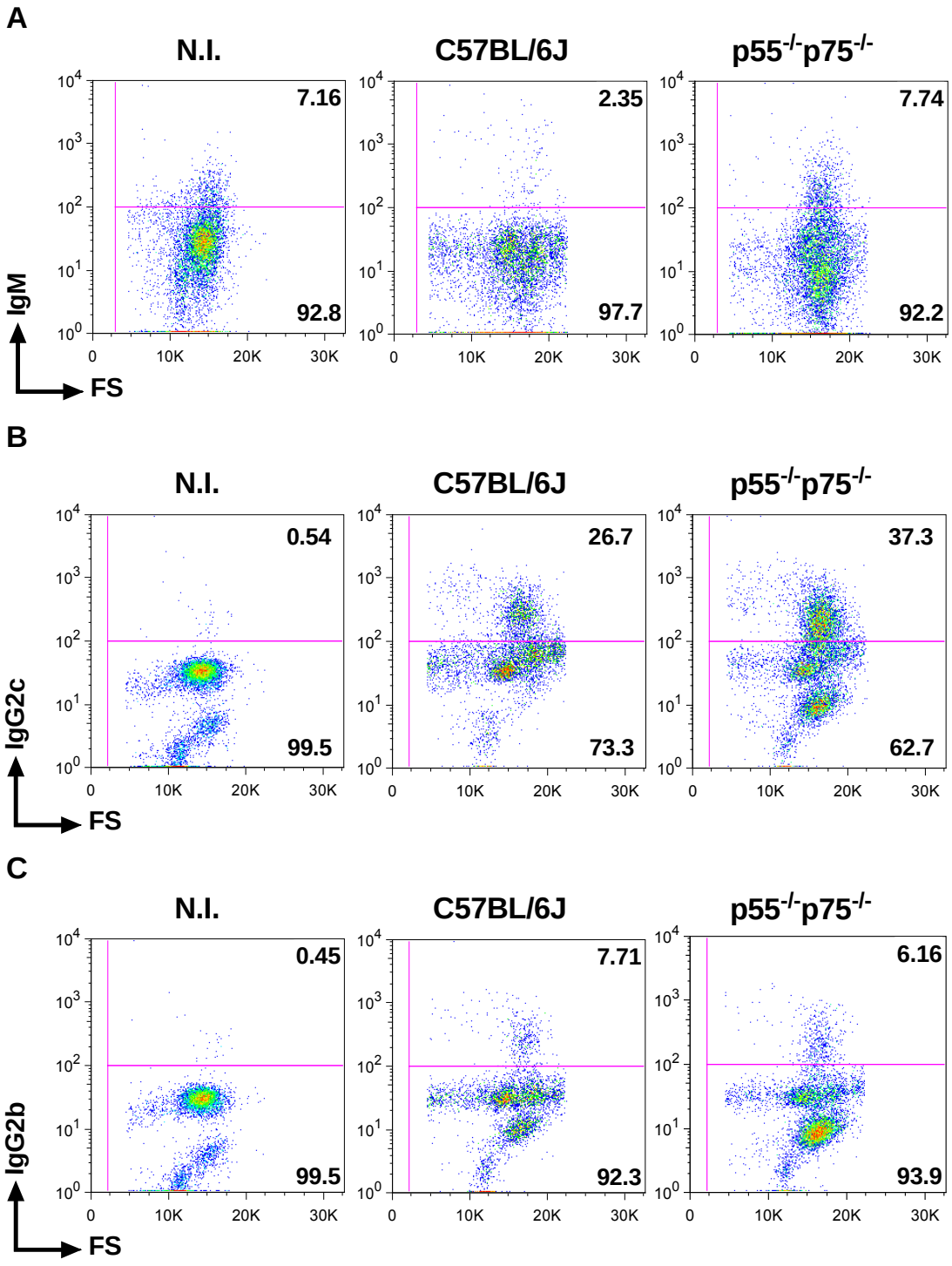

Figure 5.11:Proportion of IgM, IgG2a/c and IgG2b plasma cells in the spleens of $p55^{-/-}p75^{-/-}$ mice after infection.

C57BL/6J and $p55^{-/-}p75^{-/-}$ mice were infected for 17 days with 5 x 10^5 SL3261. Non-switched and switched plasma cells were assessed by flow cytometry. $CD138^+$ cells were selected and then gated for IgM, IgG2a/c or IgG2b. Representative plots of the proportion of $CD138^+$ **A**) IgM^+ **B**) $IgG2a/c^+$ and **C**) $IgG2b^+$ plasma cells in the spleen are shown. Data is from a single experiment with 4-5 mice per group.

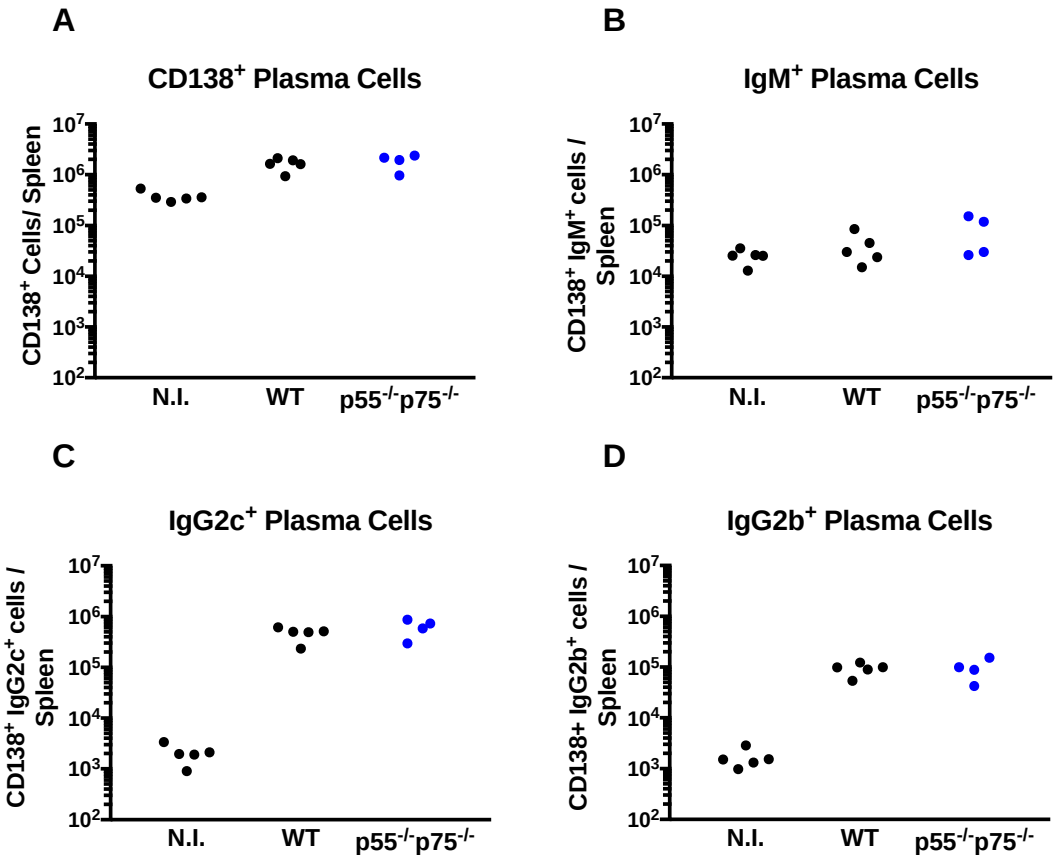

Figure 5.12:Effect of TNF deficiency on plasma cell production after infection with STm
C57BL/6J and $p55^{-/-}p75^{-/-}$ were infected for 17 days with 5 x 10^5 SL3261 and the number of non-switched and switched plasma cells in the spleen was assessed by flow cytometry. Cells were gated for CD138 and either IgM, IgG2a/c or IgG2b. Total number of **A**) $CD138^+$ or $CD138^+$ **B**) IgM^+ **C**) IgG2a/c or **D**) $IgG2b^+$ plasma cells per spleen. Data is from a single experiment with 4-5 mice per group.

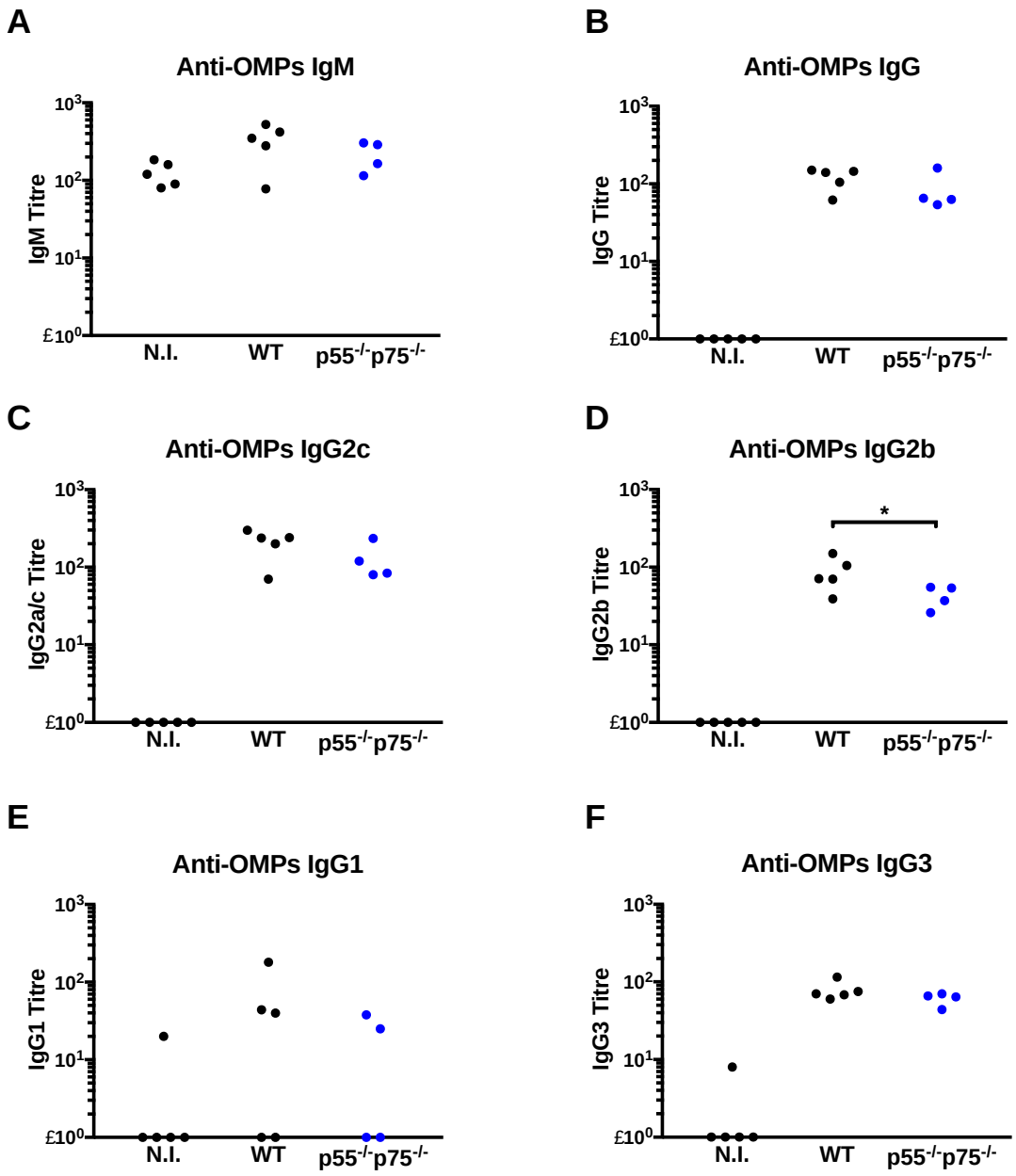

Figure 5.13:Role of TNF in the anti-OMPs antibody response after infection with STm.
C57BL/6J and $p55^{-/-}p75^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 17 days. Titres of anti-OMPs **A**) IgM, **B**) IgG, **C**) IgG2a/c, **D**) IgG2b, **E**) IgG1, and **F**) IgG3 antibodies were measured by ELISA. Data is from a single experiment with 4-5 mice per group. *(P≤0.05).

5.2.11. TNF is not required for the production of self-reactive antibodies in response to infection with STm

Although we could not detect any large differences in the titres of anti-OMP antibodies induced after infection with STm we thought that a lack of TNF could lead to loss of self-reactive antibodies. It was hypothesized that there would be a reduction in the inflammatory response and that this would lead to a loss of self-reactive antibodies. Self-reactive antibodies could be observed 17 days post-infection in **5/5** WT mice to both the stomach and underlying muscle (**Figure 5.14**). Similar observations were observed in **4/4** $p55^{-/-}p75^{-/-}$ mice. Very weak self-reactive antibody staining could be observed in the liver and kidney in the infected WT mice and $p55^{-/-}p75^{-/-}$ mice (Data not shown). This suggests that TNF is not required for the development of self-reactive antibodies in response to infection with STm.

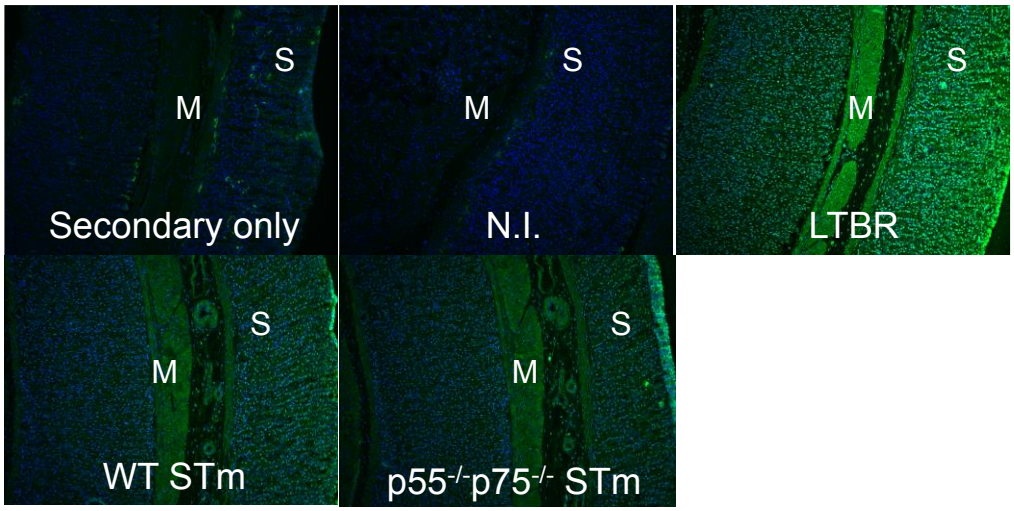

Figure 5.14: Role of TNF in the self-reactive antibody response after infection with STm
C57BL/6J and $p55^{-/-}p75^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 17 days. Rat stomach, liver and kidney slides were incubated with mouse sera and tissue specific antibodies detected using a F(ab')2 secondary antibody for IgG (Green Staining). Cell nuclei were stained with DAPI (blue). Pictures were taken at 20x magnification and images are representative of 4-6 mice from a single experiment. In total, 5/5 WT mice and 4/4 $p55^{-/-}p75^{-/-}$ mice infected were positive for self-reactive antibodies.

5.2.12. ICOSL$^{-/-}$ mice can control infection with STm

Since the correct localization of plasma cells did not seem to be important for the EFR we decided to test whether or not co-stimulation on T-cells is important. ICOSL is a co-stimulatory molecule that has been reported to be required for optimal T cell activation in a range of different *in vivo* models (286, 287, 302). It was predicted that ICOSL$^{-/-}$ mice would have reduced CD4^{+} T cell activation thus leading to poorer control of the bacterial burden in the spleen and an impaired antibody response against the pathogen and host. Mice were infected with 5 x 10^5 SL3261 for 21 days and the number of bacteria was assessed. ICOSL$^{-/-}$ mice have a marginally higher bacterial burden in both the spleen (**Figure 5.15.A**) and liver (**Figure 5.15.B**) 21 days post-infection. Splenomegaly and hepatomegaly were similar in both infected groups (**Figure 5.15.C-D**).

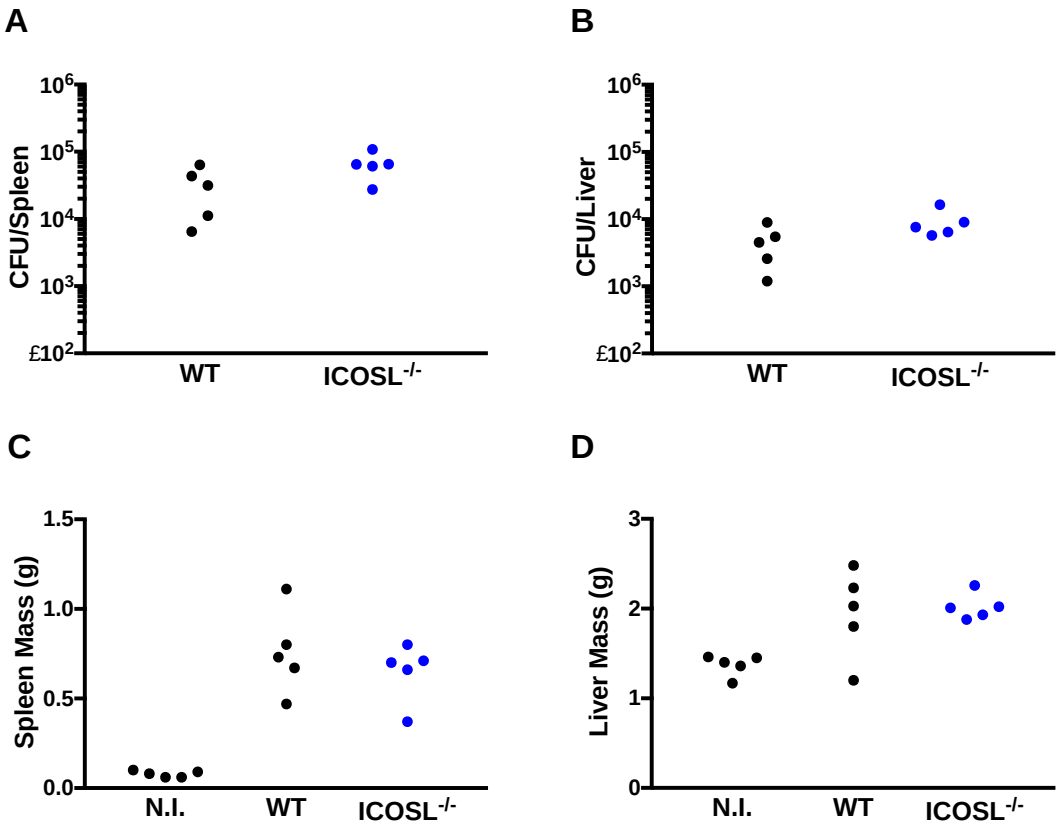

Figure 5.15:Role of ICOSL in the control of the bacterial burden in the liver and spleen
C57BL/6J and ICOSL$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 21 days. Bacterial burden in the **A**) spleen and **B**) liver was assessed. The mass of the **C**) spleen and **D**) liver was measured. Data is representative of two independent experiments with 4-5 mice per group, except for the N.I. ICOSL$^{-/-}$, which only had 1-2 mice.

5.2.13. ICOSL$^{-/-}$ mice exhibit a diminished CD4^{+} T Cell Response after infection.

Since ICOSL$^{-/-}$ mice had a similar bacterial burden to WT mice at a time when CD4^{+} T cells are known to be crucial for resolving infection, the CD4^{+} T cell populations in the spleen were assessed by flow cytometry. Cells were first gated for CD3^{+}CD4^{+} T cells and then activated/effector CD4^{+} T Cells (**Figure 5.16.A**). The total number of CD4^{+} T cells increased by 2-3 fold after three weeks of infection in WT mice, compared to N.I. controls (**Figure 5.16.B**). In contrast, infected ICOSL$^{-/-}$ mice had a similar number of CD4^{+} T cells compared to N.I. controls. There was a significantly lower number of activated CD4^{+} T cells (**Figure 5.16.C**) in the spleens of infected ICOSL$^{-/-}$ mice compared to WT mice. This appears to be due to a reduced number of CD3^{+} T cells in ICOSL$^{-/-}$ mice following infection with STm (data not shown). Therefore, ICOSL is important for optimal CD4^{+} T cell responses after infection with STm.

