Investigation of how antibody to the Outer Membrane Porin D from *Salmonella* Typhimurium binds and protects against infection

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ABSTRACT

The induction of specific-antibody that targets the surface of a pathogen or its secreted components is the basis of immunological memory to natural infection and vaccination. This antibody, induced after natural infection or vaccination, saves millions of lives every year. The few vaccines against Gram-negative bacteria either target one antigen, the surface capsular polysaccharides, or are complex vaccines involving the whole organism or complex mixtures of multiple antigens. In this project, we studied how antibody binds to the *Salmonella* outer membrane porin D (STm-OmpD) on the bacterial surface and why it is protective. Immunisation with STm-OmpD provides serovar-specific protection because: 1) IgG can access a single epitope, which is under selective pressure; 2) IgG can access the bacterial surface in the “footprint” made by the OmpD trimer; and 3) Lipopolysaccharide (LPS) O-antigen (O-Ag) influences the access of IgG to epitopes. Further, protection after immunisation with STm-OmpD is detectable by 4 hours after infection and requires $GR1^+$ cells, IFNg, and Th1 responses for optimal protection, but not IgG2a/c. These data provide insights into how antibody to the Gram-negative bacterial surface can help protect against infection and will aid in the optimisation of subunit vaccines targeting *Salmonella.*

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Ab	Antibody
Abs	Antibodies
ABC	ATP-binding cassette
ACK	Ammonium chloride potassium
Ag	Antigen
Ags	Antigens
AID	Activation induced deaminase
AP	Alternative pathway
APC	Antigen presenting cell
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-Cyanine-7
aph	Kanamycin resistance
APRIL	A proliferation-inducing ligand
ASC	Antibody secreting cell
ATP	Adenosin triphosphate
BAFF	B cell activating factor
BCA	Bicinchonic acid assay
Bcl-6	B cell lymphoma-6
BCMA	B cell maturation antigen
BCR	B cell receptor
BLIMP-1	B lymphocyte induced maturation protein 1
BM	Bone marrow

bp	Base pair
Breg	B regulatory
BSA	Bovine serum albumin
C’	Complement
cat	Chloramphenicol resistance
CD	Cluster of differentiation
CFU	Colony forming unit
CP	Classical pathway
CSR	Class switching recombination
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribunocleic acid
DZ	Dark zone
dNTP	Deoxyribunocleotide triphosphate
DT	Diphteria toxoid
EDTA	Ethylenediaminetetraacetic acid
EF	Extrafollicular
e.g.	Exempli gratia (for example)
ELISA	Enzyme-linked immunoabsorbent assay
ELISPOT	Enzyme-linked immunospot
Fab	Fragment antigen binding
FACS	Fluorescence-activated cell sorting
Fc	Fragment crystallisable

FDCs	Follicular dendritic cells
Fig.	Figure
FITC	Fluorescein Isothyocyanate
Flp	Flippase
FO	Follicular
FoxP3	Factor forkhead box P3
FPLC	Fast protein liquid chromatography
FS	Forward scatter
Fwd	Forward
g	Gram
Gal	Galactose
GATA-3	GATA-binding protein 3
GC	Germinal centre
GDP	Guanosine diphosphate
Glc	Glucose
GlcNac	N-acetylglucosamine
GMMA	General Modules for Membrane Antigens
HO-1	Enzyme heme oxygenase-1
HDP	Host defense peptide
Hi	Heat-inactivated
HiHS	Heat-inactivated human serum/sera
HiFBS	Heat-inactivated foetal bovine serum
HIV	Human immunodeficiency virus
IBD	Inflammatory bowel disease

ICOS	Inducible T cell co-stimulator
i.e.	Id est (that is)
Ig	Immunoglobulin
IFNg	Interferon gamma
IL	Interleukin
IM	Inner membrane
IMSS	Instituto Mexicano del Seguro Social (Mexican Social Security Institute)
iNTS	Invasive nontyphoidal salmonellosis
i.p.	Intraperitoneal
kb	Kilobase
kDa	Kilodalton
KO	Knockout
L	Litre
LAL	Limulus amoebocyte lysate
LB	Luria-Bertani
LN	Lymph node
log	Logarithmic
LP	Lectin pathway
LPS	Lipopolysaccharide
LZ	Light zone
mAb	Monoclonal antibody
MAC	Membrane attack complex
MAMEF	Microwave-acceleratd metal enhanced fluorescence

MBL	Mannose-binding lectin
MHC	Major histocompatibility complex
µg	Microgram
µl	Microlitre
ml	Millilitre
MLN	Mesenteric lymph node
MyD88	Myeloid differentiation primary response gene 88
MZ	Marginal zone
NK	Natural killer
NHS	Normal human serum/sera
NI	Non-immunised
NLR	NOD-like receptor
Nramp-1	Natural resistance associated macrophage protein-1
nm	Nanometre
NTS	Nontyphoidal salmonellosis
O-Ag	O-antigen
OD	Optical density
OM	Outer membrane
Omp	Outer membrane porin
OMPs	Outer membrane proteins
PAMP	Pathogen associated molecular pattern
PE	Phycoerythrin
PE Cy	Phycoerythrin Cyanin
PerCP Cy5.5	Peridinin Chlorophyll Protein (Cyanin Dye)

PBS	Phosphate buffered saine
PCP	Polysaccharide co-polymerase
PCR	Polymerase chain reaction
PEC	Peritoneal cavity
PMN	Polymorphonuclear leukocyte
pNPP	*P*-nitrophenyl phosphate
PRR	Pattern recognition receptor
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute 1640 medium
Rvs	Reverse
SBA	Serum bactericidal assay
SCV	*Salmonella* containing vacuole
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEn	*Salmonella enterica* serovar Enteritidis
SEn-OMPs	OMPs derived from SEn
SFU	Spot forming unit
STm	*Salmonella enterica* serovar Typhimurium
STm-OmpD	OmpD derived from STm
STm-OMPs	OMPs derived from STm
STm-porins	Porins derived from STm
SPI	*Salmonella* pathogenicity island
SPF	Specific pathogen free

SS	Side scatter
T3SS	Type 3 secretion system
Tbet	T box factor expressed in T cells
TBM	Tingible body macrophage
TCR	T cell receptor
TD	T-dependent
Tfh	T follicular helper
TGF	Transforming growth factor
Th	T-helper
TI	T-independent
TRIF/TICAM1	TIR domain-containing adaptor inducing factor β
TIR	Toll-interleukin-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAM/TICAM2	TRIF-related adaptor molecule
Treg	T regulatory
TT	Tetanus toxoid
UCT	University of Cape Town
UDP	Uridine phosphate
UMP	Uridine monophosphate
Und-P	Undecaprenyl phosphate
ViCPS	Vi capsular polysaccharide
WT	Wild-type

CHAPTER 1: INTRODUCTION

1.1 *Salmonella* classification

Salmonella species are Gram-negative intracellular facultative organisms that are part of the family *Enterobacteriaceae*. The genus *Salmonella* is classified into 2 different species: *Salmonella bongori* and *Salmonella enterica*. *S. enterica* serovars infect humans, other mammals and birds whereas *S. bongori* serovars principally infect cold-blooded animals (1).

Salmonella enterica is subdivided into 6 distinct subspecies (*enterica, salamae, arizonae, diarizonae, houtenae, and indica*) and >2500 serovars have been identified based on their lipopolysaccharide (O) and flagellar (H) antigens (Ags). The majority of these serovars belong to the O-antigen (O-Ag) groups A, B, C1, C2, D and E. (1-3). These serovars can be classified further into those that cause typhoidal and nontyphoidal salmonellosis. *Salmonella* Typhi and *S.* Paratyphi A are exclusively restricted to humans (4-6). However, in specific conditions, high doses of *S.* Typhi can infect higher primates (7).

1.2 Composition of the cell wall of *Salmonella*

Salmonella, as in all other Gram-negative bacteria, has a cell wall that contains an inner membrane (IM), a peptidoglycan layer, and an outer membrane (OM)(Fig. 1.1). These two membranes are separated by the periplasm, a viscous layer that contains the peptidoglycan and a high concentration of proteins. The IM, or cytoplasmic

membrane, is a phospholipid bilayer where lipid biosynthesis, adenosine triphosphate (ATP) production, secretion and transport of proteins take place (8).

1.2.1 Structure of the outer membrane (OM) of *Salmonella*

The outer membrane proteins (OMPs), lipopolysaccharide (LPS), lipoproteins and phospholipids are all part of the OM of *Salmonella* (Fig. 1.1). This asymmetric bilayer acts as a selectively permeable barrier, restricting the access of compounds larger than 700 daltons to the periplasm and IM of the bacteria (9-11). The OM is divided into an inner leaflet, containing phospholipids, and an outer leaflet containing LPS. While OM lipoproteins can be tethered to the inner leaflet or surface exposed, phospholipids are restricted to the inner leaflet. As well as this, the OM contains enzymes, receptors, autotransporters, and β-barrel proteins, which includes porins (9). Antigens found in the OM share similar characteristics between typhoidal and nontyphoidal serovars (4). However, the Vi capsular polysaccharide, which is an important virulence factor in *S.* Typhi, is not expressed in all nontyphoidal serovars, although it is present in many serovars of clinical importance such as *S.* Dublin (4).

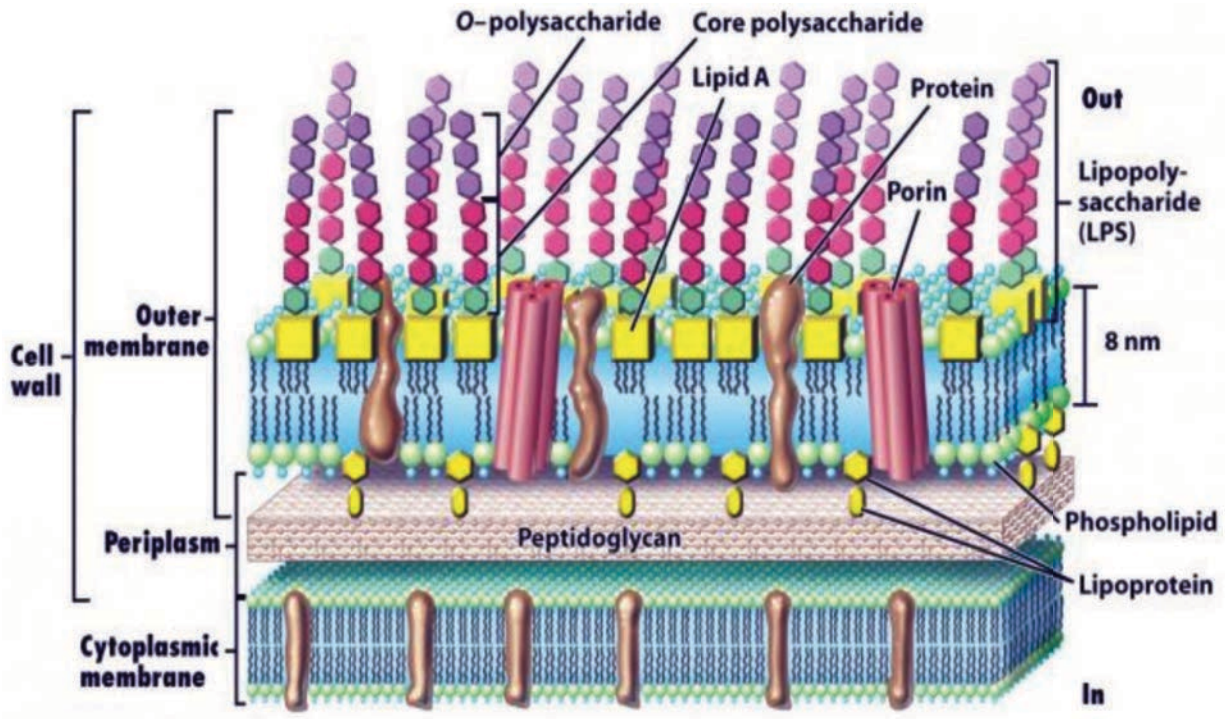

Figure 1.1 Schematic of the cell wall structure of *Salmonella*
The cell wall of *Salmonella* is conformed by 2 layers: the outer membrane (OM) and the inner membrane (IM), which are separated by a layer of peptidoglycan. Porins are found in the OM in the form of trimers. Lipopolysaccharide (LPS) constitutes the outer leaflet of the OM and is divided in: lipid A, core oligosaccharide, and O-antigen (O-Ag). Other components such as lipoproteins are also found in the OM. Figure taken from *Brock Biology of Microorganisms,* 11ed, 2006, Pearson Prentice Hall, inc.

1.2.1.1 Lipopolysaccharide (LPS)

The LPS located in the outer leaflet, closely interacts with several membrane spanning OMPs and is hypothesised to participate in the biogenesis and assembly of some OMPs (9, 12-14). LPS is comprised of lipid A, core oligosaccharide, and repeat units of O-Ag, the latter of which are only present in smooth bacterial strains (Fig.1.2A)(10, 15). Lipid A is a glucosamine disaccharide that anchors LPS into the OM (16). Lipid A has endotoxic actitvity and induces an innate immune response through the activation of macrophages and the release of pro-inflammatory cytokines via Toll-like receptor 4 (TLR4) signaling (16-18). The core is divided in inner and outer core and is conserved within *Salmonella* species (19, 20).

The O-Ag is considered a virulence factor in *Salmonella.* It is hydrophilic and consists of repeating oligosaccharide units (20). The synthesis and assembly of the O-Ag to the core follows three distinct pathways: the ATP-binding cassette (ABC) transporter pathway, the wzx/wxy pathway, and the synthase pathway. (20-25). In the ABC transporter pathway the O-Ag repeat-units are all synthetised and assembled in the cytoplasm to then be translocated across the IM for ligation by an ABC transporter (26). The synthase pathway is only present in *S. enterica* serovar Borreze O:54 and requires the participation of a glycotransferase, the synthase. However, the molecular mechanisms involved in its assembly are still unknown (25, 27). In most *Salmonella* species, the synthesis of the repeat units of O-Ag involves the wzx/wzy pathway and is determined by the gene cluster *wba.* Mutants lacking these genes produce LPS but are unable to synthetise O-Ag chains (28-30). In contrast to the ABC transporter pathway, in the wzx/wzy pathway each O-Ag repeat-unit is synthetised in the cytoplasm and transported across the IM by the flippase Wzx to

then be assembled in the periplasm by the polymerase Wzy (23). The length of the O-Ag is regulated by, the polysaccharide co-polymerase (PCP), Wzz (31, 32). Regardless of the synthesis pathway, they all require assembly on C55-undecaprenyl phosphate (Und-P)(30). The ligation of the O-Ag onto the outer core results from a bond formation between the lipid carrier, Und-P, and a uridine diphosphate (UDP) sugar with the release of UMP (uridine monophosphate)(20, 29, 33). The principal sugars involved in the biosynthesis of the polysaccharide are galactose (Gal), glucose (Glc), and N-acetylglucosamine (GlcNac). In most serotypes of *Salmonella enterica*, Gal is added as the first sugar moiety to the O-Ag via the action of a glycotransferase encoded by *wbaP* gene (23, 34-37). In the absence of *wbaP* the bacteria is unable to transfer galactosyl-1-phosphate from guanosine diphosphate (GDP)-galactose to Und-P and therefore the synthesis of O-Ag is inhibited rendering the bacteria more susceptible to host immune responses such as phagocytosis and complement mediated killing (38-41). The O-Ags of *Salmonella*e are determined by differences in the 3,6-dideoxyhexose residue of the O-Ag, which correspond to a tyvelose, abequose or paratose (e.g. abequose in *S.* Typhimurium and tyvelose in *S.* Enteritidis)(21).

1.2.1.2 Porins

Porins are β-barrels that form diffusion channels to allow the transit of hydrophilic molecules into the cell and the excretion of toxic compounds from the bacteria (10, 42). The major porins found in *Salmonella* are OmpA (37 kDa), OmpC (35 kDa), OmpF (36 KDa), and OmpD (≈34-40 KDa)(10, 11). These porins are highly permeable and possess β-strands linked by extraplasmic and periplasmic loops (42-44). Porins are abundant, contain various surface epitopes, and exist as homo- and

heterotrimers across the membrane (Fig. 1.2B and C)(44-46). Although all porins are similar in their architecture, they vary in their surface charges and loop topology (42).