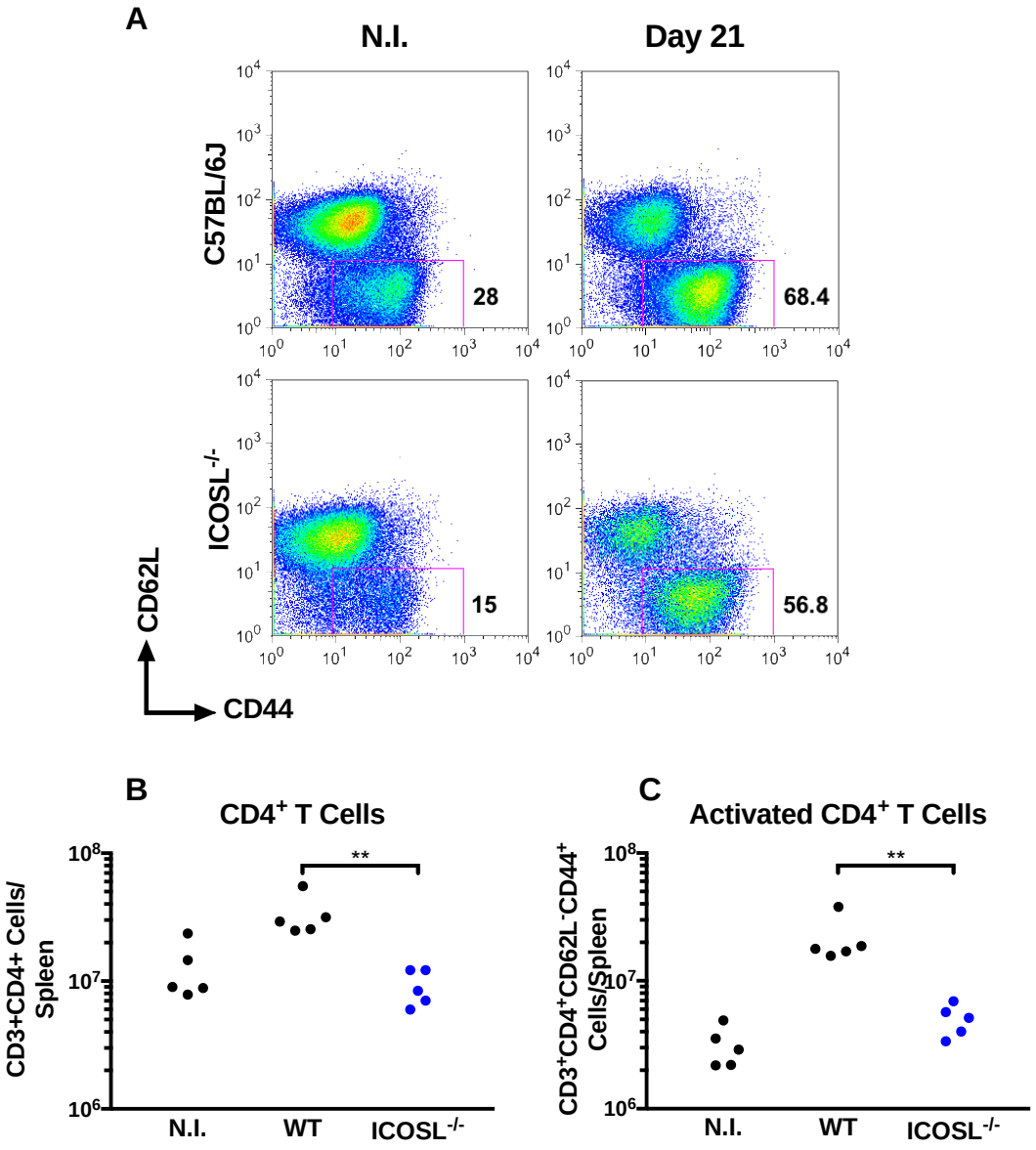

Figure 5.16:Role of ICOSL in the activation of CD4⁺ T Cells after infection with STm
C57BL/6J and ICOSL$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 21 days. **A**) Representative plots of the proportion of activated/effector CD4⁺ T cells (CD3⁺CD4⁺CD62L⁻CD44⁺). Splenocytes were gated for CD3⁺CD4⁺ T cells and then CD62L⁻CD44⁺ cells to identify activated/ effector T cells. The total number of **B**) CD3⁺CD4⁺ T cells and **C**) activated/effector CD4⁺ T Cells was assessed in the spleen by flow cytometry. Data is representative of two independent experiments with 4-5 mice per group, except for the N.I. ICOSL$^{-/-}$, which only had 1-2 mice. *(P≤0.05) & **(P≤0.01).

5.2.14. Impaired production of class switched plasma cells in the spleens of ICOSL$^{-/-}$ mice

The expression of CD40L on activated T cells has previously been shown to be important for inducing class switching in B cells in response to infection (303, 304). Given the lower numbers of activated/effector CD4^{+} T cells observed in ICOSL$^{-/-}$ mice it was expected that this would lead to a reduction in the number of plasma cells and the amount of pathogen-specific IgG antibodies against *Salmonella*. Plasma cells were selected on the basis of the expression of CD138 and then further gated for intracellular IgM (**Figure 5.17.A**), IgG2c (**Figure 5.17.B**) or IgG2b (data not shown). An increased number of plasma cells were detected in the spleens of WT infected mice, but not ICOSL$^{-/-}$ mice (**Figure 5.18.A**). No difference could be detected in the total number of IgM^{+} plasma cells between any of the time-points or strains of mice (**Figure 5.18.B**). In contrast, there were a higher number of IgG2c^{+} plasma cells in the spleen of the infected WT mice and ICOSL$^{-/-}$ mice after infection (**Figure 5.18.C**). The total number of IgG2c^{+} was significantly lower in the absence of ICOSL. Similar observations were made in the number of IgG2b^{+} plasma cells (**Figure 5.18.D**).Therefore, ICOSL is required for the optimal induction of class switched plasma cells in response to infection with STm.

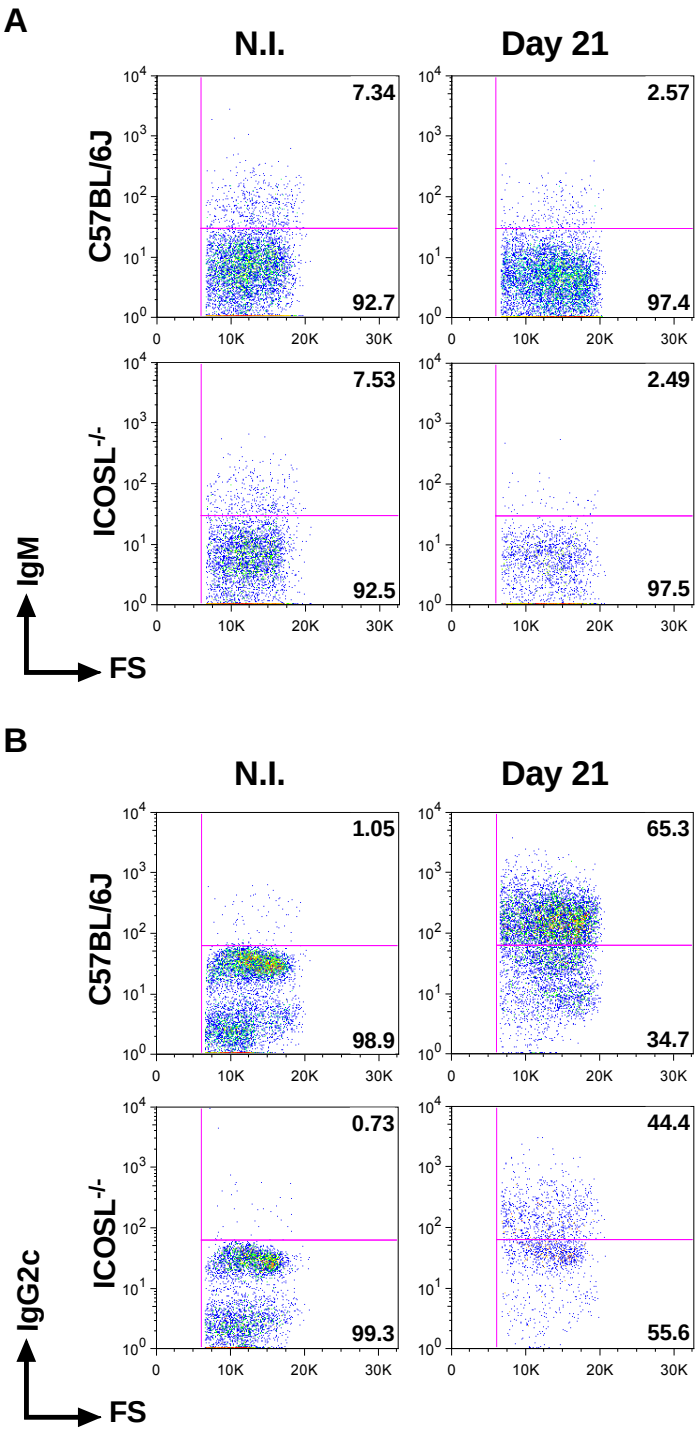

Figure 5.17:Representative Proportion of IgM and IgG2c plasma cells in ICOSL$^{-/-}$ mice after infection with STm

C57BL/6J and ICOSL$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 21 days. Spleens were stained by flow cytometry for plasma cells. CD138+ cells were gated and then further divided into **A**) IgM$^+$ or **B**) IgG2c$^+$ plasma cells. Gates were set using the N.I. mice. This figure shows representative dot plots. Data is representative of two independent experiments with 4-6 mice per group.

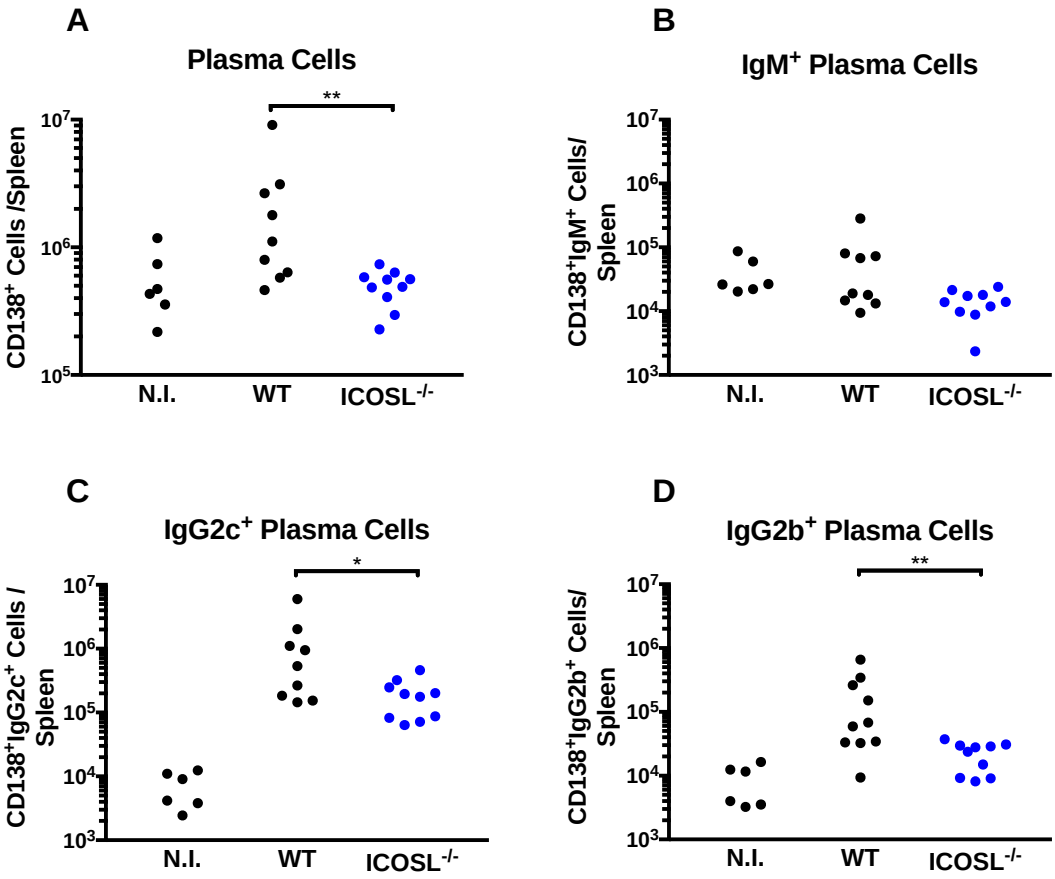

Figure 5.18:ICOSL and plasma cell production after infection with STm
C57BL/6J and ICOSL$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 21 days. The total number of **A**) CD138$^+$ and CD138$^+$ **B**) IgM$^+$, **C**) IgG2c$^+$ and **D**) IgG2b$^+$ plasma cells in the spleen was assessed by flow cytometry. Data is pooled from two independent experiments with 4-6 mice per group per experiment. It should be noted that 2 ICOSL$^{-/-}$ N.I. controls are pooled with the WT mice under the N.I. label. *(P≤0.05). & **(P≤0.01).

5.2.15. ICOSL deficiency results in a reduced *Salmonella*-specific antibody response.

Previous reports have shown that antibody responses to STm are abrogated in the absence of ICOS (305). Given that ICOSL is the only known ligand of ICOS to date we hypothesized that the *Salmonella*-specific antibody response would be impaired. After infection with STm for three weeks the antibody response was measured against OMPs by ELISA. Anti-OMPs IgM antibodies were induced 21 days post-infection in WT mice and ICOSL$^{-/-}$ mice. The titres of anti-OMPs IgM antibodies were significantly lower in the absence of ICOSL (**Figure 5.19A**). Anti-OMPs IgG, IgG2c, IgG2b and IgG3 antibodies were induced 21 days post-infection in the presence and absence of ICOSL (**Figure 5.19.B, C, D & F**). However, the titres of these antibodies were also significantly lower in the absence of ICOSL. Anti-OMPs IgG1 antibodies could not be detected in the WT or ICOSL$^{-/-}$ mice after infection (**Figure 5.19.E**). This data suggests that ICOSL is dispensable for the production of antibodies against STm, but essential for optimal antibody responses.

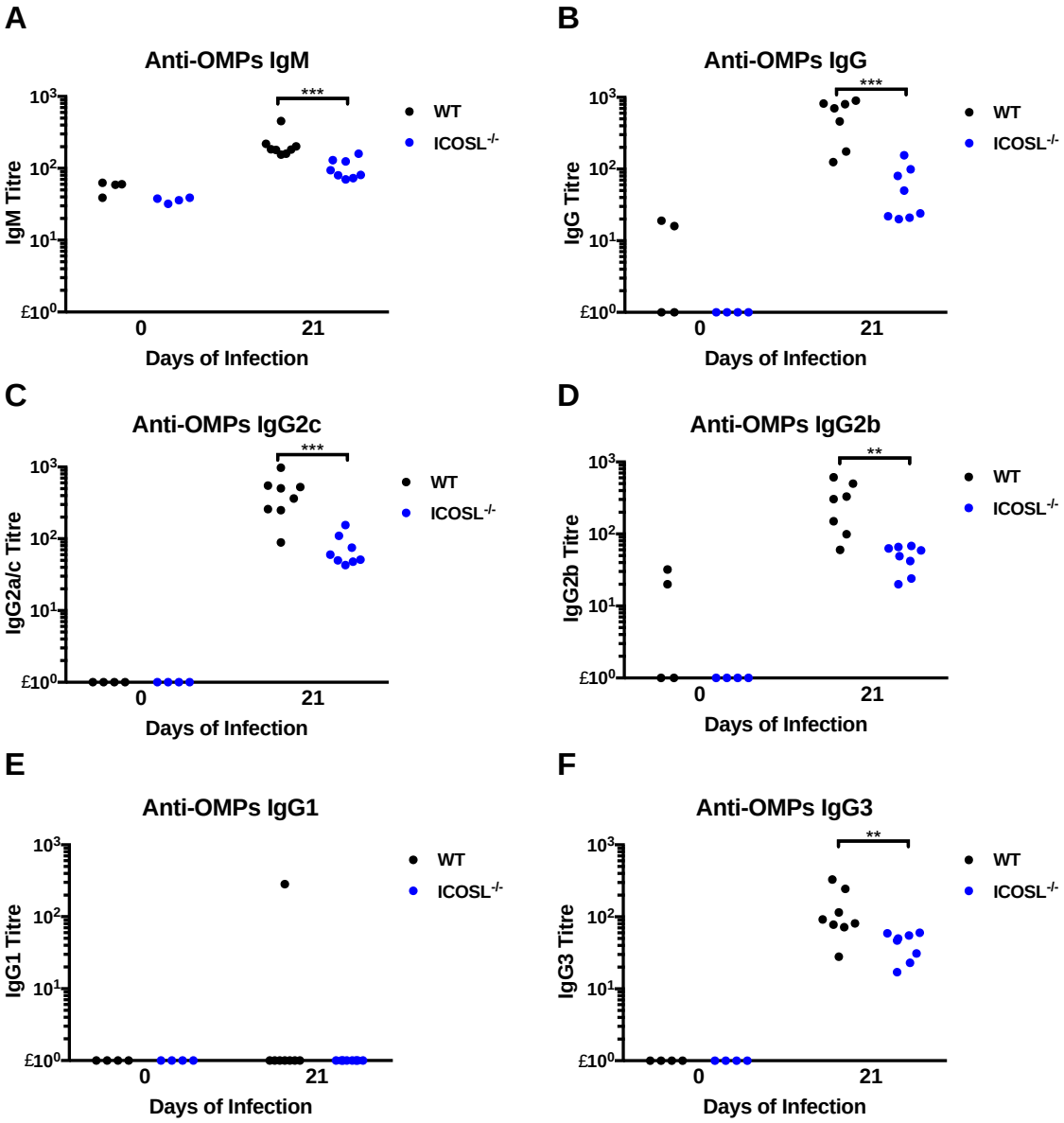

Figure 5.19:ICOSL is required for optimal pathogen-specific antibody responses during infection with STm.

C57BL/6J and ICOSL$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 21 days. Anti-OMPs **A**) IgM, **B**) IgG, **C**) IgG2c, **D**) IgG2b, **E**) IgG1 and **F**) IgG3 antibodies were measured by ELISA. Data is pooled from two independent experiments with 4-5 mice per group per time point per experiment. *(P≤0.05), **(P≤0.01) & ***(P≤0.001).

5.2.16. ICOSL$^{-/-}$ mice produce self-reactive antibodies

Since loss of ICOSL impaired CD4^{+} T cell responses and antibody production to STm, it was hypothesized that this would also cause a reduction in the self-reactive antibody response. In order to determine if ICOSL influences the production of self-reactive antibodies we stained rat kidney, liver and stomach slides for self-reactive antibodies before and after infection with SL3261. Three weeks after infection, antibodies directed against the stomach and muscle could be detected in **10/10** WT mice and **10/10** ICOSL$^{-/-}$ mice (**Figure 5.20**). There appeared to be no difference in the staining pattern between the two strains. Therefore, ICOSL is not required for the induction of self-reactive antibodies after infection with STm.

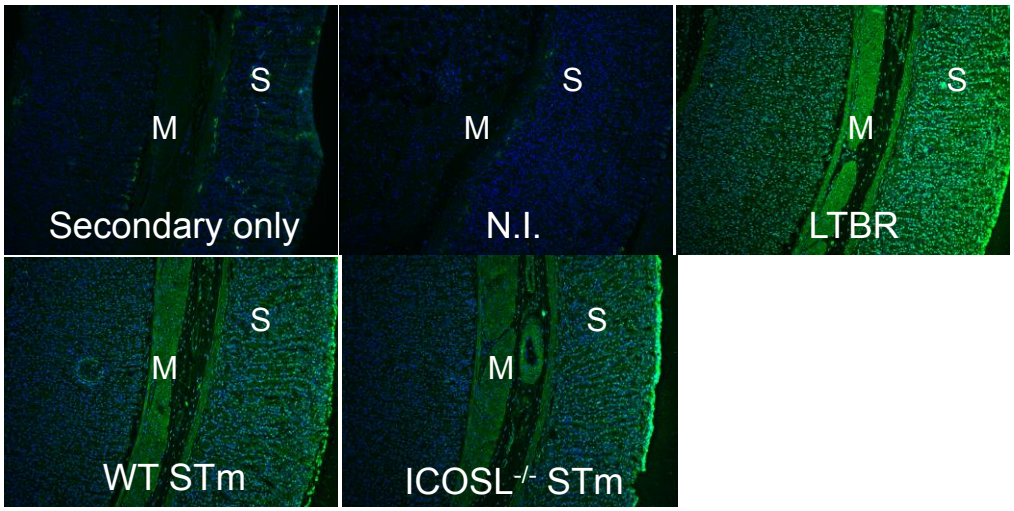

Figure 5.20:Staining for self-reactive antibodies in ICOSL$^{-/-}$ mice after infection with STm
C57BL/6J and ICOSL$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 21 days. Rat stomach, liver and kidney slides were incubated with mouse serum and tissue-specific antibodies detected with a F(ab')2 IgG-FITC secondary. Cell nuclei were counterstained with DAPI. Images were taken at a 20x magnification. Data is representative of two independent experiments with 4-6 mice per group per experiment. Note that the same secondary only, N.I. and LTBR controls are displayed as these pictures were taken at the same time as those **in Figure 5.14**.