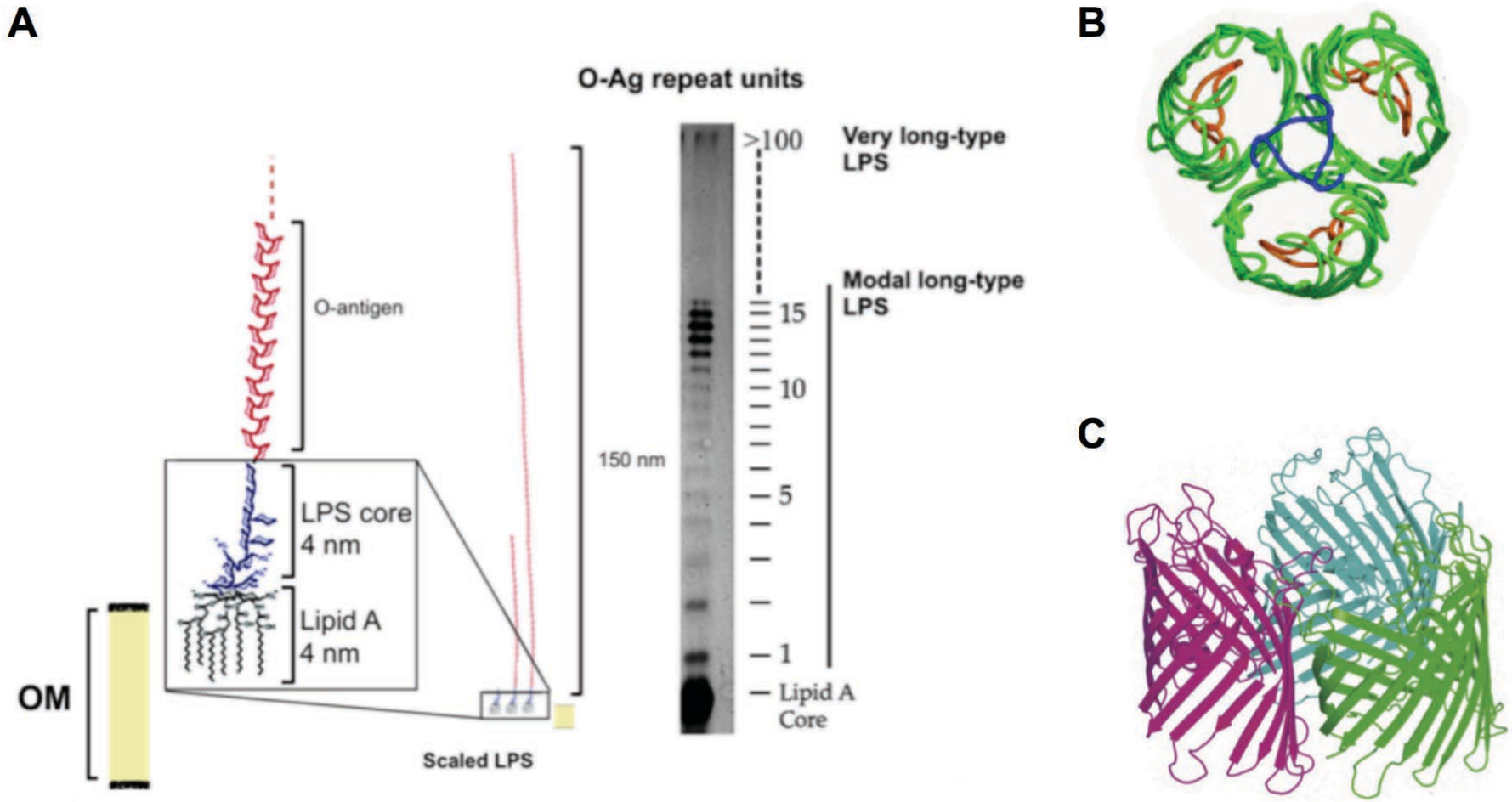

Figure 1.2 Lipopolysaccharide (LPS) and porins constitute the majority of the outer membrane (OM) of non-typhoidal *Salmonella* (NTS)

(A) Schematic of the length of O-Ag chains synthetised by STm represented on scale. Figure modified from Dr. Marcin Grabowicz (University of Princeton) original. (B) Top-view of a typical trimeric porin (OmpF from *E.* coli) found on the OM. Figure taken from (10). (C) Side-view of OmpF from *S.* Typhi. Figure taken from (47).

1.2.1.2.1 OmpA

OmpA is a pore-forming monomeric protein important for the conservation of the bacterial cell structure. It provides physical linkage between the OM and the peptidoglycan (48). OmpA is composed of two large domains: an N-terminal domain that serves as a membrane anchor; and a C-terminal domain located in the periplasmic space that interacts with the peptidoglycan layer (11, 49). OmpA is conserved amongst other *Salmonella* serovars and is >94% identical to *Escherichia coli* OmpA (50). OmpA is a target for the immune system. It can also act as an evasin, adhere to and invade epithelial cells, function as a receptor for bacteriophages, and participate in biofilm formation (51).

1.2.1.2.2 OmpC and OmpF

OmpF allows diffusion of larger molecules than OmpC (10). Both porins are recognised by phagocytes and initiate a series of signals that regulate reactive oxygen species (ROS) in the host and modulate maturation of the phagolysosome (52, 53). The locus *ompB* controls the expression of OmpC and OmpF. This locus contains the genes *envZ* and *ompR*, which have been related to virulence of the bacteria (54, 55) and regulation of *Salmonella* pathogenicity island 2 (SPI-2)(56, 57). High osmolarity favours expression of OmpC whereas OmpF is more abundant at a low osmolarity (58, 59). Purified OmpC and OmpF from *S.* Typhi have been reported to be immunogenic and induce lifelong antibody (Ab) responses (60-62). Moreover, a recent study showed that these two porins possess adjuvant properties that could be used to improve immune responses for T-dependent (TD) and T-independent (TI) antigens (63).

1.2.1.2.3 OmpD

OmpD is the most abundant porin in the OM of most *Salmonella,* but it is not present in *S.* Typhi (64). The ability of OmpD to support bacterial survival during *Salmonella* infections has been reported. Lack of OmpD diminished the virulence of *S.* Typhimurium (STm) in mice (55) whereas a similar study showed no significant difference between the *ompD* mutant and its parental strain (65). OmpD is not affected by changes in osmolarity and is not dependent on the *envZ-ompR* regulatory system (66). Transcription of the gene *ompD* increases during anaerobiosis and decreases at low pH. This gene provides the cell resistance to methyl viologen (67). Moreover, it has been reported that OmpD enhances antimicrobial stresses due to a decrease in the permeability of the cell membrane (68, 69). During the early stages of infection, OmpD is involved in the adhesion of bacteria to macrophages and enteric epithelial cells in humans (53, 70). Furthermore, OmpD may increase proliferation of *Salmonella* inside macrophages when it is down regulated (71). In addition, OmpD could be an important target of humoral immunity in mice (72).

1.2.1.3 Flagella

Flagella function as a propeller allowing the bacterium to move. Flagella are divided in three different structures: a basal body or transmembrane motor, a hook, and a filament. The basal body is where all the components for flagellar function, such as protein products and structural genes are found. The hook joins the basal body to the filament (73). The filament consists up to 20,000 subunits of flagellar proteins known as flagellin. Flagellin is encoded by *fliC* and *fljB,* expressed during flagellin phase variation (74). Flagellin can potentiate the innate immune response and is considered a target of adaptive immune responses (75-77). Nevertheless, expression of flagellin

is variable. Amino acid sequences in flagellar proteins in the surface-exposed region vary amongst serovars making it antigenically diverse (78, 79).

1.3 *Salmonella* infections in humans

1.3.1 Typhoid fever

Typhoid, also known as enteric fever, is usually associated with poor hygiene and is transmitted via faecal-oral route by the ingestion of contaminated food or water (80). Disease due to typhoid fever is more common in South and South East Asia causing approximately 21 million cases per annum with a fatality rate of 20% in untreated cases and 1% in those treated with antibiotics (81). The most affected group is children under 5 years of age (82-84). *S.* Typhi can invade the organism by infecting the intestinal microfold (M) cells and establishing an undetectable infection characterised by a lack of intestinal inflammation (85). The incubation period is normally up to 14 days after infection and symptoms can last for weeks and include cough, persisting fever, abdominal pain, anorexia, nausea, myalgia, headache, constipation or diarrhea, and hepatosplenomegaly (86, 87). Bacteraemia is common, although low grade, and *S.* Typhi can spread from the gut to the spleen, liver, bone marrow and gallbladder (Fig. 1.3)(88). In contrast to invasive nontyphoidal salmonellosis (iNTS), typhoid fever is not normally associated with the immunocompromised state (89). Typhoid fever can be treated with antibiotics, such as fluoroquinolones, and treatment is usually effective if the organism is sensitive. However, antibiotic resistance has become a problem in endemic areas and prognosis is reserved in individuals with underlying conditions, such as malaria or anaemia, and in those where treatment is delayed. Carriage of the bacteria can

persist for a long period of time in asymptomatic patients or after infection is resolved (87).