5.2.17. CD80 and CD86 are not required for the initial control of STm

Despite the impaired $CD4^+$ T cell response observed in the absence of ICOSL, no difference could be detected in the self-reactive antibody response by immunofluorescence. Attempts to identify whether this was a TI or TD antibody response by screening past experiments from the group using infected TCR-$\beta\delta^{-/-}$ mice were inconclusive (Data not shown). In addition, access to these mice was limited due to breeding problems. As a consequence we decided to investigate this indirectly by assessing whether or not APCs are required for this response. APCs are known to prime naïve T cells through the presentation of peptide associated MHC class I or II in conjunction with the delivery of co-stimulation through binding of CD80/86 to CD28 on T cells. Loss of CD80/86 should in theory lead to the loss of T cell responses and allow the assessment of whether or not the pathogen-specific and self-reactive response are both APC and T-cell dependent. In order to test this, WT mice and CD80/$86^{-/-}$ mice were first infected with SL3261 for 7 days. The bacterial burden in the spleen and liver was assessed and no difference could be detected between WT mice or CD80/$86^{-/-}$ mice (**Figure 5.21. A & B**). In addition, the loss of CD80 and CD86 did not influence the development of splenomegaly or hepatomegaly (**Figure 5.21 C & D**). CD80 and CD86 are not required for the initial control of the growth of STm in the spleen or liver.

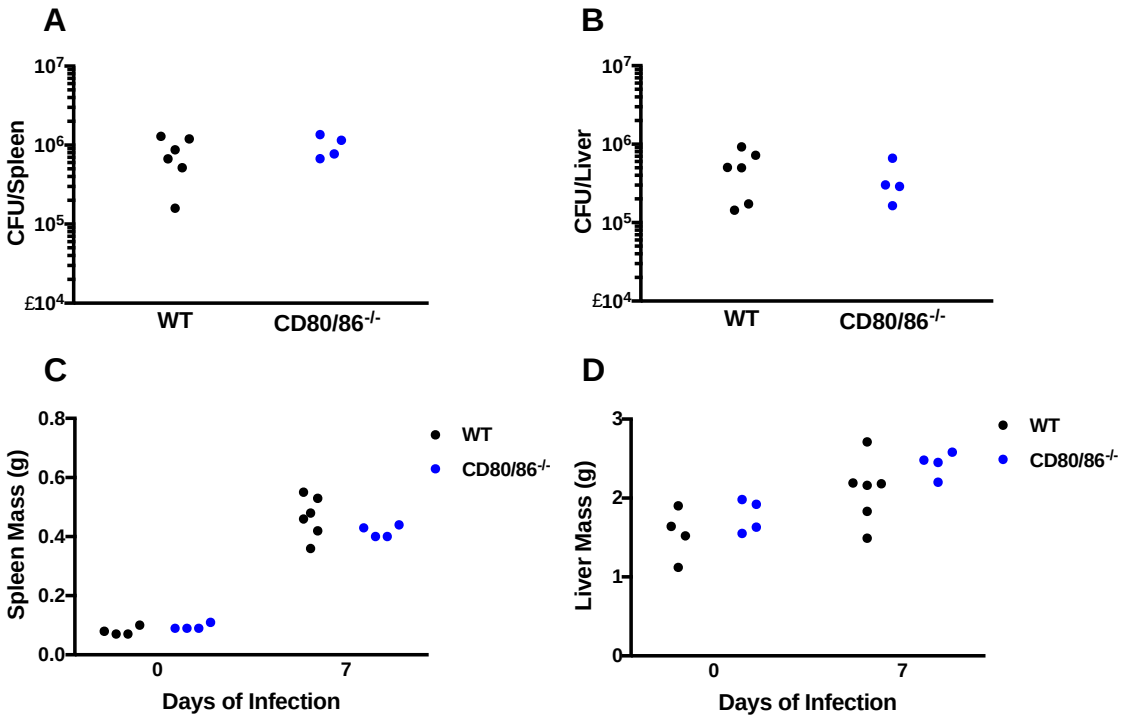

Figure 5.21:Bacterial burden in the spleen and liver of CD80/86$^{-/-}$ mice
C57BL/6J and ICOSL$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 7 days. The bacterial burden in the **A**) spleen and **B**) liver was assessed. The overall mass of the **C**) spleen and **D**) liver was measured. Data is representative of two independent experiments with 3-4 mice per group per experiment.

5.2.18. CD80 and CD86 are required for the induction of effector CD4$^+$ T Cells

Next we investigated the effects of loss of CD80 and CD86 on the CD4$^+$ T cell response to infection. It was hypothesized that during a primary infection with STm that CD80/86$^{-/-}$ mice would be unable to generate activated/effector CD4$^+$ T cells. The proportion and number of these cells was assessed by flow cytometry one week after infection. Cells were gated for CD3$^+$CD4$^+$ T cells and then the activated/effector CD4$^+$ T cells were selected on the basis of low expression of CD62L and high expression of CD44 (**Figure 5.22.A**). There were a significantly lower number of CD4$^+$ T cells in the N.I. CD80/86$^{-/-}$ mice compared to the WT controls (**Figure 5.22.B**). After infection the WT mice had a higher number of CD4$^+$ T cells compared to both the WT and CD80/86$^{-/-}$ controls. In addition, there was an increase in the number of activated/effector CD4$^+$ T cells in infected WT mice (**Figure 5.22.C**). In contrast, no difference could be detected in the number of activated/effector CD4$^+$ T cells in the infected CD80/86$^{-/-}$ mice compared to N.I. controls. Therefore, CD80 and CD86 are required for the generation of activated/effector CD4$^+$ T cells during a primary infection with STm.

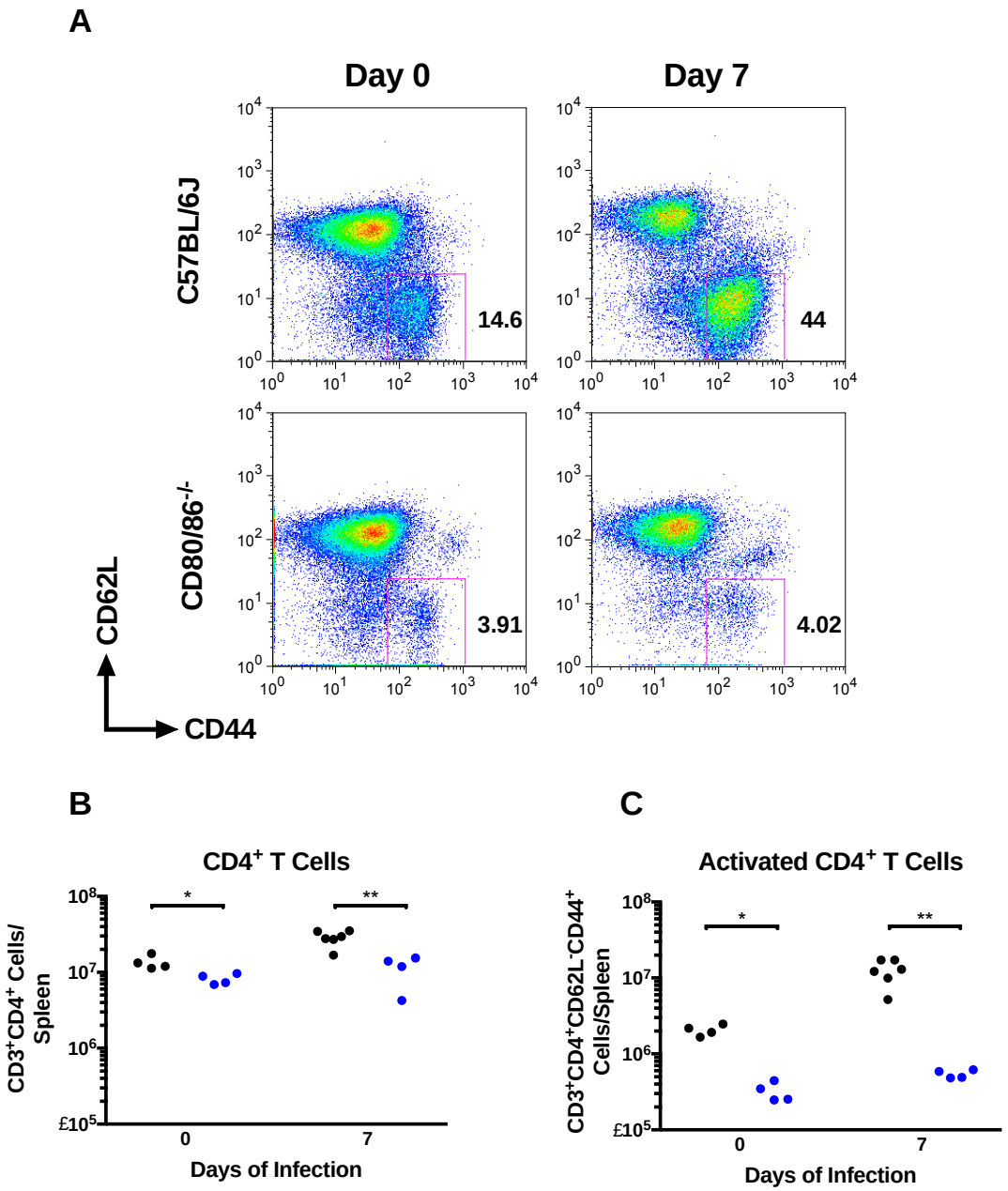

Figure 5.22:Role of CD80/86 in the activation of CD4⁺ T Cells after infection with STm.
C57BL/6J and CD80/86$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 7 days. CD4⁺ T Cells and activated/effector CD4 T cells in the spleen were studied by flow cytometry. $CD3^+CD4^+$ T cells were gated for and of activated/effector CD4⁺ T cells ($CD3^+CD4^+CD62L^-CD44^+$) was measured. **A**) Representative dot plots of the proportion of activated/effector CD4⁺ T Cells. The total number of **B**) $CD3^+CD4^+$ and **C**) activated/effector CD4⁺ T cells in the spleen is plotted. Data is representative of two independent experiments with 3-4 mice per group per experiment. *(P≤0.05). & **(P≤0.01).

5.2.19. CD80 and CD86 are required for the production of IgG2c$^+$ and IgG2b$^+$ plasma cells in the spleen

Given CD4$^+$ T cell activation is impaired in CD80/86$^{-/-}$ mice it was predicted that there would be a lower number of class switched plasma cells after infection with STm. After one week of infection the number of plasma cells in the spleen was assessed by flow cytometry. Cells were gated for CD138 and intracellular IgM, IgG2a/c (**Figure 5.23**.) or IgG2b (data not shown). To our surprise there was no difference detected in the overall number of plasma cells in the spleens of WT mice and CD80/86$^{-/-}$ mice (**Figure 5.24A**). In addition, there was a large increase in the number of IgM$^+$ plasma cells in the WT mice and to a smaller, but significant degree, in the CD80/86$^{-/-}$ mice (**Figure 5.24B**). A similar but even larger difference was observed in IgG2a and IgG2b. For example, there was a significantly lower number of IgG2a/c$^+$ and IgG2b$^+$ plasma cells in the spleens of infected mice in the absence of CD80 and CD86 (**Figure 5.24.C-D**).

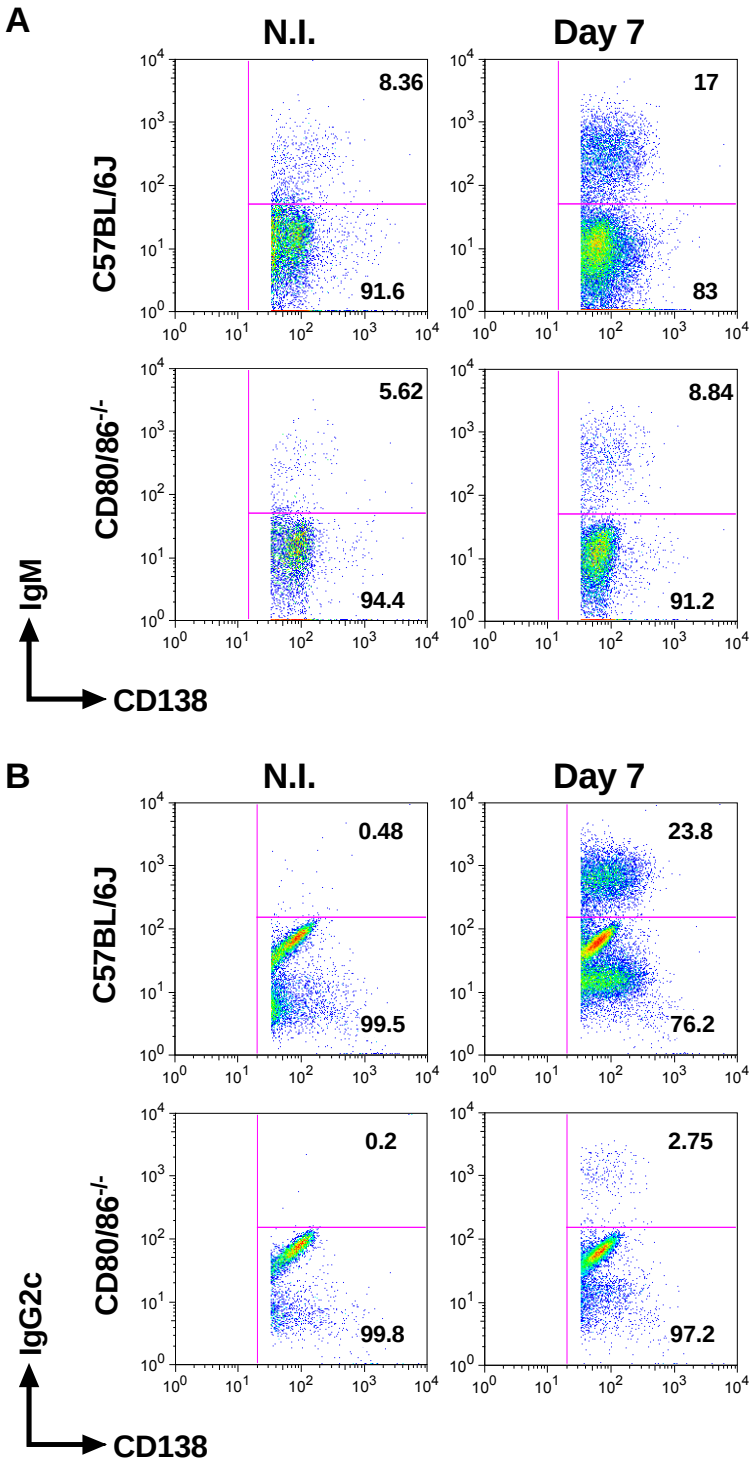

Figure 5.23: Representative Proportion of IgM and IgG2c Plasma cells after infection with STm in CD80/86$^{-/-}$ mice.

C57BL/6J and CD80/86$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 7 days. Representative dot plots of the proportion of CD138$^+$ **A**) IgM$^+$ or **B**) IgG2c$^+$ plasma cells in the spleen is shown following detection by flow cytometry. Data is representative of two independent experiments with 3-4 mice per group.

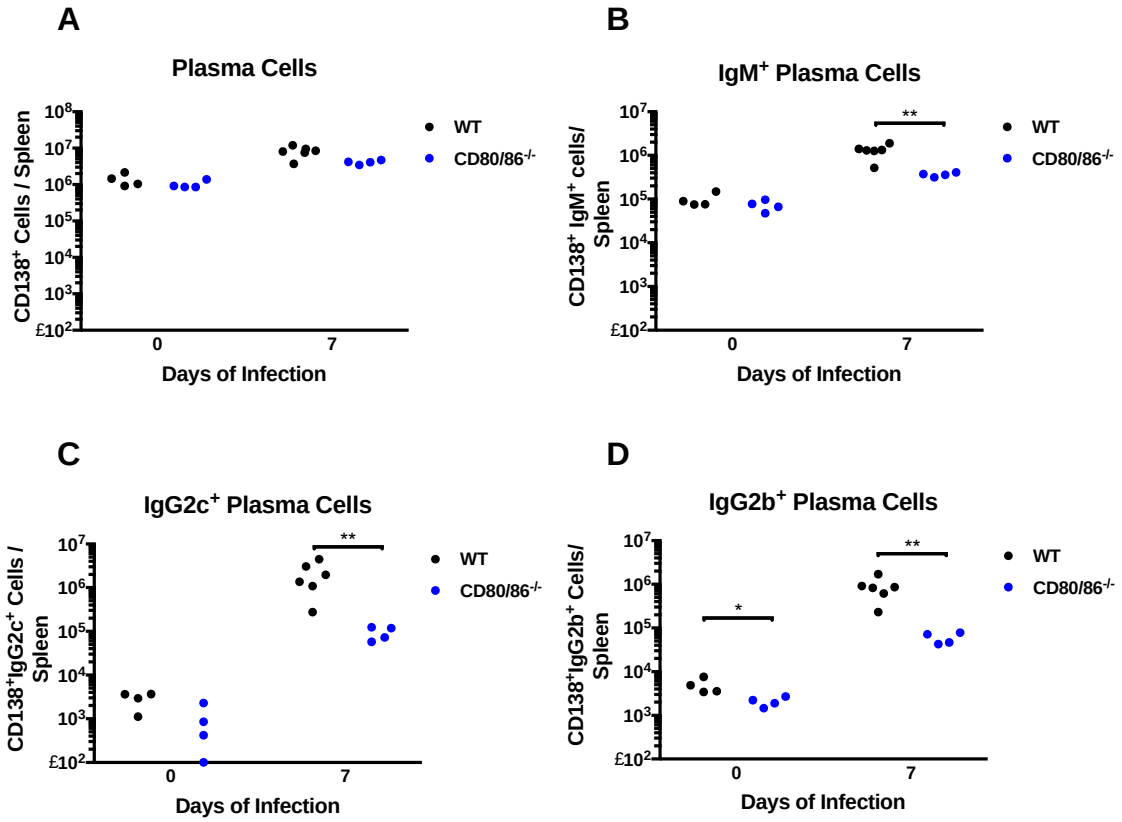

Figure 5.24: Plasma cells numbers after infection with STm in CD80/86$^{-/-}$ mice.
C57BL/6J and CD80/86$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 7 days. CD138 is a marker of plasma cells. All cells were gated and enumerated on the expression of **A**) CD138 and either intracellular **B**) IgM, **C**) IgG2c or **D**) IgG2b. Data is representative of two independent experiments with 4-6 mice per group per experiment. *(P≤0.05). & **(P≤0.01).

5.2.20. CD80 and CD86 are required for the induction of anti-OMPs IgG antibodies after infection

The anti-OMPs antibody response was assessed by ELISA 7 days after infection with STm. Anti-OMPs IgM was induced similarly in WT mice and CD80/86$^{-/-}$ mice (**Figure 5.25.A**). Anti-OMPs IgG titres were greater in WT infected mice than in CD80/86$^{-/-}$ mice, where the titres were only marginally above background (**Figure 5.25.B**). A similar pattern was observed for the anti-OMPs IgG2c response (**Figure 5.25.C**). This suggests that the majority of anti-OMPs IgG detected are of the IgG2c subclass. In contrast, IgG2b and IgG3 anti-OMPs antibodies were not significantly different between the WT mice and CD80/86$^{-/-}$ mice (**Figure 5.25.D-E**).

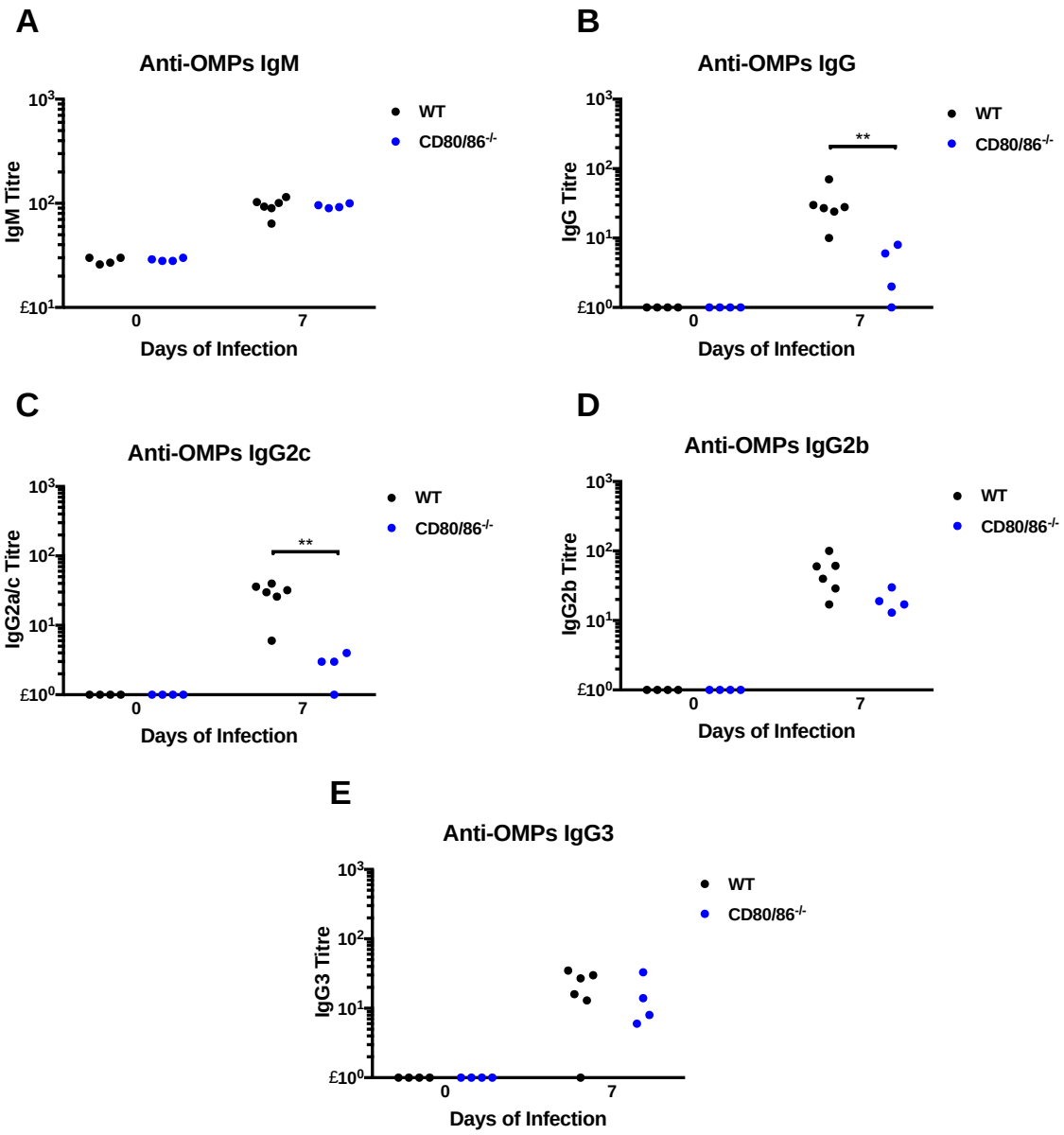

Figure 5.25: Anti-OMPs antibody responses after infection with STm in CD80/86$^{-/-}$ mice
C57BL/6J and CD80/86$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 7 days. The anti-OMPs **A**) IgM, **B**) IgG, **C**) IgG2c, **D**) IgG2b and **E**) IgG3 response was measured by ELISA. Data is representative of two independent experiments with 3-6 mice per group per experiment. *(P≤0.05) & **(P≤0.01)..

5.2.21. CD80 and CD86 are required for the production of self-reactive antibodies after infection with STm.

We previously identified that the production of self-reactive antibodies to the stomach and muscle is largely of the IgG subclass. Given that the production of anti-OMPs IgG antibodies was abrogated in the absence of CD80/86 it was hypothesized that the self-reactive antibody response would be reduced. Staining for self-reactive antibodies by immunofluorescence revealed that rat stomach and muscle antibodies could be detected 7 days post-infection in **9/10** WT mice at a 1/10 dilution (**Figure 5.26**). However, no staining of the stomach or the underlying muscle could be detected in **6/7** CD80/86$^{-/-}$ mice. This suggests that co-stimulation by CD80/86 is required for the induction of anti-self-antibodies and the activation of B cells

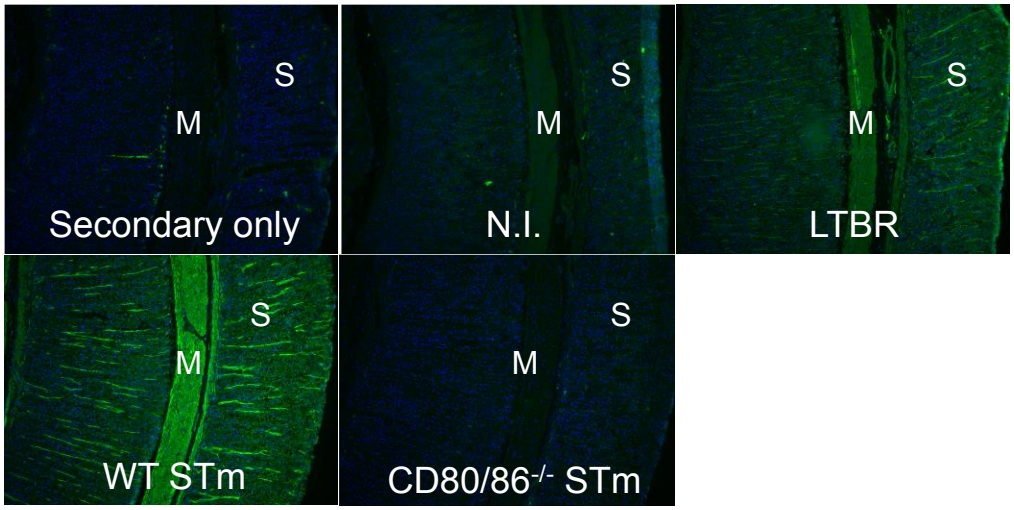

Figure 5.26:Staining for self-reactive antibodies in CD80/86$^{-/-}$ mice after infection with STm.
C57BL/6J and CD80/86$^{-/-}$ mice were infected with 5 x 10^5 SL3261 for 7 days. Rat kidney, liver and stomach slides were incubated with mouse serum and tissue-specific antibodies detected with a F(ab')2 IgG secondary antibody (Green). Cell nuclei were counterstained with DAPI (blue). Images were taken at a 20 x magnification. Data is representative of two independent experiments with 3-4 mice per group per experiment. Note that the LTBR was used as a positive control at a 1/20 dilution to ensure the assay was working, whereas all other samples were used at 1/10 dilution. This was to ensure that the WT infected mice were positive for self-reactive antibodies and could be compared directly to the CD80/86$^{-/-}$ mice

5.3. DISCUSSION

In the previous chapter, we identified that the pathogen-specific and self-reactive antibody responses develop at discordant rates. In addition, the data we obtained suggested that the initiation of this response is not dependent upon IFN-γ or T-bet. As a result we decided to investigate these two antibody responses by looking at the role of various cytokines and molecules in this process at different stages of the immune response. The aim was to identify whether or not these antibody responses are co-regulated or independently regulated.

Initially IL-6 was investigated due to its role as a B cell growth factor, and that a preliminary anti-LHK screening ELISA suggested this was essential for the self-reactive antibody response (Data not shown) (306). Infection of IL-$6^{-/-}$ mice showed that it played no role in the later control of the bacterial burden in the spleen or liver. In addition, IL-6 did not appear to be required for T cell activation. This is consistent with previous data from our laboratory, which has shown that IL-$6^{-/-}$ mice clear the infection at a similar rate to the WT mice (186). Despite no obvious effects on the T cell response, loss of IL-6 lead to a small but significant decrease in the IgG, IgG2a/c and IgG2b antibody responses to *Salmonella* three weeks post-infection. IL-6 is therefore required for the optimal production of extrafollicular derived class switched plasma cells and pathogen-specific antibodies. Whether or not this is a result of IL-6 having a direct effect on B cells or due to altered development of pre-TfH cells is not clear. Although it is possible that both have a role since the anti-OMPs IgM response, which is wholly TI, was significantly

impaired in the absence of IL-6 after 5 weeks of infection, whereas the IgG responses were equivalent to those observed in WT mice.

The production of stomach and muscle antibodies was IL-6 independent both three and five weeks post-infection. This suggests that other molecules are supporting the EFR to both the pathogen and self. It also implies that Th17 cells are not driving the response since IL-6 is required for the optimal differentiation of these cells in mice (139, 307). It appears that molecules other than IL-6 are supporting the EFR in the spleen. $CD11c^{High}$ DCs have been associated with supporting antibody-secreting cells in the EF antibody response through the provision of IL-6, BAFF and APRIL (39, 308). IL-6 from cDCs is probably less important for the pathogen-specific response since bone marrow chimeras have shown that IL-6 from stromal or haematopoietic compartment is sufficient to support antibody responses to STm (186). In addition, BAFF and APRIL can promote survival of autoreactive B cells and high expression of these molecules can correlate with increased self-reactive antibody titres and autoimmune disease (309-314). It should be noted that screening by ELISA suggests that the self-reactive antibody response is dependent upon IL-6 from stromal cells after 35 days of infection. As a consequence we do not know whether or not IL-6 has a role in the self-reactive response at later time-points without additional experiments. This is possible though since IL-6 from B cells has recently been shown to drive the formation of autoimmune GCs in mouse models of SLE (315).

Given that IL-6 did not appear to be essential for the induction of the self-reactive antibody response we decided to assess whether or not the migration of plasma cells to the splenic red pulp is important. As a consequence $p55^{-/-}p75^{-/-}$ mice were infected for 17 days. Loss of TNF activity lead to impaired control of STm infection, which was evident through the higher bacterial burdens in the spleen and liver. This was expected since TNF is known to be essential for synergizing with IFN-γ for the induction and maintenance of granulomas that help contain the infection (115, 301). TNF also drives the expression of NADPH oxidase and iNOS in monocytes/macrophages, which are essential for killing intracellular *Salmonella* (316). Loss of TNF activity resulted in smaller spleen sizes suggesting that this cytokine helps drive splenomegaly. Despite a smaller spleen size there was an equivalent number of activated $CD4^+$ T cells, non-switched and switched plasma cells. In addition, the anti-OMPs response was fully intact with only a small but significant decline in the IgG2b response. This was surprising since the plasma cells in $p55^{-/-}p75^{-/-}$ mice were retained in the T-zone and stromal niches in the red pulp are believed to be required for supporting the EFR (317, 318). However, $p55^{-/-}$ mice infected with malaria also mount normal extrafollicular antibody responses 2-3 weeks post-infection but not at later time points when germinal centres are present (217, 319). Similar observations have been observed in TNF deficient mice after infection with *Trypanosoma congolense* and TI responses to pneumococcus are not affected (320, 321). This suggests that TNF is largely important for germinal centre and memory responses given its role in promoting the formation of SLO (322-324). Consistent with this secondary responses to vaccine strains of STm are impaired in the absence of the p55 (325). However, following oral challenge with an attenuated strain of STm lower

Salmonella specific antibody responses were observed 4 weeks after infection in $p55^{-/-}$ mice (325). Unfortunately, it is not clear whether the antibody response at this time-point is derived from the EFR or GC reaction as these were not studied. Since TNF is essential for the correct organization of B cell follicles and therefore germinal centre responses, our data suggests that the extrafollicular antibody response is the source of self-reactive antibodies as no difference could be detected in the rat stomach and muscle antibody staining between WT mice and $p55^{-/-}p75^{-/-}$ mice. This was surprising since TNF is known to drive tissue damage and inflammation in a range of different models and as consequence we suspected it would promote the production of self-reactive antibodies. Evidence of this is in ReA models of *Salmonella* infection where IL-17 produced in the intestine is believed to drive the expression of TNF, which leads to joint inflammation (326). It should be noted that a study in lpr mice shows that autoreactive B cells localize in T cell rich areas in FAS deficient mouse models (327). Therefore, the impaired migration of antibody secreting cells to the splenic red pulp is not essential for the antibody response.

Since migration of plasma cells to the splenic red pulp was not required for the induction of the anti-self-antibody response we then looked at the T-B interface by investigating the role of ICOSL. $ICOSL^{-/-}$ mice had a marginally higher bacterial burden in the spleen and liver compared to WT mice after three weeks of infection. This is possibly due to the lower number of activated $CD4^+$ T cells generated in the absence of ICOSL. The ability to activate T cells did not appear to be impaired as a similar proportion of effector $CD4^+$ T cells was observed. Instead there were a lower number of $CD4^+$ T cells in the spleen.

This conflicts with the observations reported in ICOS$^{-/-}$ mice as these supposedly have more CD4^{+} T cells than there WT counterparts after infection with STm-OVA (305). Despite the difference in CD4^{+} T cell numbers the lower number of activated/effector CD4^{+} T cells in ICOSL$^{-/-}$ mice is consistent with observations reported in ICOS$^{-/-}$ mice that have been infected with STm for three weeks (305). Another similarity to ICOS$^{-/-}$ mice is that ICOSL$^{-/-}$ mice also have lower titres of IgG and IgG2a antibodies three weeks after infection with STm. However, we also observed a reduction in the IgM antibody response suggesting that a small part of this response might be T cell mediated. Preliminary data suggests that the lower IgG antibody titres are due to a reduced level of anti-LPS antibodies (data not shown). It is therefore possible that ICOSL helps diversify the antibody response by facilitating the activation of lower avidity T cells, since the porin-specific antibody responses were not impaired (data not shown).

ICOSL signaling has been reported to be responsible for hypergammaglobulinaemia and immune complex deposition in the kidneys after infection with STm (261). In our model, loss of ICOSL did not influence the rat stomach and muscle antibody response despite impaired CD4 T cell responses. One explanation for the difference in these observations is that a different strain of STm, route and source of antibodies is responsible as the kidney-specific antibodies reported in the literature are largely derived from germinal centres. Although we could detect some self-reactive antibodies against the kidneys there was no difference observed in either the WT mice or ICOSL$^{-/-}$ mice. In addition, CXCR4 expressing PSGL-1 T cells have been shown to drive autoreactive EFRs in MRL^{lpr} models of lupus in an ICOS dependent manner (253). The

ICOSL independency of the self-reactive antibody response suggests these are not important in STm infections.

Loss of ICOSL resulted in an impaired pathogen-specific response but did not affect the self-reactive antibody response. This suggests that these two responses are independently regulated. To investigate this further we decided to look at the APC-T cell interface by assessing the effects of CD80 and CD86 deficiency on the antibody response. CD80/86$^{-/-}$ mice had similar bacterial burdens to the WT mice 7 days post-infection which is consistent with the innate immune response being essential for controlling bacterial burdens at these time points. The production of activated/effector CD4^{+} T cells was completely abrogated in the absence of CD80/86. DCs are typically required to prime naïve CD4^{+} T cells and a failure to generate effector T cells is most likely a product of CD80/86$^{-/-}$ on these cells rather than B cells or macrophages (2). For example, B cell deficient mice can make normal T cell responses and actively clear a primary infection with STm (136-138). It is therefore highly unlikely that CD80/86$^{-/-}$ mice can clear an infection with STm as T cells are required to do so. Consistent with loss of T cell activation there was a significantly impaired production of IgM, IgG2a/c and IgG2b plasma cells. However, IgM, IgG2b and IgG3 anti-OMPs responses were intact suggesting that 7 days post-infection these are largely produced in a TI manner. In addition, the magnitude of the IgG2b and IgG3 response is likely to be small. Evidence supporting this is that the anti-OMPs IgG and IgG2a/c response was abolished in the absence of CD80/86, while the IgG2b and IgG3 responses were not. Consistent with these observations CD28$^{-/-}$ mice cannot clear infections with STm and have impaired

extrafollicular antibody plus effector T cell responses (137, 328). The self-reactive response to the rat stomach and muscle was abolished in the absence of CD80 and CD86. CD80 and CD86 are therefore essential for the development of the self-reactive antibody response. This implies that co-stimulation by APCs are important for the induction of self-reactive antibodies. The exact APCs responsible for this is not clear as autoreactive B cells are known to express CD80/86 and can drive the activation of autoreactive T cells in mouse models of RA (329, 330). In the presence of continuous antigen exposure, self-reactive B cells supposedly downregulate CD86 that leads to reduced T cell help and induction of apoptosis through FAS (302). B cells can be infected with STm or stimulated by porins and LPS to upregulate CD80 and CD86 (331, 332). It is therefore possible that STm is directly promoting the survival of self-reactive B cells. If this is true, then innate PRRs such as TLRs might have a role in sustaining the self-reactive antibody response.

Both the IgG pathogen-specific and self-reactive antibody response is dependent upon APCs and the expression of CD80/86. Given that T cells and CD40L are essential for the pathogen-specific response this suggests that the antibody response is T cell dependent. This is supported by the reduced anti-OMPs response observed in ICOSL$^{-/-}$ mice. Whether or not the same can be said for the self-reactive response is not clear since autoreactive B cells can be activated independently of DCs. Although these would most likely still required T cell help thus making CD80/86 a key molecule. The exact function of IL-6, ICOSL and TNF in the self-reactive antibody response is difficult to address. One reason for this is the measurement of self-reactive antibodies by

immunofluorescence is not particularly sensitive and due to the staining pattern observed it is difficult to accurately quantify the amount of self-reactive antibodies. To confirm these findings further work would really need to focus on improving our read out of self-reactive antibodies by identifying specific antigens. In addition, we cannot rule out that low affinity antibodies associated with the pathogen-specific response are simply cross-reacting with tissues and could therefore be heterophilic antibodies. Other problems are with the flow cytometry as the staining is not always clear. Additional controls, repeats and markers in these experiments such as CD19, IgM, IgD, CD5, CD21 & CD23 would help identify what B cell populations are contributing to the pathogen-specific and self-reactive response.

In conclusion, there is a differential regulation of the pathogen-specific and self-reactive antibody response as CD80/86 is essential for the development of both types of antibody responses yet IL-6 and ICOSL is only crucial for an optimal anti-*Salmonella* response.

6. CHAPTER 6 – GENERAL DISCUSSION

TLRs are common targets for the development of adjuvants to enhance immune responses, but aberrant activation of these receptors is also associated with autoimmune responses (333, 334). The expression of LPS and porins in the outer membrane of STm suggested that TLR4 could play an important role in the antibody response to this bacterium. Therefore, we initially investigated the role of this receptor in response to systemic infection with STm.

In Chapter 3, we showed that TLR4 is required for optimal induction of IgG2a class switching and antibody responses after infection in BALB/c mice. Loss of TLR4 also led to early formation of GCs during infection with STm, but little pathogen-specific antibody was detected until 5 weeks post-infection. This contrasted with C57BL/6J mice in which the antibody response developed in a similar manner to WT mice in the absence of TLR4. The limited production of antibodies from GCs in $TLR4^{-/-}$ mice on a BALB/c background implied that these were producing antibodies to unknown targets such as self. This led to the identification of self-reactive antibodies in WT mice, but not $TLR4^{-/-}$ mice in response to infection. Since LPS has previously been reported to drive non-specific polyclonal B cell activation it was suspected that this was responsible for driving the self-reactive response in infected BALB/c mice. However, this was deemed unlikely as it has been reported that the production of IgG antibody-forming cells (AFCs) after infection with STm was not impaired in $TLR2^{-/-}TLR4^{-/-}$ or $MyD88^{-/-}$ mice (214). Instead it was shown that mice with a restricted BCR repertoire have a lower number of IgG AFCs after infection with STm on both a BALB/c and C57BL/6J background (214). If the

antibody response induced by STm is truly non-specific then the number of AFCs should not be altered by changes in the diversity of the BCR. This suggested that the antibody response to STm is simultaneously giving rise to both pathogen-specific and self-reactive antibodies. It would be interesting to assess whether antibodies from infected TLR4$^{-/-}$ mice can contribute to protection as these self-reactive antibodies might form part of this response. In addition, the loss of the self-reactive response in TLR4$^{-/-}$ mice implies that this receptor may contribute to the diversification of the antibody repertoire in response to infection in BALB/c mice. This is interesting since TLRs are implicated in the function of AID in peripheral B cells and extrafollicular affinity maturation has been reported during infection with STm (214, 335). TLRs might therefore influence affinity maturation at extrafollicular sites in response to STm infection in BALB/c and C57BL/6J mice. However, given the phenotypic differences between BALB/c and C57BL/6J mice and that continuing these studies would mean we were not assessing the response to TLR4 alone and that this receptor is not expressed on naive human B cells we decided to focus on investigating the self-reactive antibody response in more detail (336).