1.3.2 Nontyphoidal salmonellosis (NTS)

In developed countries, NTS infections are limited to a gastrointestinal disease without being life-threatening. Symptoms start appearing as early as 6 hours after ingestion of the pathogen and typically last less than a week. The most common symptoms associated with NTS are gastroenteritis, diarrhea, abdominal pain, nausea, and sometimes fever. Bacteraemia is rare but it can occur in the immunocompromised, newborn and elderly patients (89). Infection can be treated with antibiotics but is normally unnecessary as it resolves after few days (86, 90). However, an invasive form of NTS is often present in developing countries, such as Sub-Saharan Africa, in HIV infected adults and children under 2 years of age, and in immunocompromised groups, such as the elderly. This presentation frequently occurs in the absence of gastrointestinal symptoms and is characterised by high fever, hepatosplenomegaly, and bacteraemia (5, 89, 91).

1.3.3 Invasive Non-Typhoidal salmonellosis (iNTS)

Invasive NTS in Sub-Saharan Africa is a neglected disease most commonly caused by *Salmonella enterica* serovars Typhimurium (STm) and Enteritidis (SEn)(92). Because of its severe presentation, it suggests that the transmission may be from human-to-human rather than through an animal reservoir (93, 94).

1.3.3.1 Pathogenesis of iNTS

NTS is typically acquired orally. Once *Salmonellae* enter the host, the bacteria reach the host intestine and outer tissues via M cells of the Peyer's patches associated with

the secretion of effector proteins by the *Salmonella* pathogenicity island 1 (SPI-1), which encodes a type 3 secretion system 1 (T3SS-1)(95, 96). Moreover, *Salmonellae* can also spread from the gut into the blood through an alternative pathway, via $CD18^{+}$ phagocytes, without colonising the Peyer's patches (97).

In the intestinal submucosa, uptake of bacteria by phagocytic cells such as macrophages, neutrophils, and dendritic cells (DCs) takes place (Fig. 1.3). SPI-2 encoded T3SS-2 prevents formation of the phagolysosome by inhibiting oxidative mechanisms to enable *Salmonellae* replicate in *Salmonella* containing vacuoles (SCVs)(98) resulting in the release of cytokines such as interleukin (IL)-17, interferon gamma (IFNg), and tumour necrosis factor (TNF)(99, 100). Additionally, phagocytic cells recognise pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) eliciting an innate immune response through Toll-like receptors (TLRs) and NOD-like receptors (NLRs). This can lead to neutrophil and macrophage recruitment, and the induction of an inflammatory response (4, 101). Migration of bacteria to the mesenteric lymph nodes (MLNs) and other tissues, such as spleen and liver, via the bloodstream occurs when the immune system fails to control infection locally (102). A summary of NTS pathogenesis is illustrated in Figure 1.3. For a more detailed review of the immune responses refer to Section 1.5.

1.3.3.2 Disease burden of iNTS

iNTS is a cause of fatal infection worldwide and severity of disease is associated with bacterial spread through the host. There are an estimated 3.4 million cases of iNTS per year globally, from which almost 2 million cases correspond to Africa (103). Fatality rates of iNTS are estimated at 20% with more than 100,000 deaths per year (5). While 90% of the cases in adults are normally associated with HIV infection, 20%

or less of children with iNTS are HIV positive (5, 104, 105). Data from a previous study recognised NTS along with *Streptococcus pneumoniae* as the most common pathogens that cause bloodstream infections in Africa (106). The global geographical distribution of iNTS is outlined in Figure 1.4A, with incidence rates of more than 100 iNTS cases per 100,000 inhabitants per year in Sub-Saharan Africa and less than 100 cases per 100,000 of the population per year in South East Asia. Previous reports have shown that more than 50% of the total iNTS cases occur in Eastern Africa and less than 10% in Northern Africa (Fig. 1.4B)(92).

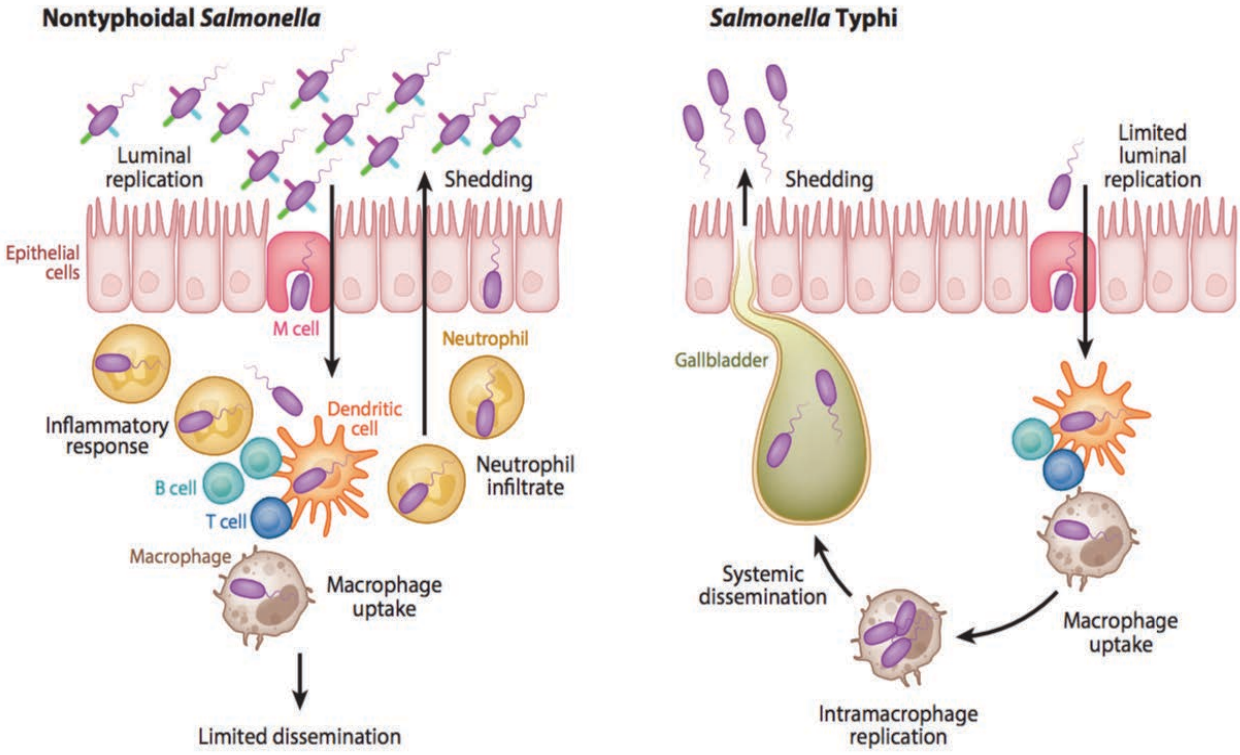

Figure 1.3 Differences in NTS and typhoid pathogenesis
NTS typically invades the organism via epithelial or M cells resulting in a self-limiting gastrointestinal disease that triggers a rapid inflammatory response characterised by massive neutrophil infiltration (left). During systemic infection the bacteria reach the Peyer's patches where they are phagocytosed by macrophages, and dendritic cells (DCs) to then be disseminated via bloodstream to secondary lymphoid tissues such as the spleen, liver, and mesenteric lymph nodes (MLN). In contrast, *S.* Typhi infects the M cells resulting in a systemic disseminated disease with the absence of intestinal inflammation (right). Picture taken from (80).