Autoimmune diseases have classically been associated with high affinity GC derived autoantibodies. However, EFRs can also give rise to autoreactive plasmablasts with limited involvement of GCs (337). In chapter 4, we characterised the kinetics of the pathogen-specific and self-reactive antibody response after infection with STm. The peak of the self-reactive antibody response was detected three weeks after infection, a time when the EFR dominates. This response marginally declined by 5 weeks of infection but persisted above background levels for 110 days. Whether or not the

persistence of this response is due to activation/maintenance of autoreactive B cells and plasma cells in extrafollicular sites or due to germinal centres is unknown. However, this data suggests that either autoreactive or cross-reactive B and T cells are induced and ultimately persist after infection with STm. The long-term implications of this are unidentified but it could mean that repeated infections might enhance the expansion of autoreactive cells and increase the titres of self-reactive antibodies in healthy hosts. This could potentially influence an individual's susceptibility to developing autoimmune disease as it might enhance the likelihood of inducing an autoimmune response. In addition, it is not known whether the self-reactive antibodies induced by infection with STm are pathogenic or whether other infections exhibit the same response. If these antibodies induce destruction of host tissue they could potentially cause tissue inflammation that overtime could alter tissue architecture. Although the exact source and significance of the self-reactive antibodies is not clear the induction of these follows similar but differential kinetics to the pathogen-specific response. This is supported by the detection of IgM, IgG and IgG2a/c self-reactive antibodies. The induction of these types of antibodies to STm involves both TI and TD components that suggest this may also apply to the self-reactive antibody response (47, 67). One possible explanation for these observations is that heterophilic antibodies are being induced and that our assays cannot distinguish between the two responses. However, in Chapter 5 we showed that loss of ICOSL results in an impaired antibody responses to the pathogen but not self, which suggests that this is not the case and that these responses are independently regulated. Additional evidence supporting this is that assessment of the self-reactive response in other genetically modified mice (**Table 6.1**) showed that Th1 responses and

TNF are not required for the induction of self-reactive antibodies, yet these are essential for protective immune responses against the pathogen. This was surprising as these cytokines are pro-inflammatory and can induce systemic autoimmune diseases in genetically susceptible mice (269). An explanation for this is that other T cell subsets such as Th17 cells could be driving the self-reactive response. However, IL-6 is required for Th17 cell differentiation in mice, yet infected IL-$6^{-/-}$ mice only had a modest reduction in the self-reactive antibody response (338, 339). Therefore, other pathways may be important and it cannot be ruled out that IL-6 is having a direct affect on B cells since stromal cells derived IL-6 seems to be important. Hence, additional experiments need to be done to confirm the role of IL-6 in the self-reactive response. The most striking observation from this project was that loss of CD80 and CD86 resulted in the abolishment of self-reactive antibodies and IgG class switched pathogen-specific antibodies. The pathogen-specific response is most likely impaired due to loss of these co-stimulatory molecules from DCs and the impaired $CD4^+$ T cell response, but whether this applies to the self-reactive response is less clear as B cells may be important. In addition, the role of DCs in extrafollicular responses is complex as they are implicated in supporting the expansion of plasmablasts but also regulating the production of autoreactive plasmablasts (39, 340).

Table 6.1. Summary of observations comparing knockout mice to wild-type mice after infection with STm.

Mouse Strain	CFU per spleen				Spleen Mass (g)				Number of CD4+ T Cells				Number of Activated CD4+ T Cells				Germinal Centres				CD138+ Plasma Cells				IgM+ Plasma Cells				IgG2c+ Plasma Cells				Anti-OMPs IgM ELISA				Anti-OMPs IgG ELISA				Anti-OMPs IgG2c ELISA				Self-Reactive Antibodies			
	7	14	21	35	7	14	21	35	7	14	21	35	7	14	21	35	7	14	21	35	7	14	21	35	7	14	21	35	7	14	21	35	7	14	21	35	7	14	21	35	7	14	21	35	7	14	21	35
TLR4$^{-/-}$ (BALB/c)																																															
TLR4$^{-/-}$ (C57BL/6J)									N/A				N/A																														N/A			
IFN-γ-/-									N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A							
IFN-γ$^{-/-}$ (Stromal only)	N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A							
IFN-γ$^{-/-}$ (Haematopoietic Only)	N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A							
IL-6$^{-/-}$																	N/A																														
-IL-6$^{-/-}$ (Stromal Only)	N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A							
IL-6$^{-/-}$ (Haematopoietic Only)	N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A							
T-bet$^{-/-}$	N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A				N/A							
p55$^{-/-}$p75$^{-/-}$ (*)																	N/A																														
ICOSL$^{-/-}$																	N/A																														
CD80/86$^{-/-}$																	N/A																														

A summary table of the data presented throughout this thesis showing the changes in the STm infected knockout (KO) mice relative to their infected wild-type (WT) counterpart. The number of days after infection is displayed under each column heading as day 7, 14, 21 and 35.
The colour in the cells represents the following: Red - Lower in the KO mice compared to WT mice. Yellow - No difference between KO mice and WT mice. Green - Higher/Present in KO mice but not WT mice & White or N/A – Parameter not measured or non-applicable, respectively.
*Represent experiments where the knockout (KO) mice and WT mice were infected for 17 days.

6.1. Limitations

There are a range of different limitations to this work which largely derive from limited access to certain genetically modified mice due to breeding problems combined with a short time for experiments and writing up, since the entire PhD project had to be completed within 3 years due to the requirements set out by the funding body. In chapter 4 and chapter 5 we attempted to characterise the self-reactive response in C57BL/6j mice and identify what molecules are required for the self-reactive antibody response. Initially a screening assay was used to test for self-reactive antibodies but one weakness of this method is that the antibodies detected will not just be against self but also the pathogen and possibly other unidentified targets. This probably even more evident in the IgM anti-LHK response due to high avidity of these antibodies compared to IgG antibodies. One of the key limitations of this work is the staining for self-reactive antibodies using rat stomach, liver and kidney slides. The reason for this is our data is largely giving us qualitative results thus making it difficult to say how big or small the self-reactive response is. Attempts to quantify this response through the use of ImageJ were unsuccessful due to the variation in staining intensity and patterns. This made it difficult to compare the intensity of the response particularly in the stomach. Additional problems were in acquiring a consistent positive control to validate the assay. However, with more time and optimization of the assay quantification of self-reactive antibodies through ImageJ may be possible. Finally it should also be noted that with regards to the flow cytometry data presented throughout this thesis, one of the key limitations was the quality of the staining. As a consequence it was sometimes difficult to distinguish between two different populations using the laboratories current flow cytometry

procedures and gating strategies, which relies on using the N.I. mice or infected WT mice to set the gates to allow a direct comparison to the KO mice. The suboptimal staining could be due to technical errors or that the infection itself is reducing the expression of certain cell markers. To help account for this, in future experiments these flow cytometry panels should all include both isotype controls and fluorescence minus one controls in order to accurately place the gate and minimize the chances of non-specific binding. In addition, staining panels could be further improved by incorporating new markers. For example, in order to clearly distinguish between certain cell types such as plasmablasts and plasma cells an additional antibody for CD19 could be used. This would help distinguish in conjunction with controls whether or not intermediate levels of intracellular IgM, IgG2a/c & IgG2c is due to the accumulation of plasmablasts.

6.2. Future Directions

6.2.1. Why does TLR4 suppress the germinal centre response in BALB/c mice?

TLR4$^{-/-}$ mice from a BALB/c background have a reduced IgG2a/c antibody response and show early formation of germinal centres in response to infection with STm. This phenotype could be due to a decreased production of IFN-γ or B cell sensitivity to it in BALB/c mice. It would be interesting to infect BALB/c IFN-γ$^{-/-}$ mice to check whether this is responsible for the phenotype observed in TLR4 knockouts. Alternatively, we could make mixed bone marrow chimaeras in which B cells are deficient in IFN-γ receptor. If IFN-γ is not responsible it would suggest that TLR4 has a suppressive function that works through other pathways or that there is an intrinsic difference in the B cells of BALB/c and C57BL/6J mice. Future work could then focus on making mixed bone chimaeras in which only B cells are deficient in TLR4. If loss of TLR4 on B cells in BALB/c mice gives rise to the same phenotype as that in TLR4$^{-/-}$ mice we could then transfer TLR4$^{-/-}$ B cells from C57BL/6J mice into B cell deficient BALB/c mice and vice versa. This would tell us whether the phenotype is due to difference in the intrinsic quality of the B cells or stromal cell compartment of BALB/c mice and C57BL/6J mice.

6.2.2. Heterophilic antibodies & the source of self-reactive antibodies at later time points

Antibodies that are produced by extrafollicular derived antibody secreting cells are known to be of a lower affinity compared to those that originate from the germinal centre reaction. In addition, antibodies that can bind to poorly defined antigens may give rise to multi-specific binding. One possibility is that the self-reactive antibody response we have

been measuring three weeks post-infection is actually a product of a cross-reactive antibodies induced/directed against *Salmonella*. To test this we should adsorb *Salmonella* with mouse serum from mice that have and have not been infected for three weeks and then collect the unbound antibody fraction and use this to stain for self-reactive antibodies. In addition, we should also check whether the self-reactive antibodies detected at 35 and 110 days are derived from the germinal centre reaction. Since ICOSL is required for the maintenance of the germinal centre response we could test this by infecting WT mice and ICOSL$^{-/-}$ mice for 35 and 110 days. The self-reactive antibody response could then be measured and in theory this response should be absent at 110 days in the absence of ICOSL if it is derived from the germinal centre.

6.2.3. Are the self-reactive antibodies induced by infection with STm pathogenic?

Most pathogenic antibodies associated with clinically detectable autoimmune disease have a high affinity for their antigen. To test whether or not the self-reactive antibodies induced by STm are pathogenic the ability of these antibodies to kill other cells could be assayed *in vitro*. One possible means of achieving this would be to label host cells from individual tissues with radioactive chromium. Cells could then be incubated with sera from non-infected and infected mice in the presence and absence of complement. The amount of radioactive chromium released into medium could then be used to measure the amount of killing.

6.2.4. Is there a better way of measuring the self-reactive antibody response?

Self-reactive antibodies were detected against rat stomach, muscle, kidney and liver. One possible way to enhance the quantification of these antibodies is to measure titres through ELISA to known autoantigens that are associated with these tissues. In addition, the production of IgM self-reactive antibodies needs to be characterised in more detail alongside the B cell subsets.

6.3. Final Conclusions

The experiments in this project have shown that there is differential role for TLR4 and the mouse genetic background in the antibody response to STm. This has important implications for vaccine development as BALB/c and C57BL/6J mice are regularly used to study the role of TLRs in the antibody response. On top of this we have identified the simultaneous but independent production of antibodies against both the pathogen and self in WT mice after infection with STm. Both IgM and IgG self-reactive antibodies are induced in response to infection. In addition, the production of self-reactive antibodies persists above background levels long after the infection is cleared from the spleen and liver. Although ICOSL and Th1 associated molecules are vital for optimal pathogen-specific antibody responses they are not necessary for the induction of self-reactive antibodies. The data from this project suggests that pathogen-specific and anti-self-antibody responses are independently regulated. The findings in this thesis has potential implications in understanding how self-reactive responses can be modulated while enhancing optimal pathogen-specific responses.

7. APPENDIX A: BUFFERS, SUBSTRATES & MEDIA

Agar Plates

20 g of LB agar was dissolved in 1L of dH_2O, autoclaved and poured into plates.

Coating Buffer

500 mL of dH_2O
0.83g of Na_2CO_3
1.42g of $NaHCO_3$
0.1 % NaN_3

FACs Buffer

500 mL of sterile Dulbecco's PBS without $CaCl_2$ and $MgCl_2$.
2% FBS
5 mM EDTA

Luria Bertani Medium

20 g of LB dissolved in 1L of dH_2O, autoclaved and poured into plates.

Tris Buffer pH 7.6.

Make 1L of 200 mM Tris Base (6.075g) in dH_2O
Add 1.5L of 154 mM NaCl
Add 1L of 0.1 N HCl.

Tris Buffer pH 9.2

Made according to Tris Buffer pH 7.6. The pH was then adjusted by adding 1M NaOH one drop at a time.

ELISA Substrate

Dissolve 1 SIGMAFAST p-Nitrophenyl phosphate and 1 Tris buffer tablet in 20 mL of dH_2O.

B Cell ELISPOT Substrate

Dissolve 1 SIGMAFAST BCIP/NBT tablet in 10 mL of dH_2O.

Immunohistochemistry Substrates

Substrate for Alkaline Phosphatase:
Dissolve 8 mgs of Levamisole in 10 mL of Tris buffer (pH 9.2). Then dissolve 4 mgs of Naphthol-AS-MX-phosphate in 380 µL of N,N-dimethyl-formide and add to the solution of levamisole. Add 10mg of Fast Blue BB Salt and then filter.

Substrate for Horseradish Peroxidase:
1 3,3'-diaminobenzidine tetrahydrochloride (DAB) tablet was dissolved in 15 mL of Tris Buffer pH 7.6. This was then filtered and 50 µl of hydrogen peroxide was added.

R10 Medium

500 mL of RPMI-1640 with L-glutamine and $NaHCO_3$ was purchased from Sigma-Aldrich. Remove 55 mL of medium and replace this with 50 mL of FBS/FCS and 5mL of Penicillin/Streptomycin. To give R10 Medium (RPMI-1640 supplemented with 10% FBS/FCS and 1% Peniciliin/Streptomycin).

8. REFERENCES

1. Turvey SE, Broide DH. Innate immunity. J Allergy Clin Immunol. 2010;125(2 Suppl 2):S24-32.
2. Murphy K, Weaver C. Janeway's immunobiology. 9th edition. ed. New York, NY: Garland Science/Taylor & Francis Group, LLC; 2017. xx, 904 pages p.
3. Kim YK, Shin JS, Nahm MH. NOD-Like Receptors in Infection, Immunity, and Diseases. Yonsei Med J. 2016;57(1):5-14.
4. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010;140(6):805-20.
5. Medzhitov R, Janeway CA, Jr. Decoding the patterns of self and nonself by the innate immune system. Science. 2002;296(5566):298-300.
6. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011;34(5):637-50.
7. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124(4):783-801.
8. Yarovinsky F, Zhang D, Andersen JF, Bannenberg GL, Serhan CN, Hayden MS, et al. TLR11 activation of dendritic cells by a protozoan profilin-like protein. Science. 2005;308(5728):1626-9.
9. Yarovinsky F. Innate immunity to Toxoplasma gondii infection. Nat Rev Immunol. 2014;14(2):109-21.
10. Shi Z, Cai Z, Yu J, Zhang T, Zhao S, Smeds E, et al. Toll-like receptor 11 (TLR11) prevents Salmonella penetration into the murine Peyer patches. J Biol Chem. 2012;287(52):43417-23.
11. Zhang D, Zhang G, Hayden MS, Greenblatt MB, Bussey C, Flavell RA, et al. A toll-like receptor that prevents infection by uropathogenic bacteria. Science. 2004;303(5663):1522-6.
12. West AP, Koblansky AA, Ghosh S. Recognition and signaling by toll-like receptors. Annu Rev Cell Dev Biol. 2006;22:409-37.
13. Kawasaki T, Kawai T. Toll-like receptor signaling pathways. Front Immunol. 2014;5:461.
14. InvivoGen. Toll-Like Receptors: InvivoGen; 2012 [
15. Park BS, Lee JO. Recognition of lipopolysaccharide pattern by TLR4 complexes. Exp Mol Med. 2013;45:e66.
16. Maeshima N, Fernandez RC. Recognition of lipid A variants by the TLR4-MD-2 receptor complex. Front Cell Infect Microbiol. 2013;3:3.
17. Gangaraju VK, Lin H. MicroRNAs: key regulators of stem cells. Nat Rev Mol Cell Biol. 2009;10(2):116-25.
18. Ashley NT, Weil ZM, Nelson RJ. Inflammation: Mechanisms, Costs, and Natural Variation. Annual Review of Ecology, Evolution, and Systematics. 2012;43(1):385-406.
19. Baggiolini M, Clark-Lewis I. Interleukin-8, a chemotactic and inflammatory cytokine. FEBS Lett. 1992;307(1):97-101.
20. Ingulli E, Mondino A, Khoruts A, Jenkins MK. In vivo detection of dendritic cell antigen presentation to CD4(+) T cells. J Exp Med. 1997;185(12):2133-41.

21. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392(6673):245-52.
22. Dieu MC, Vanbervliet B, Vicari A, Bridon JM, Oldham E, Ait-Yahia S, et al. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. J Exp Med. 1998;188(2):373-86.
23. Sozzani S, Allavena P, D'Amico G, Luini W, Bianchi G, Kataura M, et al. Differential regulation of chemokine receptors during dendritic cell maturation: a model for their trafficking properties. J Immunol. 1998;161(3):1083-6.
24. Forster R, Davalos-Misslitz AC, Rot A. CCR7 and its ligands: balancing immunity and tolerance. Nat Rev Immunol. 2008;8(5):362-71.
25. Yanagihara S, Komura E, Nagafune J, Watarai H, Yamaguchi Y. EBI1/CCR7 is a new member of dendritic cell chemokine receptor that is up-regulated upon maturation. J Immunol. 1998;161(6):3096-102.
26. Hogquist KA, Baldwin TA, Jameson SC. Central tolerance: learning self-control in the thymus. Nat Rev Immunol. 2005;5(10):772-82.
27. Nemazee D. Mechanisms of central tolerance for B cells. Nat Rev Immunol. 2017;17(5):281-94.
28. Germain RN. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell. 1994;76(2):287-99.
29. Zhang N, Bevan MJ. CD8(+) T cells: foot soldiers of the immune system. Immunity. 2011;35(2):161-8.
30. Zhang Y, Zhang Y, Gu W, Sun B. TH1/TH2 cell differentiation and molecular signals. Adv Exp Med Biol. 2014;841:15-44.
31. Vinuesa CG, Linterman MA, Yu D, MacLennan IC. Follicular Helper T Cells. Annu Rev Immunol. 2016;34:335-68.
32. Caza T, Landas S. Functional and Phenotypic Plasticity of CD4(+) T Cell Subsets. Biomed Res Int. 2015;2015:521957.
33. Toellner KM, Luther SA, Sze DM, Choy RK, Taylor DR, MacLennan IC, et al. T helper 1 (Th1) and Th2 characteristics start to develop during T cell priming and are associated with an immediate ability to induce immunoglobulin class switching. J Exp Med. 1998;187(8):1193-204.
34. Sethi A, Kulkarni N, Sonar S, Lal G. Role of miRNAs in CD4 T cell plasticity during inflammation and tolerance. Front Genet. 2013;4:8.
35. Allman D, Pillai S. Peripheral B cell subsets. Curr Opin Immunol. 2008;20(2):149-57.
36. Martin F, Kearney JF. Marginal-zone B cells. Nat Rev Immunol. 2002;2(5):323-35.
37. Hayakawa K, Hardy RR, Parks DR, Herzenberg LA. The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. J Exp Med. 1983;157(1):202-18.
38. Baumgarth N. B-1 Cell Heterogeneity and the Regulation of Natural and Antigen-Induced IgM Production. Front Immunol. 2016;7:324.
39. MacLennan IC, Toellner KM, Cunningham AF, Serre K, Sze DM, Zuniga E, et al. Extrafollicular antibody responses. Immunol Rev. 2003;194:8-18.
40. Montecino-Rodriguez E, Dorshkind K. B-1 B cell development in the fetus and adult. Immunity. 2012;36(1):13-21.

41. Cariappa A, Chase C, Liu H, Russell P, Pillai S. Naive recirculating B cells mature simultaneously in the spleen and bone marrow. Blood. 2007;109(6):2339-45.
42. MacLennan IC. Germinal centers. Annu Rev Immunol. 1994;12:117-39.
43. De Silva NS, Klein U. Dynamics of B cells in germinal centres. Nat Rev Immunol. 2015;15(3):137-48.
44. Paus D, Phan TG, Chan TD, Gardam S, Basten A, Brink R. Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. J Exp Med. 2006;203(4):1081-91.
45. O'Connor BP, Vogel LA, Zhang W, Loo W, Shnider D, Lind EF, et al. Imprinting the fate of antigen-reactive B cells through the affinity of the B cell receptor. J Immunol. 2006;177(11):7723-32.
46. Kurosaki T, Kometani K, Ise W. Memory B cells. Nat Rev Immunol. 2015;15(3):149-59.
47. Cunningham AF, Gaspal F, Serre K, Mohr E, Henderson IR, Scott-Tucker A, et al. Salmonella Induces a Switched Antibody Response without Germinal Centers That Impedes the Extracellular Spread of Infection. The Journal of Immunology. 2007;178(10):6200-7.
48. Vinuesa CG, Cyster JG. How T cells earn the follicular rite of passage. Immunity. 2011;35(5):671-80.
49. Aguzzi A, Kranich J, Krautler NJ. Follicular dendritic cells: origin, phenotype, and function in health and disease. Trends Immunol. 2014;35(3):105-13.
50. Ballesteros-Tato A, Randall TD. Priming of T follicular helper cells by dendritic cells. Immunol Cell Biol. 2014;92(1):22-7.
51. Crotty S. Follicular helper CD4 T cells (TFH). Annu Rev Immunol. 2011;29:621-63.
52. Chu VT, Beller A, Nguyen TT, Steinhauser G, Berek C. The long-term survival of plasma cells. Scand J Immunol. 2011;73(6):508-11.
53. Cassese G, Arce S, Hauser AE, Lehnert K, Moewes B, Mostarac M, et al. Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. J Immunol. 2003;171(4):1684-90.
54. Benson MJ, Dillon SR, Castigli E, Geha RS, Xu S, Lam KP, et al. Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL. J Immunol. 2008;180(6):3655-9.
55. Chu VT, Frohlich A, Steinhauser G, Scheel T, Roch T, Fillatreau S, et al. Eosinophils are required for the maintenance of plasma cells in the bone marrow. Nat Immunol. 2011;12(2):151-9.
56. Martin F, Oliver AM, Kearney JF. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. Immunity. 2001;14(5):617-29.
57. Mond JJ, Lees A, Snapper CM. T cell-independent antigens type 2. Annu Rev Immunol. 1995;13:655-92.
58. Mosier DE, Mond JJ, Goldings EA. The ontogeny of thymic independent antibody responses in vitro in normal mice and mice with an X-linked B cell defect. J Immunol. 1977;119(6):1874-8.
59. Vinuesa CG, Chang PP. Innate B cell helpers reveal novel types of antibody responses. Nat Immunol. 2013;14(2):119-26.

60. Bekeredjian-Ding I, Jego G. Toll-like receptors--sentries in the B-cell response. Immunology. 2009;128(3):311-23.
61. Peng SL. Signaling in B cells via Toll-like receptors. Curr Opin Immunol. 2005;17(3):230-6.
62. Garcia de Vinuesa C, O'Leary P, Sze DM, Toellner KM, MacLennan IC. T-independent type 2 antigens induce B cell proliferation in multiple splenic sites, but exponential growth is confined to extrafollicular foci. Eur J Immunol. 1999;29(4):1314-23.
63. Alugupalli KR, Gerstein RM, Chen J, Szomolanyi-Tsuda E, Woodland RT, Leong JM. The resolution of relapsing fever borreliosis requires IgM and is concurrent with expansion of B1b lymphocytes. J Immunol. 2003;170(7):3819-27.
64. Alugupalli KR, Leong JM, Woodland RT, Muramatsu M, Honjo T, Gerstein RM. B1b lymphocytes confer T cell-independent long-lasting immunity. Immunity. 2004;21(3):379-90.
65. Colombo MJ, Alugupalli KR. Complement factor H-binding protein, a putative virulence determinant of Borrelia hermsii, is an antigenic target for protective B1b lymphocytes. J Immunol. 2008;180(7):4858-64.
66. Cunningham AF, Flores-Langarica A, Bobat S, Dominguez Medina CC, Cook CN, Ross EA, et al. B1b cells recognize protective antigens after natural infection and vaccination. Front Immunol. 2014;5:535.
67. Gil-Cruz C, Bobat S, Marshall JL, Kingsley RA, Ross EA, Henderson IR, et al. The porin OmpD from nontyphoidal Salmonella is a key target for a protective B1b cell antibody response. Proc Natl Acad Sci U S A. 2009;106(24):9803-8.
68. Haury M, Sundblad A, Grandien A, Barreau C, Coutinho A, Nobrega A. The repertoire of serum IgM in normal mice is largely independent of external antigenic contact. Eur J Immunol. 1997;27(6):1557-63.
69. Panda S, Ding JL. Natural antibodies bridge innate and adaptive immunity. J Immunol. 2015;194(1):13-20.
70. Schultz M. Theobald Smith. Emerg Infect Dis. 2008;14(12):1940-2.
71. Knodler LA. Salmonella enterica: living a double life in epithelial cells. Current Opinion in Microbiology. 2015;23(0):23-31.
72. Fookes M, Schroeder GN, Langridge GC, Blondel CJ, Mammina C, Connor TR, et al. <italic>Salmonella bongori</italic> Provides Insights into the Evolution of the Salmonellae. PLoS Pathog. 2011;7(8):e1002191.
73. Andino A, Hanning I. Salmonella enterica: Survival, Colonization, and Virulence Differences among Serovars. The Scientific World Journal. 2015;2015.
74. Eng S-K, Pusparajah P, Ab Mutalib N-S, Ser H-L, Chan K-G, Lee L-H. Salmonella: A review on pathogenesis, epidemiology and antibiotic resistance. Frontiers in Life Science. 2015;8(3):284-93.
75. Guibourdenche M, Roggentin P, Mikoleit M, Fields PI, Bockemuhl J, Grimont PA, et al. Supplement 2003-2007 (No. 47) to the White-Kauffmann-Le Minor scheme. Res Microbiol. 2010;161(1):26-9.
76. Brenner FW, Villar RG, Angulo FJ, Tauxe R, Swaminathan B. Salmonella nomenclature. J Clin Microbiol. 2000;38(7):2465-7.

77. Ranieri ML, Shi C, Moreno Switt AI, den Bakker HC, Wiedmann M. Comparison of Typing Methods with a New Procedure Based on Sequence Characterization for Salmonella Serovar Prediction. J Clin Microbiol. 2013;51(6):1786-97.
78. Hurley D, McCusker MP, Fanning S, Martins M. Salmonella–Host Interactions – Modulation of the Host Innate Immune System. Front Immunol. 2014;5.
79. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, et al. The Global Burden of Nontyphoidal Salmonella Gastroenteritis. Clinical Infectious Diseases. 2010;50(6):882-9.
80. MacLennan CA, Tennant SM. Comparing the Roles of Antibodies to Nontyphoidal Salmonella enterica in High- and Low-Income Countries and Implications for Vaccine Development. Clin Vaccine Immunol. 2013;20(10):1487-90.
81. MacLennan CA, Gondwe EN, Msefula CL, Kingsley RA, Thomson NR, White SA, et al. The neglected role of antibody in protection against bacteremia caused by nontyphoidal strains of Salmonella in African children. J Clin Invest. 2008;118(4):1553-62.
82. Feasey NA, Dougan G, Kingsley RA, Heyderman RS, Gordon MA. Invasive non-typhoidal salmonella disease: an emerging and neglected tropical disease in Africa. Lancet. 2012;379(9835):2489-99.
83. Gilchrist JJ, MacLennan CA, Hill AV. Genetic susceptibility to invasive Salmonella disease. Nat Rev Immunol. 2015;15(7):452-63.
84. Brown L, Wolf JM, Prados-Rosales R, Casadevall A. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. Nat Rev Microbiol. 2015;13(10):620-30.
85. Beveridge TJ. Structures of gram-negative cell walls and their derived membrane vesicles. J Bacteriol. 1999;181(16):4725-33.
86. Muhlradt PF, Golecki JR. Asymmetrical distribution and artifactual reorientation of lipopolysaccharide in the outer membrane bilayer of Salmonella typhimurium. Eur J Biochem. 1975;51(2):343-52.
87. Madigan MT, Brock TD. Brock biology of microorganisms. 13th ed. Boston: Benjamin Cummings; 2012. xxviii, 1043, 77 p. p.
88. Michels J, Geyer A, Mocanu V, Welte W, Burlingame AL, Przybylski M. Structure and functional characterization of the periplasmic N-terminal polypeptide domain of the sugar-specific ion channel protein (ScrY porin). Protein Sci. 2002;11(6):1565-74.
89. Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. Annu Rev Biochem. 2002;71:635-700.
90. Wang X, Quinn PJ. Lipopolysaccharide: Biosynthetic pathway and structure modification. Prog Lipid Res. 2010;49(2):97-107.
91. Kusumoto S, Fukase K, Shiba T. Key structures of bacterial peptidoglycan and lipopolysaccharide triggering the innate immune system of higher animals: chemical synthesis and functional studies. Proc Jpn Acad Ser B Phys Biol Sci. 2010;86(4):322-37.
92. Galanos C, Luderitz O, Rietschel ET, Westphal O, Brade H, Brade L, et al. Synthetic and natural Escherichia coli free lipid A express identical endotoxic activities. Eur J Biochem. 1985;148(1):1-5.

93. Kawasaki K, Ernst RK, Miller SI. 3-O-deacylation of lipid A by PagL, a PhoP/PhoQ-regulated deacylase of Salmonella typhimurium, modulates signaling through Toll-like receptor 4. J Biol Chem. 2004;279(19):20044-8.
94. Steimle A, Autenrieth IB, Frick JS. Structure and function: Lipid A modifications in commensals and pathogens. Int J Med Microbiol. 2016;306(5):290-301.
95. Qiao S, Luo Q, Zhao Y, Zhang XC, Huang Y. Structural basis for lipopolysaccharide insertion in the bacterial outer membrane. Nature. 2014;511(7507):108-11.
96. Lai MA, Quarles EK, Lopez-Yglesias AH, Zhao X, Hajjar AM, Smith KD. Innate immune detection of flagellin positively and negatively regulates salmonella infection. PLoS One. 2013;8(8):e72047.
97. Stecher B, Hapfelmeier S, Muller C, Kremer M, Stallmach T, Hardt WD. Flagella and chemotaxis are required for efficient induction of Salmonella enterica serovar Typhimurium colitis in streptomycin-pretreated mice. Infect Immun. 2004;72(7):4138-50.
98. Mimori-Kiyosue Y, Yamashita I, Fujiyoshi Y, Yamaguchi S, Namba K. Role of the outermost subdomain of Salmonella flagellin in the filament structure revealed by electron cryomicroscopy. J Mol Biol. 1998;284(2):521-30.
99. Aizawa SI. Flagellar assembly in Salmonella typhimurium. Mol Microbiol. 1996;19(1):1-5.
100. Wyant TL, Tanner MK, Sztein MB. Salmonella typhi flagella are potent inducers of proinflammatory cytokine secretion by human monocytes. Infect Immun. 1999;67(7):3619-24.
101. Ikeda JS, Schmitt CK, Darnell SC, Watson PR, Bispham J, Wallis TS, et al. Flagellar phase variation of Salmonella enterica serovar Typhimurium contributes to virulence in the murine typhoid infection model but does not influence Salmonella-induced enteropathogenesis. Infect Immun. 2001;69(5):3021-30.
102. Cunningham AF, Khan M, Ball J, Toellner KM, Serre K, Mohr E, et al. Responses to the soluble flagellar protein FliC are Th2, while those to FliC on Salmonella are Th1. Eur J Immunol. 2004;34(11):2986-95.
103. Bobat S, Flores-Langarica A, Hitchcock J, Marshall JL, Kingsley RA, Goodall M, et al. Soluble flagellin, FliC, induces an Ag-specific Th2 response, yet promotes T-bet-regulated Th1 clearance of Salmonella typhimurium infection. Eur J Immunol. 2011;41(6):1606-18.
104. Flores-Langarica A, Bobat S, Marshall JL, Yam-Puc JC, Cook CN, Serre K, et al. Soluble flagellin coimmunization attenuates Th1 priming to Salmonella and clearance by modulating dendritic cell activation and cytokine production. Eur J Immunol. 2015;45(8):2299-311.
105. Flores-Langarica A, Marshall JL, Hitchcock J, Cook C, Jobanputra J, Bobat S, et al. Systemic flagellin immunization stimulates mucosal CD103+ dendritic cells and drives Foxp3+ regulatory T cell and IgA responses in the mesenteric lymph node. J Immunol. 2012;189(12):5745-54.
106. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature. 2001;410(6832):1099-103.
107. Delcour AH. Outer membrane permeability and antibiotic resistance. Biochim Biophys Acta. 2009;1794(5):808-16.

108. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev. 2003;67(4):593-656.
109. Galdiero S, Falanga A, Cantisani M, Tarallo R, Della Pepa ME, D'Oriano V, et al. Microbe-host interactions: structure and role of Gram-negative bacterial porins. Curr Protein Pept Sci. 2012;13(8):843-54.
110. Forst D, Welte W, Wacker T, Diederichs K. Structure of the sucrose-specific porin ScrY from Salmonella typhimurium and its complex with sucrose. Nat Struct Biol. 1998;5(1):37-46.
111. Santiviago CA, Toro CS, Bucarey SA, Mora GC. A chromosomal region surrounding the ompD porin gene marks a genetic difference between Salmonella typhi and the majority of Salmonella serovars. Microbiology. 2001;147(Pt 7):1897-907.
112. Pfeifer CG, Marcus SL, Steele-Mortimer O, Knodler LA, Finlay BB. Salmonella typhimurium virulence genes are induced upon bacterial invasion into phagocytic and nonphagocytic cells. Infect Immun. 1999;67(11):5690-8.
113. Lahiri A, Lahiri A, Iyer N, Das P, Chakravortty D. Visiting the cell biology of Salmonella infection. Microbes Infect. 2010;12(11):809-18.
114. Monack DM, Mueller A, Falkow S. Persistent bacterial infections: the interface of the pathogen and the host immune system. Nat Rev Microbiol. 2004;2(9):747-65.
115. Mastroeni P, Bryant C. Cytokines in Salmonellosis. EcoSal Plus. 2004;1(1).
116. Talbot S, Tötemeyer S, Yamamoto M, Akira S, Hughes K, Gray D, et al. Toll-like receptor 4 signalling through MyD88 is essential to control Salmonella enterica serovar Typhimurium infection, but not for the initiation of bacterial clearance. Immunology. 2009;128(4):472-83.
117. Royle MC, Totemeyer S, Alldridge LC, Maskell DJ, Bryant CE. Stimulation of Toll-like receptor 4 by lipopolysaccharide during cellular invasion by live Salmonella typhimurium is a critical but not exclusive event leading to macrophage responses. J Immunol. 2003;170(11):5445-54.
118. Arpaia N, Godec J, Lau L, Sivick KE, McLaughlin LM, Jones MB, et al. TLR signaling is required for virulence of an intracellular pathogen. Cell. 2011;144(5):675-88.
119. Marcus SL, Brumell JH, Pfeifer CG, Finlay BB. Salmonella pathogenicity islands: big virulence in small packages. Microbes Infect. 2000;2(2):145-56.
120. Carden SE, Walker GT, Honeycutt J, Lugo K, Pham T, Jacobson A, et al. Pseudogenization of the Secreted Effector Gene sseI Confers Rapid Systemic Dissemination of S. Typhimurium ST313 within Migratory Dendritic Cells. Cell Host Microbe. 2017;21(2):182-94.
121. Kaur J, Jain SK. Role of antigens and virulence factors of Salmonella enterica serovar Typhi in its pathogenesis. Microbiological Research. 2012;167(4):199-210.
122. Kirby AC, Yrlid U, Wick MJ. The innate immune response differs in primary and secondary Salmonella infection. J Immunol. 2002;169(8):4450-9.
123. Mastroeni P. Immunity to systemic Salmonella infections. Curr Mol Med. 2002;2(4):393-406.
124. Kagaya K, Watanabe K, Fukazawa Y. Capacity of recombinant gamma interferon to activate macrophages for Salmonella-killing activity. Infect Immun. 1989;57(2):609-15.

125. Ravindran R, McSorley SJ. Tracking the dynamics of T-cell activation in response to Salmonella infection. Immunology. 2005;114(4):450-8.
126. Ravindran R, Foley J, Stoklasek T, Glimcher LH, McSorley SJ. Expression of T-bet by CD4 T cells is essential for resistance to Salmonella infection. J Immunol. 2005;175(7):4603-10.
127. Hughes EA, Galan JE. Immune response to Salmonella: location, location, location? Immunity. 2002;16(3):325-8.
128. Nairz M, Fritsche G, Brunner P, Talasz H, Hantke K, Weiss G. Interferon-γ limits the availability of iron for intramacrophage Salmonella typhimurium. European Journal of Immunology. 2008;38(7):1923-36.
129. Henard CA, Vazquez-Torres A. Nitric oxide and salmonella pathogenesis. Front Microbiol. 2011;2:84.
130. Mastroeni P, Grant A, Restif O, Maskell D. A dynamic view of the spread and intracellular distribution of Salmonella enterica. Nat Rev Microbiol. 2009;7(1):73-80.
131. Behnsen J, Perez-Lopez A, Nuccio SP, Raffatellu M. Exploiting host immunity: the Salmonella paradigm. Trends Immunol. 2015;36(2):112-20.
132. Muotiala A, Makela PH. Role of gamma interferon in late stages of murine salmonellosis. Infect Immun. 1993;61(10):4248-53.
133. Cleary AM, Tu W, Enright A, Giffon T, Dewaal-Malefyt R, Gutierrez K, et al. Impaired accumulation and function of memory CD4 T cells in human IL-12 receptor beta 1 deficiency. J Immunol. 2003;170(1):597-603.
134. Jouanguy E, Doffinger R, Dupuis S, Pallier A, Altare F, Casanova JL. IL-12 and IFN-gamma in host defense against mycobacteria and salmonella in mice and men. Curr Opin Immunol. 1999;11(3):346-51.
135. Hess J, Ladel C, Miko D, Kaufmann SH. Salmonella typhimurium aroA- infection in gene-targeted immunodeficient mice: major role of CD4+ TCR-alpha beta cells and IFN-gamma in bacterial clearance independent of intracellular location. J Immunol. 1996;156(9):3321-6.
136. Mastroeni P, Simmons C, Fowler R, Hormaeche CE, Dougan G. Igh-6(-/-) (B-cell-deficient) mice fail to mount solid acquired resistance to oral challenge with virulent Salmonella enterica serovar typhimurium and show impaired Th1 T-cell responses to Salmonella antigens. Infect Immun. 2000;68(1):46-53.
137. McSorley SJ, Jenkins MK. Antibody is required for protection against virulent but not attenuated Salmonella enterica serovar typhimurium. Infect Immun. 2000;68(6):3344-8.
138. Nanton MR, Way SS, Shlomchik MJ, McSorley SJ. Cutting edge: B cells are essential for protective immunity against Salmonella independent of antibody secretion. The Journal of Immunology. 2012;189(12):5503-7.
139. Barr TA, Brown S, Mastroeni P, Gray D. TLR and B cell receptor signals to B cells differentially program primary and memory Th1 responses to Salmonella enterica. J Immunol. 2010;185(5):2783-9.
140. Nanton MR, Way SS, Shlomchik MJ, McSorley SJ. Cutting edge: B cells are essential for protective immunity against Salmonella independent of antibody secretion. J Immunol. 2012;189(12):5503-7.