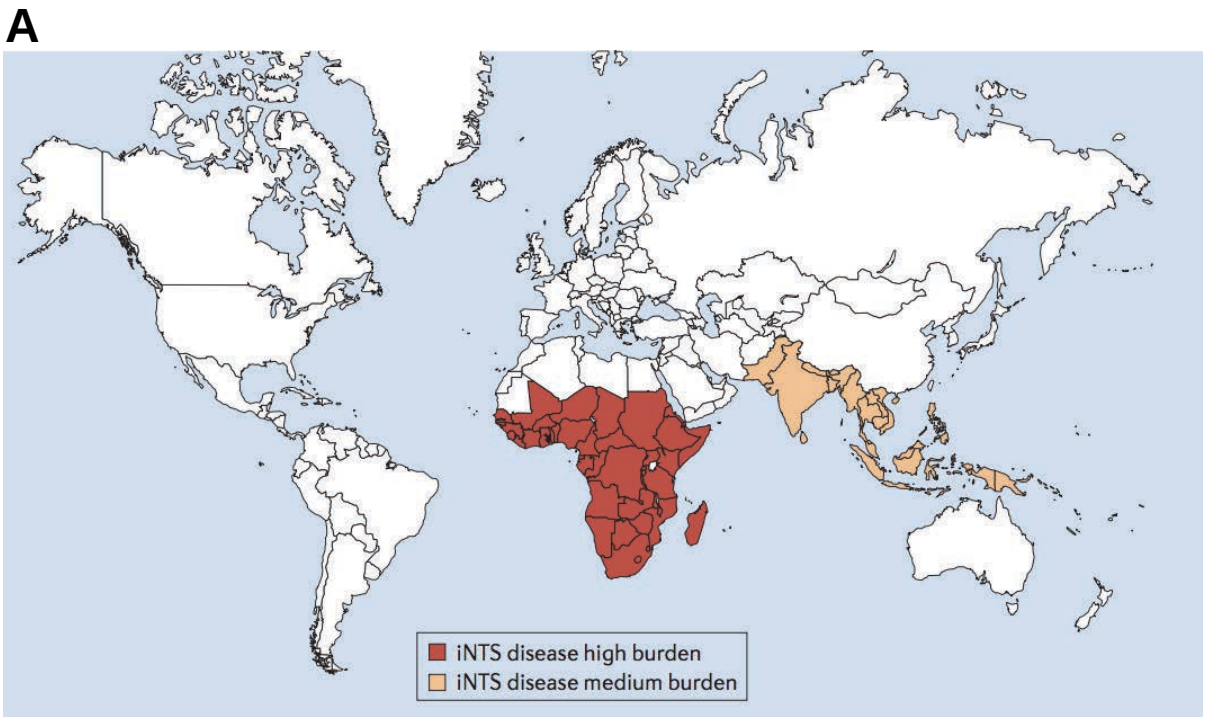

Figure 1.4 Global burden and frequency of iNTS cases reported in Africa
(A) Geographical distribution worldwide of iNTS (102). (B) Distribution of iNTS cases in different regions in Africa. Figure taken from (92).

1.3.3.3 Risk factors associated with iNTS

1.3.3.3.1 HIV infection

In HIV infected adults, iNTS infections have been associated with depletion of $CD4^+$ T cells in the gastrointestinal tract, especially T-helper 17 (Th-17) cells. This is associated with defects in the gut mucosa and a decrease in the production of antimicrobial peptides and neutrophil recruitment (107). Moreover, an inhibition of proinflammatory responses through cytokine dysregulation such as defects in production of IL-12, IL-23, and IFNg increase susceptibility to iNTS (108). Presence of high amounts of anti-LPS immunoglobulin (Ig) G Ab in HIV positive adults has also been reported to cause impaired immunity through an inability to kill bacteria in serum, highlighting the importance of humoral responses in iNTS (109).

1.3.3.3.2 Young age and other conditions

In children, iNTS infections often occur in the young, with the majority of bacteraemia cases found in infants between 6-24 months old (110). However, in children under 4 months old, maternal IgG specific to *Salmonella* may play an important role in conferring protection against iNTS (38). Other risk factors can contribute to susceptibility. These include anaemia, severe malaria infection, and malnutrition, which are more common than HIV infections in infants (111-114). In malaria infections, haemolysis cause high levels of free heme by activation of the enzyme heme oxygenase 1 (HO-1). The cytotoxic activity of HO-1 results in impaired of oxidative burst by polymorphonuclear leukocytes (PMNs) and increases *Salmonella* survival and replication in the host (115). Furthermore, children homozygous for sickle-cell disease are more susceptible to iNTS, increasing the mortality associated with this illness (116).

1.3.3.4 Clinical presentation

Invasive NTS is a febrile illness similar where diarrhea is normally absent (114). Patients with iNTS can develop hepatosplenomegaly and pneumonia, which is commonly associated with co-infections such as *Mycobacterium tuberculosis* and *Streptococcus pneumoniae* (117-119). In addition, iNTS has been associated with meningitis and schistosomiasis in children (120-122). Other non-specific symptoms include myalgia, abdominal pain, headaches, and nausea (119).

1.3.3.5 Diagnosis and treatment

Due to the lack of specific symptoms, diagnosis of iNTS is difficult. *Salmonella* can be detected by blood cultures but the lack of appropriate facilities in Africa makes diagnosis complicated. Furthermore, symptoms are often confused with severe pneumonia and malaria (123, 124). The Widal test and bone marrow culture have been applied to detect *S.* Typhi and polymerase chain reaction (PCR) from stool samples has also been used (125, 126). Nevertheless, iNTS is present at levels of 1 colony forming unit per millilitre (CFU/ml) of blood, which may correlate with a low sensitivity in blood cultures by PCR (127). Further, new techniques like microwave-accelerated metal-enhanced fluorescence (MAMEF) to detect the *Salmonella oriC* chromosomal locus looks promising yet validation is still needed (128).

Antibiotics such as ampicillin, chloramphenicol, fluoroquinolones and cephalosporins have been used in the past to treat iNTS. However, the increase in multi-drug resistance has reduced the effectiveness of many treatments leading to attempts to develop effective vaccines to prevent and protect against iNTS in developing countries (110, 129).

1.4 Vaccines against iNTS

The vaccines that are currently available for humans against *Salmonella* infections are only directed against S. Typhi. These are an inactivated whole cell vaccine, the Ty21a (live attenuated vaccine) and the Vi capsular polysaccharide (ViCPS) vaccine (130-132). The inactivated whole cell vaccine is as effective as the other vaccines, however, due to its high reactogenicity it is no longer used (132, 133). Live attenuated Ty21a lacks expression of the Vi-Ag (134) but still induces a T cell response and Ab to other antigens. There is evidence that it may be cross-protective against non-Typhi serovars such as *S.* Paratyphi A and B (135-137). In contrast, the Vi CPS vaccine is conformed by purified Vi polysaccharide and generates a T-independent type 2 (TI-2) response. Both have a similar 3-year efficacy; three oral doses of Ty21a confer 51% whereas only one intramuscular dose of Vi CPS corresponds to 55% of efficacy (131, 132). However, none of them are effective in children under 2 years of age. A Vi glycoconjugate vaccine consisting of Vi-OAg conjugated to *Pseudomona aeruginosa* exoprotein A (Vi-*r*EPA), appears to be more effective in younger children (138).

At the moment, no vaccines are available for humans against NTS but various candidates against STm and SEn are under development (139). Some of the vaccine candidates currently going under pre-clinical research include: live attenuated oral vaccines, glycoconjugate vaccines, protein based subunit vaccines, and Generalised Modules for Membrane Antigens (GMMA)(72, 139-142).

Live-attenuated vaccines elicit a TD response and can be given orally as they can induce effective mucosal immunity (143). However, the disadvantage of these

vaccines involves a compromised level in their immunogenicity and a higher number of doses needed as a consequence of attenuating the bacteria (144, 145). These include 4 vaccines under clinical trial: CVD 909 and Ty800 against *S.* Typhi (phase 2); CVD 1902 against *S.* Paratyphi A (phase 2); and the WT05 against STm (phase 1)(144, 146, 147).

Glycoconjugate vaccines induce a T cell mediated response by using proteins as carriers to induce the production of antibodies towards the O-Ag or Vi-Ag (in *S.* Typhi) and transforming these TI antigens into TD antigens Carrier proteins include diphtheria toxoid (DT), tetanus toxoid (TT), porins, and flagellin. Examples of these types of vaccines are the Vi-TT and Vi-*r*EPA, both already licensed for use in humans (138, 144, 148).

Protein antigens such as flagellin and porins have been studied as possible vaccine candidates. These types of vaccines can induce B and T cell responses (144). Porins, such as OmpC and OmpF, from *S.* Typhi induce a long-lasting Ab response (60) and are safe when used as a vaccine in humans (61). Another porin, OmpD, has also showed to produce a protective response against STm in mice (72). GMMA consist in small OM blebs of 50-90 nm diametre that consist in periplasm encapsulated by the OM (149). GMMA are highly immunogenic although their reactogenicity in humans is still unknown and more studies need to be conducted.