141. Brown A, Hormaeche CE. The antibody response to salmonellae in mice and humans studied by immunoblots and ELISA. Microb Pathog. 1989;6(6):445-54.
142. Mittrucker HW, Raupach B, Kohler A, Kaufmann SH. Cutting edge: role of B lymphocytes in protective immunity against Salmonella typhimurium infection. J Immunol. 2000;164(4):1648-52.
143. Mastroeni P, Villarreal-Ramos B, Hormaeche CE. Adoptive transfer of immunity to oral challenge with virulent salmonellae in innately susceptible BALB/c mice requires both immune serum and T cells. Infect Immun. 1993;61(9):3981-4.
144. Smith KGC, Light A, Nossal GJV, Tarlinton DM. The extent of affinity maturation differs between the memory and antibody - forming cell compartments in the primary immune response1997 1997-06-01 00:00:00. 2996-3006 p.
145. Toellner KM, Gulbranson-Judge A, Taylor DR, Sze DM, MacLennan IC. Immunoglobulin switch transcript production in vivo related to the site and time of antigen-specific B cell activation. The Journal of Experimental Medicine. 1996;183(5):2303-12.
146. Nanton MR, Lee SJ, Atif SM, Nuccio SP, Taylor JJ, Baumler AJ, et al. Direct visualization of endogenous Salmonella-specific B cells reveals a marked delay in clonal expansion and germinal center development. Eur J Immunol. 2015;45(2):428-41.
147. Thomas SL, Griffiths C, Smeeth L, Rooney C, Hall AJ. Burden of mortality associated with autoimmune diseases among females in the United Kingdom. Am J Public Health. 2010;100(11):2279-87.
148. Walsh SJ, Rau LM. Autoimmune diseases: a leading cause of death among young and middle-aged women in the United States. Am J Public Health. 2000;90(9):1463-6.
149. Ngo ST, Steyn FJ, McCombe PA. Gender differences in autoimmune disease. Front Neuroendocrinol. 2014;35(3):347-69.
150. Pociot F, McDermott MF. Genetics of type 1 diabetes mellitus. Genes Immun. 2002;3(5):235-49.
151. Ebers GC, Bulman DE, Sadovnick AD, Paty DW, Warren S, Hader W, et al. A population-based study of multiple sclerosis in twins. N Engl J Med. 1986;315(26):1638-42.
152. Deapen D, Escalante A, Weinrib L, Horwitz D, Bachman B, Roy-Burman P, et al. A revised estimate of twin concordance in systemic lupus erythematosus. Arthritis Rheum. 1992;35(3):311-8.
153. Silman AJ, MacGregor AJ, Thomson W, Holligan S, Carthy D, Farhan A, et al. Twin concordance rates for rheumatoid arthritis: results from a nationwide study. Br J Rheumatol. 1993;32(10):903-7.
154. Rook GA. Hygiene hypothesis and autoimmune diseases. Clin Rev Allergy Immunol. 2012;42(1):5-15.
155. Colafrancesco S, Agmon-Levin N, Perricone C, Shoenfeld Y. Unraveling the soul of autoimmune diseases: pathogenesis, diagnosis and treatment adding dowels to the puzzle. Immunol Res. 2013;56(2-3):200-5.
156. Marrie RA. Environmental risk factors in multiple sclerosis aetiology. Lancet Neurol. 2004;3(12):709-18.

157. Cooper GS, Stroehla BC. The epidemiology of autoimmune diseases. Autoimmun Rev. 2003;2(3):119-25.
158. Karvonen M, Viik-Kajander M, Moltchanova E, Libman I, LaPorte R, Tuomilehto J. Incidence of childhood type 1 diabetes worldwide. Diabetes Mondiale (DiaMond) Project Group. Diabetes Care. 2000;23(10):1516-26.
159. Smallwood TB, Giacomin PR, Loukas A, Mulvenna JP, Clark RJ, Miles JJ. Helminth Immunomodulation in Autoimmune Disease. Front Immunol. 2017;8:453.
160. Sfriso P, Ghirardello A, Botsios C, Tonon M, Zen M, Bassi N, et al. Infections and autoimmunity: the multifaceted relationship. J Leukoc Biol. 2010;87(3):385-95.
161. Wucherpfennig KW. Mechanisms for the induction of autoimmunity by infectious agents. J Clin Invest. 2001;108(8):1097-104.
162. Packman CH. The Clinical Pictures of Autoimmune Hemolytic Anemia. Transfus Med Hemother. 2015;42(5):317-24.
163. Janney FA, Lee LT, Howe C. Cold hemagglutinin cross-reactivity with Mycoplasma pneumoniae. Infect Immun. 1978;22(1):29-33.
164. Croker BA, Lawson BR, Rutschmann S, Berger M, Eidenschenk C, Blasius AL, et al. Inflammation and autoimmunity caused by a SHP1 mutation depend on IL-1, MyD88, and a microbial trigger. Proc Natl Acad Sci U S A. 2008;105(39):15028-33.
165. Santos RL, Zhang S, Tsolis RM, Kingsley RA, Adams LG, Baumler AJ. Animal models of Salmonella infections: enteritis versus typhoid fever. Microbes Infect. 2001;3(14-15):1335-44.
166. Hormaeche CE, Mastroeni P, Arena A, Uddin J, Joysey HS. T cells do not mediate the initial suppression of a Salmonella infection in the RES. Immunology. 1990;70(2):247-50.
167. Plant J, Glynn AA. Genetics of resistance to infection with Salmonella typhimurium in mice. J Infect Dis. 1976;133(1):72-8.
168. Vidal SM, Pinner E, Lepage P, Gauthier S, Gros P. Natural resistance to intracellular infections: Nramp1 encodes a membrane phosphoglycoprotein absent in macrophages from susceptible (Nramp1 D169) mouse strains. J Immunol. 1996;157(8):3559-68.
169. Hoiseth SK, Stocker BAD. Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines. Nature. 1981;291(5812):238-9.
170. Rawlings DJ, Schwartz MA, Jackson SW, Meyer-Bahlburg A. Integration of B cell responses through Toll-like receptors and antigen receptors. Nat Rev Immunol. 2012;12(4):282-94.
171. Nemazee D, Gavin A, Hoebe K, Beutler B. Immunology: Toll-like receptors and antibody responses. Nature. 2006;441(7091):E4; discussion E.
172. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol. 2004;5(10):987-95.
173. Liu Y, Yin H, Zhao M, Lu Q. TLR2 and TLR4 in autoimmune diseases: a comprehensive review. Clin Rev Allergy Immunol. 2014;47(2):136-47.
174. Cervantes-Barragan L, Gil-Cruz C, Pastelin-Palacios R, Lang KS, Isibasi A, Ludewig B, et al. TLR2 and TLR4 signaling shapes specific antibody responses to Salmonella typhi antigens. Eur J Immunol. 2009;39(1):126-35.

175. Moreno-Eutimio MA, Tenorio-Calvo A, Pastelin-Palacios R, Perez-Shibayama C, Gil-Cruz C, Lopez-Santiago R, et al. Salmonella Typhi OmpS1 and OmpS2 porins are potent protective immunogens with adjuvant properties. Immunology. 2013;139(4):459-71.
176. Garin A, Meyer-Hermann M, Contie M, Figge MT, Buatois V, Gunzer M, et al. Toll-like receptor 4 signaling by follicular dendritic cells is pivotal for germinal center onset and affinity maturation. Immunity. 2010;33(1):84-95.
177. Hwang I-Y, Park C, Harrison K, Kehrl JH. TLR4 signaling augments B lymphocyte migration and overcomes the restriction that limits access to germinal center dark zones. The Journal of Experimental Medicine. 2009;206(12):2641-57.
178. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, et al. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol. 1999;162(7):3749-52.
179. Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A, Stewart TA. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. Science. 1993;259(5102):1739-42.
180. Szabo SJ, Sullivan BM, Stemmann C, Satoskar AR, Sleckman BP, Glimcher LH. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. Science. 2002;295(5553):338-42.
181. Kopf M, Baumann H, Freer G, Freudenberg M, Lamers M, Kishimoto T, et al. Impaired immune and acute-phase responses in interleukin-6-deficient mice. Nature. 1994;368(6469):339-42.
182. Peschon JJ, Torrance DS, Stocking KL, Glaccum MB, Otten C, Willis CR, et al. TNF receptor-deficient mice reveal divergent roles for p55 and p75 in several models of inflammation. J Immunol. 1998;160(2):943-52.
183. Mak TW, Shahinian A, Yoshinaga SK, Wakeham A, Boucher LM, Pintilie M, et al. Costimulation through the inducible costimulator ligand is essential for both T helper and B cell functions in T cell-dependent B cell responses. Nat Immunol. 2003;4(8):765-72.
184. Borriello F, Sethna MP, Boyd SD, Schweitzer AN, Tivol EA, Jacoby D, et al. B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. Immunity. 1997;6(3):303-13.
185. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. Cell. 1992;68(5):869-77.
186. Coughlan RE. Regulation of B cell responses during *Salmonella* Typhimurium infection. University of Birmingham: University of Birmingham; 2012.
187. Cunningham AF, Serre K, Mohr E, Khan M, Toellner KM. Loss of CD154 impairs the Th2 extrafollicular plasma cell response but not early T cell proliferation and interleukin-4 induction. Immunology. 2004;113(2):187-93.
188. Cunningham AF, Fallon PG, Khan M, Vacheron S, Acha-Orbea H, MacLennan IC, et al. Th2 activities induced during virgin T cell priming in the absence of IL-4, IL-13, and B cells. J Immunol. 2002;169(6):2900-6.
189. Salazar-Gonzalez RM, Maldonado-Bernal C, Ramirez-Cruz NE, Rios-Sarabia N, Beltran-Nava J, Castanon-Gonzalez J, et al. Induction of cellular immune response and anti-Salmonella enterica serovar typhi bactericidal antibodies in healthy volunteers by

immunization with a vaccine candidate against typhoid fever. Immunol Lett. 2004;93(2-3):115-22.
190. Vazquez-Torres A, Vallance BA, Bergman MA, Finlay BB, Cookson BT, Jones-Carson J, et al. Toll-like receptor 4 dependence of innate and adaptive immunity to Salmonella: importance of the Kupffer cell network. J Immunol. 2004;172(10):6202-8.
191. Wieland CW, Florquin S, Maris NA, Hoebe K, Beutler B, Takeda K, et al. The MyD88-dependent, but not the MyD88-independent, pathway of TLR4 signaling is important in clearing nontypeable haemophilus influenzae from the mouse lung. J Immunol. 2005;175(9):6042-9.
192. Wang X, Moser C, Louboutin JP, Lysenko ES, Weiner DJ, Weiser JN, et al. Toll-like receptor 4 mediates innate immune responses to Haemophilus influenzae infection in mouse lung. J Immunol. 2002;168(2):810-5.
193. Albiger B, Dahlberg S, Henriques-Normark B, Normark S. Role of the innate immune system in host defence against bacterial infections: focus on the Toll-like receptors. J Intern Med. 2007;261(6):511-28.
194. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science. 1998;282(5396):2085-8.
195. O'Brien AD, Rosenstreich DL, Scher I, Campbell GH, MacDermott RP, Formal SB. Genetic control of susceptibility to Salmonella typhimurium in mice: role of the LPS gene. J Immunol. 1980;124(1):20-4.
196. Woods JP, Frelinger JA, Warrack G, Cannon JG. Mouse genetic locus Lps influences susceptibility to Neisseria meningitidis infection. Infect Immun. 1988;56(8):1950-5.
197. Molteni M, Gemma S, Rossetti C. The Role of Toll-Like Receptor 4 in Infectious and Noninfectious Inflammation. Mediators Inflamm. 2016;2016:6978936.
198. Weiss DS, Raupach B, Takeda K, Akira S, Zychlinsky A. Toll-like receptors are temporally involved in host defense. J Immunol. 2004;172(7):4463-9.
199. Feuillet V, Medjane S, Mondor I, Demaria O, Pagni PP, Galan JE, et al. Involvement of Toll-like receptor 5 in the recognition of flagellated bacteria. Proc Natl Acad Sci U S A. 2006;103(33):12487-92.
200. Arpaia N, Godec J, Lau L, Sivick KE, McLaughlin LM, Jones MB, et al. TLR signaling is required for Salmonella typhimurium virulence. Cell. 2011;144(5):675-88.
201. Bhuvanendran S, Hussin HM, Meran LP, Anthony AA, Zhang L, Burch LH, et al. Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms and typhoid susceptibility in Asian Malay population in Malaysia. Microbes Infect. 2011;13(10):844-51.
202. Nguyen TH, Mai NL, Le TP, Ha V, Nguyen TC, Tran TH, et al. Toll-like receptor 4 (TLR4) and typhoid fever in Vietnam. PLoS One. 2009;4(3):e4800.
203. Mastroeni P, Menager N. Development of acquired immunity to Salmonella. J Med Microbiol. 2003;52(Pt 6):453-9.
204. McAleer JP, Vella AT. Understanding how lipopolysaccharide impacts CD4 T-cell immunity. Crit Rev Immunol. 2008;28(4):281-99.
205. O'Donnell H, Pham OH, Li LX, Atif SM, Lee SJ, Ravesloot MM, et al. Toll-like receptor and inflammasome signals converge to amplify the innate bactericidal capacity of T helper 1 cells. Immunity. 2014;40(2):213-24.

206. Seibert SA, Mex P, Köhler A, Kaufmann SHE, Mittrücker H-W. TLR2-, TLR4- and Myd88-independent acquired humoral and cellular immunity against Salmonella enterica serovar Typhimurium. Immunology Letters. 2010;127(2):126-34.
207. Neves P, Lampropoulou V, Calderon-Gomez E, Roch T, Stervbo U, Shen P, et al. Signaling via the MyD88 Adaptor Protein in B Cells Suppresses Protective Immunity during Salmonella typhimurium Infection. Immunity. 2010;33(5):777-90.
208. Guay HM, Andreyeva TA, Garcea RL, Welsh RM, Szomolanyi-Tsuda E. MyD88 is required for the formation of long-term humoral immunity to virus infection. J Immunol. 2007;178(8):5124-31.
209. Barr TA, Brown S, Mastroeni P, Gray D. B cell intrinsic MyD88 signals drive IFN-gamma production from T cells and control switching to IgG2c. J Immunol. 2009;183(2):1005-12.
210. Issac JM, Sarawathiamma D, Al-Ketbi MI, Azimullah S, Al-Ojali SM, Mohamed YA, et al. Differential outcome of infection with attenuated Salmonella in MyD88-deficient mice is dependent on the route of administration. Immunobiology. 2013;218(1):52-63.
211. Raetz CR. Biochemistry of endotoxins. Annu Rev Biochem. 1990;59:129-70.
212. Renshaw BR, Fanslow WC, 3rd, Armitage RJ, Campbell KA, Liggitt D, Wright B, et al. Humoral immune responses in CD40 ligand-deficient mice. J Exp Med. 1994;180(5):1889-900.
213. M.Gaspal F, Withers D, Saini M, Bekiaris V, McConnell FM, White A, et al. Abrogation of CD30 and OX40 signals prevents autoimmune disease in FoxP3-deficient mice. The Journal of Experimental Medicine. 2011;208(8):1579-84.
214. Di Niro R, Lee SJ, Vander Heiden JA, Elsner RA, Trivedi N, Bannock JM, et al. Salmonella Infection Drives Promiscuous B Cell Activation Followed by Extrafollicular Affinity Maturation. Immunity. 2015;43(1):120-31.
215. Hsieh CS, Macatonia SE, O'Garra A, Murphy KM. T cell genetic background determines default T helper phenotype development in vitro. J Exp Med. 1995;181(2):713-21.
216. Sellers RS, Clifford CB, Treuting PM, Brayton C. Immunological variation between inbred laboratory mouse strains: points to consider in phenotyping genetically immunomodified mice. Vet Pathol. 2012;49(1):32-43.
217. Ryg-Cornejo V, Ioannidis LJ, Ly A, Chiu CY, Tellier J, Hill DL, et al. Severe Malaria Infections Impair Germinal Center Responses by Inhibiting T Follicular Helper Cell Differentiation. Cell Rep. 2016;14(1):68-81.
218. Broz P, Ohlson MB, Monack DM. Innate immune response to Salmonella typhimurium, a model enteric pathogen. Gut Microbes. 2012;3(2):62-70.
219. Kupz A, Scott TA, Belz GT, Andrews DM, Greyer M, Lew AM, et al. Contribution of Thy1+ NK cells to protective IFN-gamma production during Salmonella typhimurium infections. Proc Natl Acad Sci U S A. 2013;110(6):2252-7.
220. Jackson A, Nanton MR, O'Donnell H, Akue AD, McSorley SJ. Innate Immune Activation during Salmonella Infection Initiates Extramedullary Erythropoiesis and Splenomegaly. J Immunol. 2010;185(10):6198-204.

221. Li LX, Benoun JM, Weiskopf K, Garcia KC, McSorley SJ. Salmonella Infection Enhances Erythropoietin Production by the Kidney and Liver, Which Correlates with Elevated Bacterial Burdens. Infect Immun. 2016;84(10):2833-41.
222. Ross EA, Flores-Langarica A, Bobat S, Coughlan RE, Marshall JL, Hitchcock JR, et al. Resolving Salmonella infection reveals dynamic and persisting changes in murine bone marrow progenitor cell phenotype and function. Eur J Immunol. 2014;44(8):2318-30.
223. Bobat S, University of B. Characterising the immune response to Salmonella and Salmonella surface antigens during a systemic infection2011.
224. Lee SJ, O'Donnell H, McSorley SJ. B7-H1 (programmed cell death ligand 1) is required for the development of multifunctional Th1 cells and immunity to primary, but not secondary, Salmonella infection. J Immunol. 2010;185(4):2442-9.
225. Genestier L, Taillardet M, Mondiere P, Gheit H, Bella C, Defrance T. TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses. J Immunol. 2007;178(12):7779-86.
226. Barnett BE, Staupe RP, Odorizzi PM, Palko O, Tomov VT, Mahan AE, et al. Cutting Edge: B Cell-Intrinsic T-bet Expression Is Required To Control Chronic Viral Infection. J Immunol. 2016;197(4):1017-22.
227. Rubtsov AV, Marrack P, Rubtsova K. T-bet expressing B cells - Novel target for autoimmune therapies? Cell Immunol. 2017.
228. Lee SK, Rigby RJ, Zotos D, Tsai LM, Kawamoto S, Marshall JL, et al. B cell priming for extrafollicular antibody responses requires Bcl-6 expression by T cells. J Exp Med. 2011;208(7):1377-88.
229. St John AL, Abraham SN. Salmonella disrupts lymph node architecture by TLR4-mediated suppression of homeostatic chemokines. Nat Med. 2009;15(11):1259-65.
230. Zeng M, Nourishirazi E, Guinet E, Nouri-Shirazi M. The genetic background influences the cellular and humoral immune responses to vaccines. Clin Exp Immunol. 2016;186(2):190-204.
231. Weisel F, Wellmann U, Winkler TH. Autoreactive B cells get activated in extrafollicular sites. Eur J Immunol. 2007;37(12):3330-3.
232. Ercolini AM, Miller SD. The role of infections in autoimmune disease. Clin Exp Immunol. 2009;155(1):1-15.
233. Fujinami RS, Oldstone MB. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. Science. 1985;230(4729):1043-5.
234. Cusick MF, Libbey JE, Fujinami RS. Molecular mimicry as a mechanism of autoimmune disease. Clin Rev Allergy Immunol. 2012;42(1):102-11.
235. Vanderlugt CL, Miller SD. Epitope spreading in immune-mediated diseases: implications for immunotherapy. Nat Rev Immunol. 2002;2(2):85-95.
236. Cunningham MW, McCormack JM, Talaber LR, Harley JB, Ayoub EM, Muneer RS, et al. Human monoclonal antibodies reactive with antigens of the group A Streptococcus and human heart. J Immunol. 1988;141(8):2760-6.
237. Yacoub M, Mayosi B, ElGuindy A, Carpentier A, Yusuf S. Eliminating acute rheumatic fever and rheumatic heart disease. Lancet. 2017;390(10091):212-3.