1.5 The mouse as a model to study *Salmonella* infection

Murine models are used to study the immune responses to *Salmonella* infections, including systemic infections. Typhoid fever is restricted to humans and higher primates, however, in mice, STm can cause a fever with symptoms similar to those observed in iNTS and typhoid disease in humans (150). Additionally, it has been reported that in mice pre-treated with streptomycin, STm can also induce enterocolitis accompanied by inflammation and ulceration of the intestinal epithelium due to infiltration of granulocytes and $CD18^{+}$ cells (151).

The severity of infection depends on the virulence of the *Salmonella* strain used, the dose, route of infection, and mouse strain (152, 153). Approximately 10^{8} is the maximum bacterial load in tissues in mice before they succumb to infection (154). However, with a non-fatal dose mice can survive for weeks to several months and recover (155). The intraperitoneal and intravenous routes are often used, which enables better systemic dosing and therefore a synchronised and disseminated infection is established (88).

Laboratory mouse strains, such as C57BL/6 and BALB/c, are hyper susceptible to wild-type STm and develop a systemic disease due to a mutation in the natural resistance associated macrophage protein-1 (Nramp-1) encoded by the *SLC11α1* gene (156, 157). Nramp-1 is a divalent cation transporter that is recruited to the membrane of macrophages upon phagocytosis (158). Therefore, these mice do not survive for long after systemic challenge with invasive strains. In contrast, resistant Nramp-$1^{+/+}$ mice, are able to survive after challenge with virulent *Salmonella* and therefore chronic infections can be studied (155).

Infection with *aroA* mutants allows clearance of the bacteria within weeks in susceptible mice, with the establishment of different innate and acquired immune responses (159). These attenuated *aroA* mutants, have a defect in the aromatic amino acid synthesis that reduces the virulence of the bacteria but still makes it able to colonise the host (159). This metabolic mutation makes *Salmonella* auxotrophic for aromatic amino acids, which are not accessible in the host (160, 161). Furthermore, when adaptive immune responses to attenuated STm are established, susceptible mice are able to cope with subsequent virulent *Salmonella* infections (88).

1.6 Innate immunity

Innate immunity is the initial line of defense against Gram-negative bacterial infections and is independent of T and B cells. Elements of the innate system include: anatomical barriers (skin, mucosae, gastric pH, antimicrobial peptides, and enzymes), humoral barriers (complement system and cytokines), and innate cells. Recognition of *Salmonella* at this stage is dependent on TLRs, NLRs, pro-inflammatory cytokines and activation of effector mechanisms for the control of both extracellular and intracellular bacteria (162).

1.6.1 Toll-like and NOD-like receptors

The innate system is non-specific and therefore in order to distinguish self from non-self it requires recognition of pathogen components, PAMPs and DAMPs, which are recognised by pattern recognition receptors (PRRs) such as TLRs and NLRs (163). TLRs are present as homo- or heterodimers that detect PAMPs (Fig. 1.5). A total of 10 functional TLRs have been recognised in humans and 12 in mice. TLRs 1 to 9 are conserved in both mice and humans (101, 102, 164). TLR4 is necessary to control

early *Salmonella* infections (165). TLRs are expressed in the cell wall or in intracellular vesicles. They are composed of ectodomains (which are involved in PAMPs recognition), a transmembrane region, and Toll-interleukin-1 receptor (TIR) domains (which regulate signaling pathways). TLRs activate adaptor proteins in the cytoplasm such as myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor inducing interferon β (TRIF or TICAM1), TRIF-related adaptor molecule (TRAM or TICAM2)(164). Upon activation of these molecules, a cascade of signals leads to the release of pro-inflammatory cytokines, chemokines, interleukins (IL), and host defense peptides (HDP). Furthermore, these responses give rise to the recruitment and activation of innate cells such as neutrophils and macrophages (101). During *Salmonella* infections, activation of TLRs leads to production of IL-18 and IL-23, which at the same time promote release of IFNg, IL-17, and IL-22 by mucosal resident T cells. The combination of these cytokines results in an inflammatory response in the intestine and a massive production of HDPs (IL-22 and IL-23) and neutrophil recruitment (IL-17 and IL-23)(162). NLRs are mainly expressed in neutrophils, macrophages, and epithelial cells. NLRs detect the presence of PAMPs in the cytosol and initiate a variety of signals for the activation of IL-1β, IL-18, and inflammatory caspases (166). While both TLRs and NLRs play an important role in initiating an innate immune response, for the purpose of this project they will not be discussed in more detail.

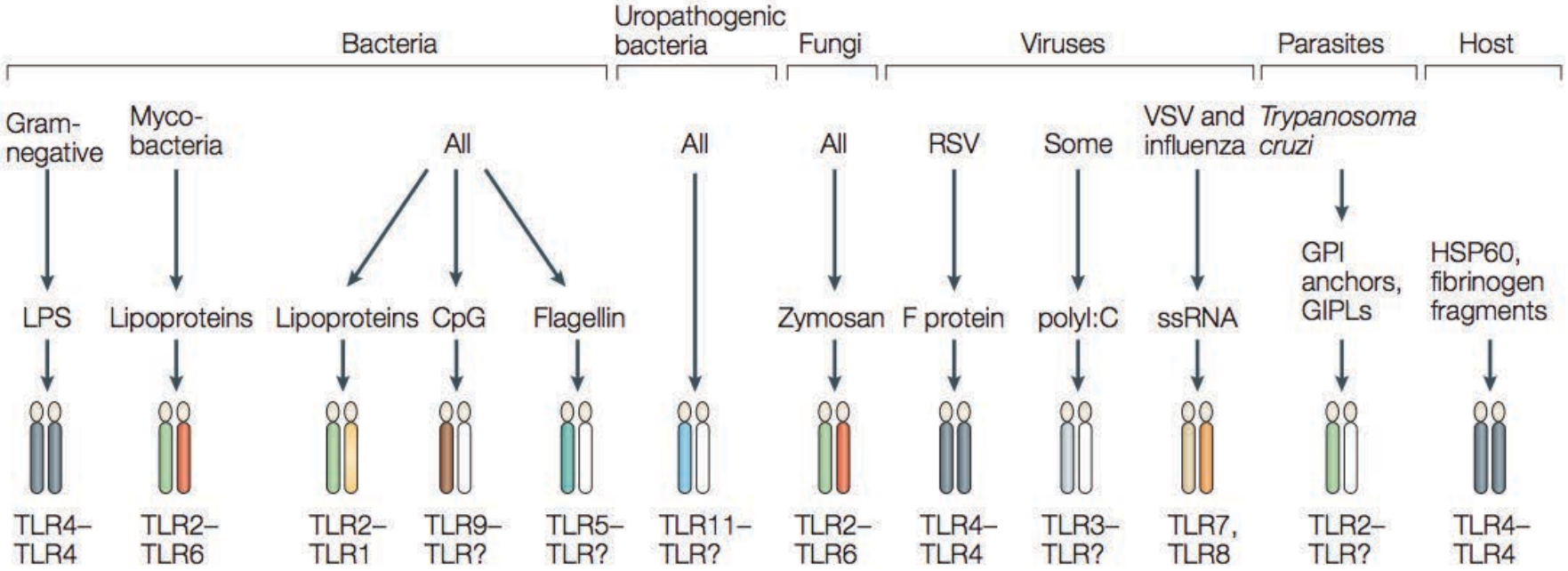

Figure 1.5 Toll-like receptors (TLRs) recognise pathogen associated molecular patterns (PAMPs) from mice and humans

In both species, TLRs show specificity to a certain type of ligand/PAMPs. TLRs that recognise extracellular components of the pathogen are localised in the surface of the host cell whereas TLRs that recognise viral/bacterial nucleic acids are expressed inside vesicles in the endosome. Figure taken from (167).

1.6.2 Complement activation

The complement system is a cascade of plasma proteins that are capable of increasing the opsonisation of bacteria in order to control infection (168). This system can be activated via three different pathways (classical, lectin, and alternative pathway) all of which result in the activation and formation of the convertase C3 (Fig. 1.6)(169, 170). The activation of the classical pathway (CP) is antibody-dependent and requires binding of C1 (first protein of the complement cascade) to IgM or IgG Ab or to the cell membrane of the bacteria (171). Moreover, formation of IgG hexamers after antigen (Ag) binding on the cell surface results in complement activation (172). The lectin pathway (LP) initiates when mannose-binding lectin (MBL) and ficolins bound to carbohydrates on microbial surfaces (173, 174). The alternative pathway (AP) occurs by spontaneous activation of C3 or by the classical or lectin pathways (175).