238. Marijon E, Mirabel M, Celermajer DS, Jouven X. Rheumatic heart disease. Lancet. 2012;379(9819):953-64.
239. Steelman AJ. Infection as an Environmental Trigger of Multiple Sclerosis Disease Exacerbation. Front Immunol. 2015;6:520.
240. Principi N, Berioli MG, Bianchini S, Esposito S. Type 1 diabetes and viral infections: What is the relationship? J Clin Virol. 2017;96:26-31.
241. Coppieters KT, Boettler T, von Herrath M. Virus infections in type 1 diabetes. Cold Spring Harb Perspect Med. 2012;2(1):a007682.
242. Browning JL. B cells move to centre stage: novel opportunities for autoimmune disease treatment. Nat Rev Drug Discov. 2006;5(7):564-76.
243. Domeier PP, Chodisetti SB, Soni C, Schell SL, Elias MJ, Wong EB, et al. IFN-gamma receptor and STAT1 signaling in B cells are central to spontaneous germinal center formation and autoimmunity. J Exp Med. 2016;213(5):715-32.
244. Lee SK, Silva DG, Martin JL, Pratama A, Hu X, Chang PP, et al. Interferon-gamma excess leads to pathogenic accumulation of follicular helper T cells and germinal centers. Immunity. 2012;37(5):880-92.
245. Rubtsova K, Marrack P, Rubtsov AV. TLR7, IFNgamma, and T-bet: their roles in the development of ABCs in female-biased autoimmunity. Cell Immunol. 2015;294(2):80-3.
246. Rubtsova K, Rubtsov AV, Thurman JM, Mennona JM, Kappler JW, Marrack P. B cells expressing the transcription factor T-bet drive lupus-like autoimmunity. J Clin Invest. 2017;127(4):1392-404.
247. Gurcan HM, Keskin DB, Stern JN, Nitzberg MA, Shekhani H, Ahmed AR. A review of the current use of rituximab in autoimmune diseases. Int Immunopharmacol. 2009;9(1):10-25.
248. Tracy KE, Baumgarth N. Borrelia burgdorferi Manipulates Innate and Adaptive Immunity to Establish Persistence in Rodent Reservoir Hosts. Front Immunol. 2017;8:116.
249. Tunev SS, Hastey CJ, Hodzic E, Feng S, Barthold SW, Baumgarth N. Lymphoadenopathy during lyme borreliosis is caused by spirochete migration-induced specific B cell activation. PLoS Pathog. 2011;7(5):e1002066.
250. Hastey CJ, Elsner RA, Barthold SW, Baumgarth N. Delays and diversions mark the development of B cell responses to Borrelia burgdorferi infection. J Immunol. 2012;188(11):5612-22.
251. Singh SK, Girschick HJ. Lyme borreliosis: from infection to autoimmunity. Clin Microbiol Infect. 2004;10(7):598-614.
252. Poholek AC, Hansen K, Hernandez SG, Eto D, Chandele A, Weinstein JS, et al. In vivo regulation of Bcl6 and T follicular helper cell development. J Immunol. 2010;185(1):313-26.
253. Odegard JM, Marks BR, DiPlacido LD, Poholek AC, Kono DH, Dong C, et al. ICOS-dependent extrafollicular helper T cells elicit IgG production via IL-21 in systemic autoimmunity. J Exp Med. 2008;205(12):2873-86.
254. Campbell AW. Autoimmunity and the gut. Autoimmune Dis. 2014;2014:152428.
255. Nikkari S, Rantakokko K, Ekman P, Mottonen T, Leirisalo-Repo M, Virtala M, et al. Salmonella-triggered reactive arthritis: use of polymerase chain reaction, immunocytochemical staining, and gas chromatography-mass spectrometry in the detection of bacterial components from synovial fluid. Arthritis Rheum. 1999;42(1):84-9.

256. von Hirschhausen R, Rohrs E, Clemens M. [Autoimmune hemolytic anemia following recurrent infections with Salmonella typhimurium and E. coli]. Internist (Berl). 1990;31(12):773-5.
257. Kobayashi K, Hamaki T, Ohwada A, Tomiyama J, Sakuma R, Mizuta Y, et al. [Low-titer cold agglutinin disease following Salmonella gastroenteritis]. Rinsho Ketsueki. 2011;52(1):32-6.
258. Pourhassan A, Shoja MM, Tubbs RS, Sadeghilar A, Kerr D. Acute transverse myelitis secondary to Salmonella paratyphi B infection. Infection. 2008;36(2):170-3.
259. Khan FY, Kamha AA, Abbas MT, Miyares F, Elshafie SS. Guillain-Barre syndrome associated with Salmonella paratyphi A. Clin Neurol Neurosurg. 2007;109(5):452-4.
260. Tripathi MK, Pratap CB, Dixit VK, Singh TB, Shukla SK, Jain AK, et al. Ulcerative Colitis and Its Association with Salmonella Species. Interdiscip Perspect Infect Dis. 2016;2016:5854285.
261. Ko HJ, Yang H, Yang JY, Seo SU, Chang SY, Seong JK, et al. Expansion of Tfh-like cells during chronic Salmonella exposure mediates the generation of autoimmune hypergammaglobulinemia in MyD88-deficient mice. Eur J Immunol. 2012;42(3):618-28.
262. Zaccone P, Raine T, Sidobre S, Kronenberg M, Mastroeni P, Cooke A. Salmonella typhimurium infection halts development of type 1 diabetes in NOD mice. Eur J Immunol. 2004;34(11):3246-56.
263. Newland SA, Phillips JM, Mastroeni P, Azuma M, Zaccone P, Cooke A. PD-L1 blockade overrides Salmonella typhimurium-mediated diabetes prevention in NOD mice: no role for Tregs. Eur J Immunol. 2011;41(10):2966-76.
264. Wu W, Shi Y, Xia H, Chai Q, Jin C, Ren B, et al. Epithelial LTbetaR signaling controls the population size of the progenitors of medullary thymic epithelial cells in neonatal mice. Sci Rep. 2017;7:44481.
265. Zhu M, Fu Y. The complicated role of NF-kappaB in T-cell selection. Cell Mol Immunol. 2010;7(2):89-93.
266. Vinuesa CG, Cook MC, Angelucci C, Athanasopoulos V, Rui L, Hill KM, et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. Nature. 2005;435(7041):452-8.
267. Kupz A, Bedoui S, Strugnell RA. Cellular requirements for systemic control of Salmonella enterica serovar Typhimurium infections in mice. Infect Immun. 2014;82(12):4997-5004.
268. Peng SL, Li J, Lin L, Gerth A. The role of T-bet in B cells. Nat Immunol. 2003;4(11):1041-.
269. Pollard KM, Cauvi DM, Toomey CB, Morris KV, Kono DH. Interferon-gamma and systemic autoimmunity. Discov Med. 2013;16(87):123-31.
270. Fournie GJ, Lambert PH, Meischer PA. Release of DNA in circulating blood and induction of anti-DNA antibodies after injection of bacterial lipopolysaccharides. J Exp Med. 1974;140(5):1189-206.
271. Irvine WJ, Davies SH, Teitelbaum S, Delamore IW, Williams AW. The clinical and pathological significance of gastric parietal cell antibody. Ann N Y Acad Sci. 1965;124(2):657-91.

272. Chanarin I, James D. Humoral and cell-mediated intrinsic-factor antibody in pernicious anaemia. Lancet. 1974;1(7866):1078-80.
273. Appelmelk BJ, Simoons-Smit I, Negrini R, Moran AP, Aspinall GO, Forte JG, et al. Potential role of molecular mimicry between Helicobacter pylori lipopolysaccharide and host Lewis blood group antigens in autoimmunity. Infect Immun. 1996;64(6):2031-40.
274. Amedei A, Bergman MP, Appelmelk BJ, Azzurri A, Benagiano M, Tamburini C, et al. Molecular mimicry between Helicobacter pylori antigens and H+, K+ --adenosine triphosphatase in human gastric autoimmunity. J Exp Med. 2003;198(8):1147-56.
275. Lunardi C, Bason C, Dolcino M, Navone R, Simone R, Saverino D, et al. Antiflagellin antibodies recognize the autoantigens Toll-Like Receptor 5 and Pals 1-associated tight junction protein and induce monocytes activation and increased intestinal permeability in Crohn's disease. J Intern Med. 2009;265(2):250-65.
276. Shoenfeld Y, Gershwin ME, Meroni P-L. Autoantibodies. 3rd edition / ed. Amsterdam: Elsevier; 2014. lx, 855 pages p.
277. Stemerowicz R, Hopf U, Moller B, Wittenbrink C, Rodloff A, Reinhardt R, et al. Are antimitochondrial antibodies in primary biliary cirrhosis induced by R(rough)-mutants of enterobacteriaceae? Lancet. 1988;2(8621):1166-70.
278. Nickowitz RE, Worman HJ. Autoantibodies from patients with primary biliary cirrhosis recognize a restricted region within the cytoplasmic tail of nuclear pore membrane glycoprotein Gp210. J Exp Med. 1993;178(6):2237-42.
279. Zeman MV, Hirschfield GM. Autoantibodies and liver disease: uses and abuses. Can J Gastroenterol. 2010;24(4):225-31.
280. Tursi SA, Lee EY, Medeiros NJ, Lee MH, Nicastro LK, Buttaro B, et al. Bacterial amyloid curli acts as a carrier for DNA to elicit an autoimmune response via TLR2 and TLR9. PLoS Pathog. 2017;13(4):e1006315.
281. Gallo PM, Rapsinski GJ, Wilson RP, Oppong GO, Sriram U, Goulian M, et al. Amyloid-DNA Composites of Bacterial Biofilms Stimulate Autoimmunity. Immunity. 2015;42(6):1171-84.
282. Vieira P, Rajewsky K. The half-lives of serum immunoglobulins in adult mice. Eur J Immunol. 1988;18(2):313-6.
283. Seery JP. IFN-gamma transgenic mice: clues to the pathogenesis of systemic lupus erythematosus? Arthritis Res. 2000;2(6):437-40.
284. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. Annu Rev Immunol. 2005;23:515-48.
285. Webb LMC, Linterman MA. Signals that drive T follicular helper cell formation. Immunology. 2017.
286. Wikenheiser DJ, Stumhofer JS. ICOS Co-Stimulation: Friend or Foe? Front Immunol. 2016;7:304.
287. Dong C, Juedes AE, Temann UA, Shresta S, Allison JP, Ruddle NH, et al. ICOS co-stimulatory receptor is essential for T-cell activation and function. Nature. 2001;409(6816):97-101.
288. Dong C, Temann UA, Flavell RA. Cutting edge: critical role of inducible costimulator in germinal center reactions. J Immunol. 2001;166(6):3659-62.

289. Wong SC, Oh E, Ng CH, Lam KP. Impaired germinal center formation and recall T-cell-dependent immune responses in mice lacking the costimulatory ligand B7-H2. Blood. 2003;102(4):1381-8.
290. Yu D, Tan AH, Hu X, Athanasopoulos V, Simpson N, Silva DG, et al. Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. Nature. 2007;450(7167):299-303.
291. Hunter CA, Jones SA. IL-6 as a keystone cytokine in health and disease. Nat Immunol. 2015;16(5):448-57.
292. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta. 2011;1813(5):878-88.
293. Hirano T, Taga T, Nakano N, Yasukawa K, Kashiwamura S, Shimizu K, et al. Purification to homogeneity and characterization of human B-cell differentiation factor (BCDF or BSFp-2). Proc Natl Acad Sci U S A. 1985;82(16):5490-4.
294. Suematsu S, Matsuda T, Aozasa K, Akira S, Nakano N, Ohno S, et al. IgG1 plasmacytosis in interleukin 6 transgenic mice. Proc Natl Acad Sci U S A. 1989;86(19):7547-51.
295. Fornek JL, Tygrett LT, Waldschmidt TJ, Poli V, Rickert RC, Kansas GS. Critical role for Stat3 in T-dependent terminal differentiation of IgG B cells. Blood. 2006;107(3):1085-91.
296. Dienz O, Eaton SM, Bond JP, Neveu W, Moquin D, Noubade R, et al. The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by CD4+ T cells. J Exp Med. 2009;206(1):69-78.
297. Linterman MA, Beaton L, Yu D, Ramiscal RR, Srivastava M, Hogan JJ, et al. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. J Exp Med. 2010;207(2):353-63.
298. Tanaka T, Narazaki M, Kishimoto T. Therapeutic targeting of the interleukin-6 receptor. Annu Rev Pharmacol Toxicol. 2012;52:199-219.
299. Kalliolias GD, Ivashkiv LB. TNF biology, pathogenic mechanisms and emerging therapeutic strategies. Nat Rev Rheumatol. 2016;12(1):49-62.
300. Bradley JR. TNF-mediated inflammatory disease. J Pathol. 2008;214(2):149-60.
301. Mastroeni P, Skepper JN, Hormaeche CE. Effect of anti-tumor necrosis factor alpha antibodies on histopathology of primary Salmonella infections. Infect Immun. 1995;63(9):3674-82.
302. Rathmell JC, Fournier S, Weintraub BC, Allison JP, Goodnow CC. Repression of B7.2 on self-reactive B cells is essential to prevent proliferation and allow Fas-mediated deletion by CD4(+) T cells. J Exp Med. 1998;188(4):651-9.
303. Grewal IS, Flavell RA. The role of CD40 ligand in costimulation and T-cell activation. Immunol Rev. 1996;153:85-106.
304. Elgueta R, Benson MJ, de Vries VC, Wasiuk A, Guo Y, Noelle RJ. Molecular mechanism and function of CD40/CD40L engagement in the immune system. Immunol Rev. 2009;229(1):152-72.
305. Vidric M, Bladt AT, Dianzani U, Watts TH. Role for inducible costimulator in control of Salmonella enterica serovar Typhimurium infection in mice. Infect Immun. 2006;74(2):1050-61.

306. Hirano T, Yasukawa K, Harada H, Taga T, Watanabe Y, Matsuda T, et al. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature. 1986;324(6092):73-6.
307. Zhou L, Ivanov, II, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol. 2007;8(9):967-74.
308. Mohr E, Serre K, Manz RA, Cunningham AF, Khan M, Hardie DL, et al. Dendritic cells and monocyte/macrophages that create the IL-6/APRIL-rich lymph node microenvironments where plasmablasts mature. J Immunol. 2009;182(4):2113-23.
309. Tan SM, Xu D, Roschke V, Perry JW, Arkfeld DG, Ehresmann GR, et al. Local production of B lymphocyte stimulator protein and APRIL in arthritic joints of patients with inflammatory arthritis. Arthritis Rheum. 2003;48(4):982-92.
310. Groom J, Kalled SL, Cutler AH, Olson C, Woodcock SA, Schneider P, et al. Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjogren's syndrome. J Clin Invest. 2002;109(1):59-68.
311. Mackay F, Woodcock SA, Lawton P, Ambrose C, Baetscher M, Schneider P, et al. Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. J Exp Med. 1999;190(11):1697-710.
312. Marino E, Walters SN, Villanueva JE, Richards JL, Mackay CR, Grey ST. BAFF regulates activation of self-reactive T cells through B-cell dependent mechanisms and mediates protection in NOD mice. Eur J Immunol. 2014;44(4):983-93.
313. Cheema GS, Roschke V, Hilbert DM, Stohl W. Elevated serum B lymphocyte stimulator levels in patients with systemic immune-based rheumatic diseases. Arthritis Rheum. 2001;44(6):1313-9.
314. Stohl W. B lymphocyte stimulator protein levels in systemic lupus erythematosus and other diseases. Curr Rheumatol Rep. 2002;4(4):345-50.
315. Arkatkar T, Du SW, Jacobs HM, Dam EM, Hou B, Buckner JH, et al. B cell-derived IL-6 initiates spontaneous germinal center formation during systemic autoimmunity. J Exp Med. 2017.
316. Vazquez-Torres A, Fantuzzi G, Edwards CK, 3rd, Dinarello CA, Fang FC. Defective localization of the NADPH phagocyte oxidase to Salmonella-containing phagosomes in tumor necrosis factor p55 receptor-deficient macrophages. Proc Natl Acad Sci U S A. 2001;98(5):2561-5.
317. den Haan JM, Mebius RE, Kraal G. Stromal cells of the mouse spleen. Front Immunol. 2012;3:201.
318. Ellyard JI, Avery DT, Mackay CR, Tangye SG. Contribution of stromal cells to the migration, function and retention of plasma cells in human spleen: potential roles of CXCL12, IL-6 and CD54. Eur J Immunol. 2005;35(3):699-708.
319. Li C, Langhorne J. Tumor necrosis factor alpha p55 receptor is important for development of memory responses to blood-stage malaria infection. Infect Immun. 2000;68(10):5724-30.
320. Takashima K, Tateda K, Matsumoto T, Iizawa Y, Nakao M, Yamaguchi K. Role of tumor necrosis factor alpha in pathogenesis of pneumococcal pneumonia in mice. Infect Immun. 1997;65(1):257-60.

321. Naessens J, Kitani H, Momotani E, Sekikawa K, Nthale JM, Iraqi F. Susceptibility of TNF-alpha-deficient mice to Trypanosoma congolense is not due to a defective antibody response. Acta Trop. 2004;92(3):193-203.
322. Randall TD, Carragher DM, Rangel-Moreno J. Development of secondary lymphoid organs. Annu Rev Immunol. 2008;26:627-50.
323. Pasparakis M, Alexopoulou L, Episkopou V, Kollias G. Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. J Exp Med. 1996;184(4):1397-411.
324. Pasparakis M, Alexopoulou L, Grell M, Pfizenmaier K, Bluethmann H, Kollias G. Peyer's patch organogenesis is intact yet formation of B lymphocyte follicles is defective in peripheral lymphoid organs of mice deficient for tumor necrosis factor and its 55-kDa receptor. Proc Natl Acad Sci U S A. 1997;94(12):6319-23.
325. Everest P, Roberts M, Dougan G. Susceptibility to Salmonella typhimurium infection and effectiveness of vaccination in mice deficient in the tumor necrosis factor alpha p55 receptor. Infect Immun. 1998;66(7):3355-64.
326. Noto Llana M, Sarnacki SH, Vazquez MV, Gartner AS, Giacomodonato MN, Cerquetti MC. Salmonella enterica induces joint inflammation and expression of interleukin-17 in draining lymph nodes early after onset of enterocolitis in mice. Infect Immun. 2012;80(6):2231-9.
327. Jacobson BA, Panka DJ, Nguyen KA, Erikson J, Abbas AK, Marshak-Rothstein A. Anatomy of autoantibody production: dominant localization of antibody-producing cells to T cell zones in Fas-deficient mice. Immunity. 1995;3(4):509-19.
328. Mittrucker HW, Kohler A, Mak TW, Kaufmann SH. Critical role of CD28 in protective immunity against Salmonella typhimurium. J Immunol. 1999;163(12):6769-76.
329. Dorner T, Jacobi AM, Lipsky PE. B cells in autoimmunity. Arthritis Res Ther. 2009;11(5):247.
330. O'Neill SK, Cao Y, Hamel KM, Doodes PD, Hutas G, Finnegan A. Expression of CD80/86 on B cells is essential for autoreactive T cell activation and the development of arthritis. J Immunol. 2007;179(8):5109-16.
331. Galdiero M, Pisciotta MG, Galdiero E, Carratelli CR. Porins and lipopolysaccharide from Salmonella typhimurium regulate the expression of CD80 and CD86 molecules on B cells and macrophages but not CD28 and CD152 on T cells. Clin Microbiol Infect. 2003;9(11):1104-11.
332. Rosales-Reyes R, Perez-Lopez A, Sanchez-Gomez C, Hernandez-Mote RR, Castro-Eguiluz D, Ortiz-Navarrete V, et al. Salmonella infects B cells by macropinocytosis and formation of spacious phagosomes but does not induce pyroptosis in favor of its survival. Microb Pathog. 2012;52(6):367-74.
333. Lee S, Nguyen MT. Recent advances of vaccine adjuvants for infectious diseases. Immune Netw. 2015;15(2):51-7.
334. Mohammad Hosseini A, Majidi J, Baradaran B, Yousefi M. Toll-Like Receptors in the Pathogenesis of Autoimmune Diseases. Adv Pharm Bull. 2015;5(Suppl 1):605-14.
335. Giltiay NV, Chappell CP, Clark EA. B-cell selection and the development of autoantibodies. Arthritis Res Ther. 2012;14 Suppl 4:S1.

336. Chiron D, Bekeredjian-Ding I, Pellat-Deceunynck C, Bataille R, Jego G. Toll-like receptors: lessons to learn from normal and malignant human B cells. Blood. 2008;112(6):2205-13.
337. Shlomchik MJ. Sites and stages of autoreactive B cell activation and regulation. Immunity. 2008;28(1):18-28.
338. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity. 2006;24(2):179-89.
339. Stockinger B, Veldhoen M. Differentiation and function of Th17 T cells. Curr Opin Immunol. 2007;19(3):281-6.
340. Ols ML, Cullen JL, Turqueti-Neves A, Giles J, Shlomchik MJ. Dendritic Cells Regulate Extrafollicular Autoreactive B Cells via T Cells Expressing Fas and Fas Ligand. Immunity. 2016;45(5):1052-65.