There are three different outcomes as a result of complement activation. First, C3 convertases promote binding of C3b molecules to the membrane of the bacteria, where they act as opsonins promoting phagocytosis and killing of the pathogen (176). Second, C3a which is also activated during this process; is a chemoattractant and anaphilatoxin mediator of inflammation that recruits and activates phagocytes (170). And third, the C5 convertase, formed during the binding of C3b to C3, initiates the last steps of complement activation with C5a and C5b produced and the formation of a membrane attack complex (MAC) that creates pores in the bacterial membrane causing bacterial cell lysis (168).

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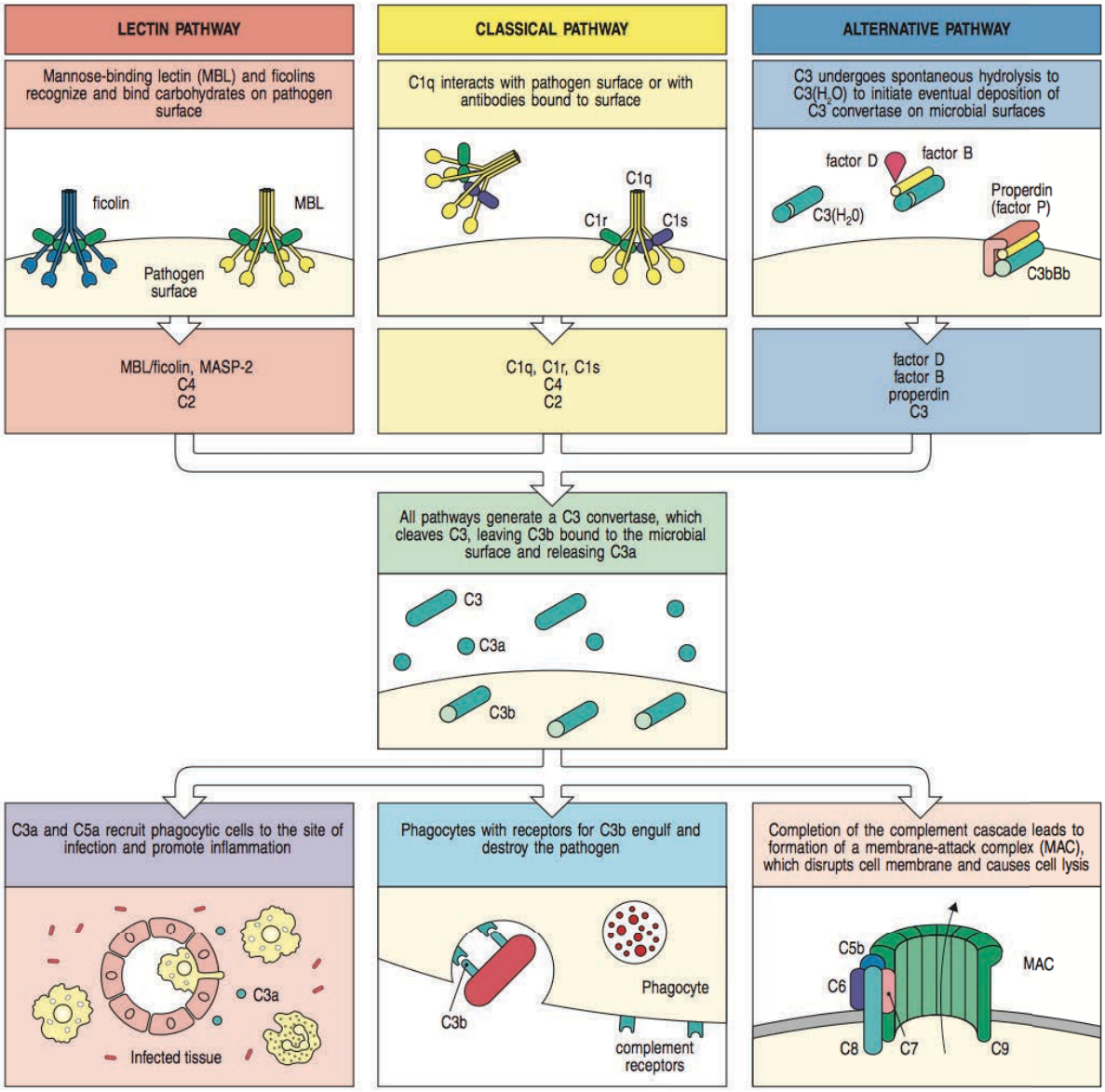


Figure 1.6 The complement cascade
The complement system can be activated via the classical, lectin, or alternative pathway. All pathways converge in the formation of the C3 convertase but each differs in the way complement activation is initiated. The classical pathway (CP) requires binding of the C1q molecule to the surface of the bacteria or to opsonised Ab. The lectin pathway (LP) is activated by binding of mannose-binding lectin (MBL) or ficolins to carbohydrates in the cell wall. The alternative pathway (AP) is triggered when by spontaneous activation of C3 by hydrolysis. Figure taken from *The Immune system,* 9th edition, Garland Science 2016.

1.6.3 Innate immune cells and their role in *Salmonella* infection

The innate immune system includes cells such as neutrophils, macrophages, dendritic cells (DCs), natural killer (NK) cells, basophils and eosinophils. These cells derive from a common myeloid haematopoietic progenitor with the exception of NK cells, which are derived from lymphoid stem cells (Fig. 1.7)(163). Moreover, innate cells develop into different subpopulations that express a variety of specific phenotypic markers and functional characteristics (177).

During infection, neutrophils and macrophages play an important role in controlling the dissemination of bacteria to other tissues via phagocytosis and releasing reactive oxygen species (ROS)(178-180). Macrophages induce production of IL-12 and TNF by TLR stimulation (181, 182). Depletion experiments in mice indicate the importance of both macrophages and neutrophils during primary *Salmonella* infections (183-188). Moreover, in HIV positive adults, neutropenia is a risk factor for bacteraemia caused by NTS (189). Additionally, depletion of neutrophils in mice with enterocolitis suggested the important role of neutrophils as a source of IFNg in the intestinal mucosa (190). Furthermore, IL-12 and IL-18 enhance cytotoxicity and IFNg production by NK cells (182), particularly $Thy1^+$ NK cells during the early stages of *Salmonella* infection (191). NK cells recognise virulent and neoplastic cells by producing α-defensins and also contribute to controlling infection by secreting IL-10 and TNF (192, 193). Additionally, DCs function as antigen presenting cells (APCs) and induce cytokine production. DCs can recognise LPS and flagellin, and enhance expression of CD80, CD86, and CD40 (194, 195). DCs mature and migrate to secondary lymphoid tissues to initiate T cell responses (196).

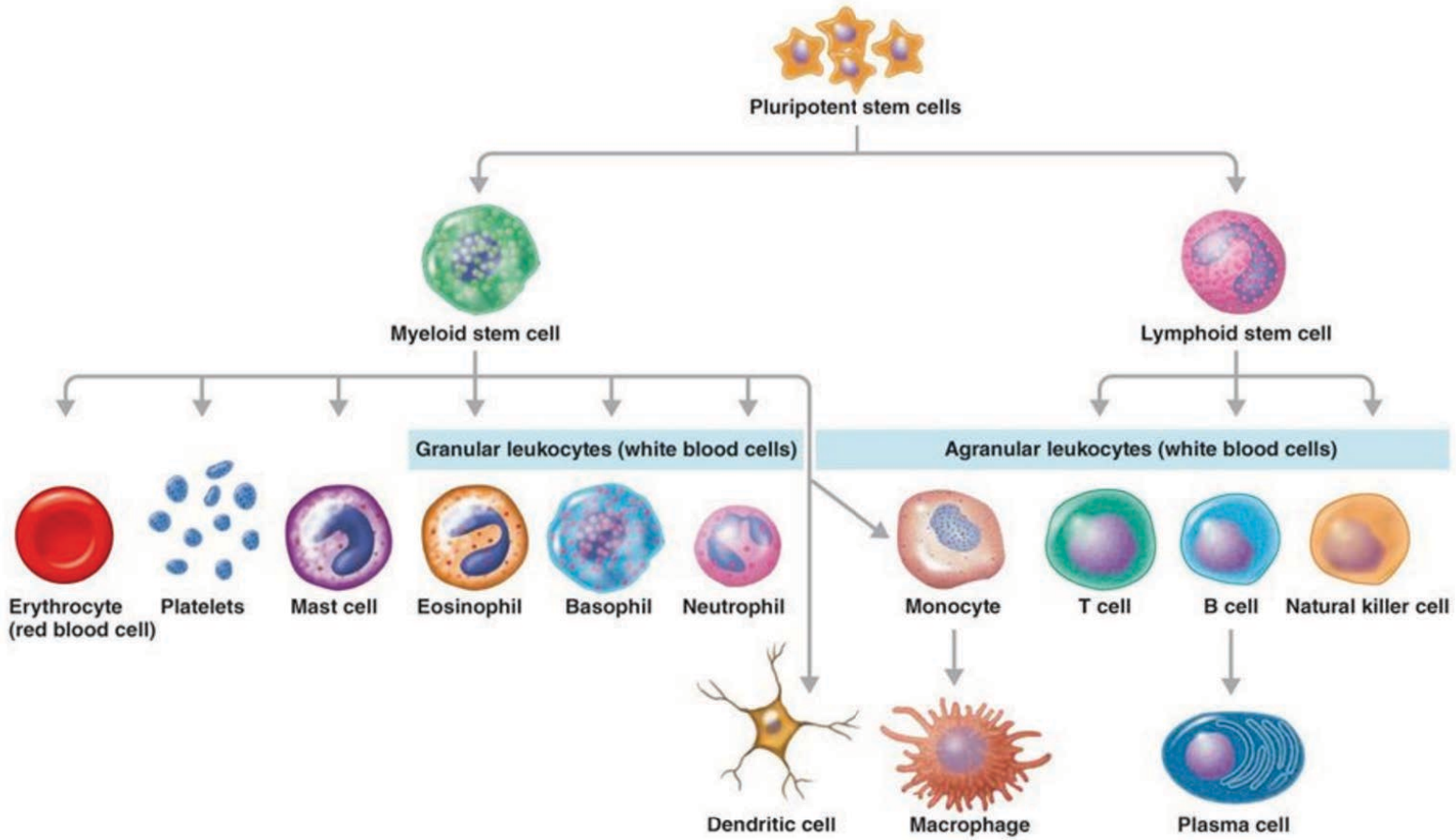


Figure 1.7 Overview of haematopoiesis
Haematopoiesis is the process by which new blood cells are created. Pluripotent stem cells give rise to 2 more specialised cell progenitors in the bone marrow: myeloid and lymphoid stem cells. Myeloid stem cells give rise to white blood cells (leukocytes), red blood cells (erythrocytes), and megakaryocytes (producers of platelets). The erythrocytes include mast cells, monocytes, eosinophils, basophils, and neutrophils (the latter 3 also known as granulocytes). On the other hand, lymphoid stem cells give rise to T and B cells. NK cells are also originated from the same lymphoid progenitor but lack Ag specificity, which is a key feature of adaptive immunity. Image taken from *Microbiology: An Introduction,* 12th edition, Pearson Education, Inc 2016.

1.7 The adaptive immune response

If innate cells fail to control infection then an adaptive response is required. These responses, also called acquired immune responses, are important for providing protection upon a secondary infection (197). T and B lymphocytes are both involved in adaptive immune responses against *Salmonella*. These two lineages of lymphocytes are derived from a common haematopoietic lymphoid progenitor. However, T cells mature in the thymus and B cells in the bone marrow (BM) (Fig. 1.7). These antigen-specific cells differentiate into several subsets, each of them specialised in triggering different memory and effector responses (198).

1.7.1 T cell-mediated responses

T cell-mediated immunity is based on Ag recognition via T cell receptors (TCRs). The majority of TCRs express α-β chains that recognise antigens presented by major histocompatibility complexes (MHC) present on the surface of antigen presenting cells (APCs)(199, 200). In addition, a small number of TCRs, found in the epithelial tissues, express γ-δ chains, and do not necessarily require activation by MHC (200). Naïve T cells reside in secondary lymphoid tissues and circulate via the blood and lymph until encounter with an antigenic peptide activates them. A schematic overview of the structure of the spleen is shown in Figure 1.8. Activation of naïve T cells requires two signals (Fig. 1.9A). The first signal is generated through interaction of the TCR with the peptide-MHC complex on the APCs. The second signal is delivered through co-stimulatory molecules by binding of CD28 on TCRs with B7-1 (CD80) and B7-2 (CD86) on APCs (201, 202).

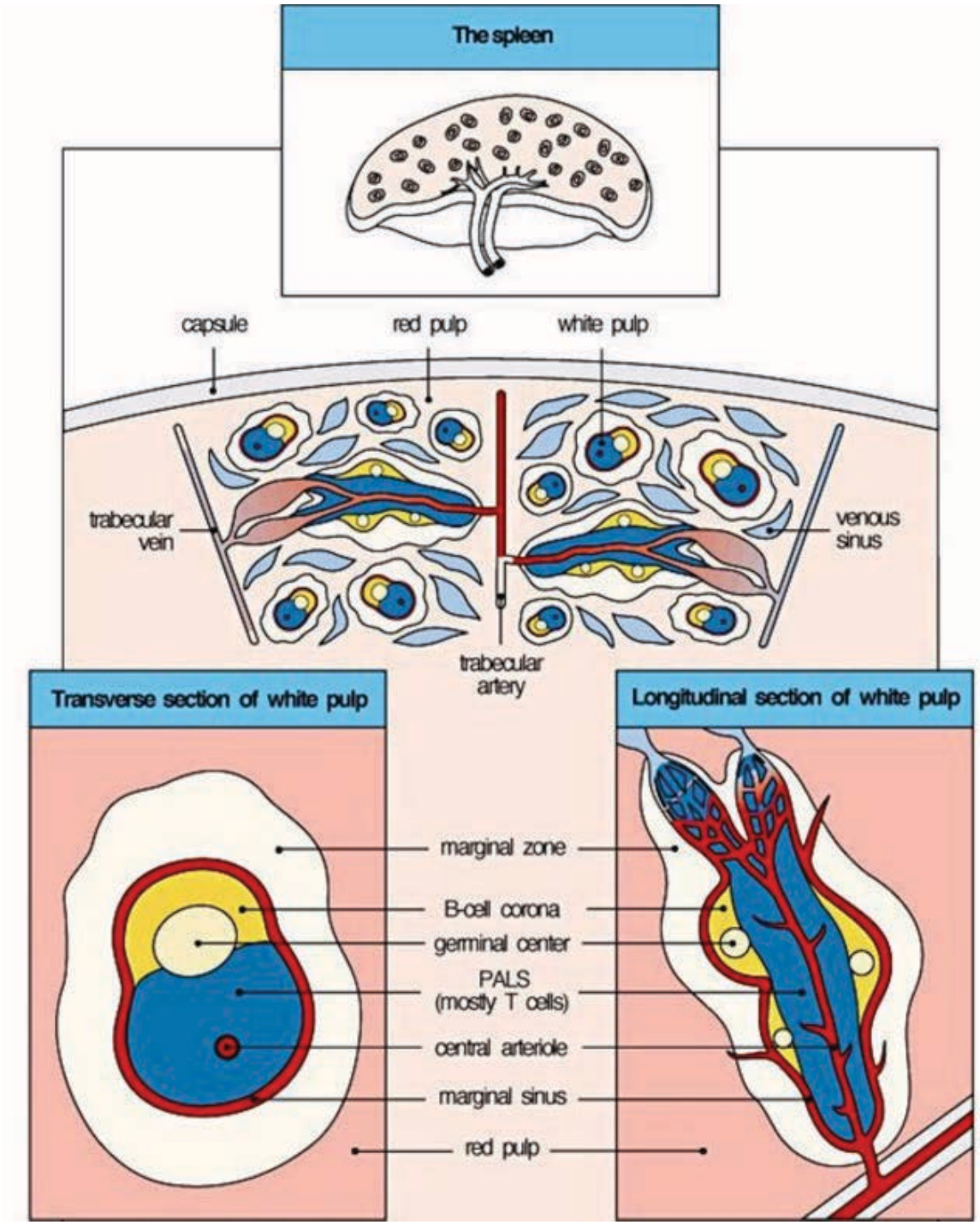


Figure 1.8 Schematic of the structure of the spleen
The spleen is delimited by a capsule and composed by the red pulp and white pulp. The white pulp consists in the T zone or periarteriolar lymphoid sheath (PALS), arterioles, and B cell follicles. Recirculating T and B cells can be found in the white pulp whereas marginal zone (MZ) B cells reside in the marginal zone of the white pulp. The white pulp is surrounded by arterioles that lead to cords in the red pulp. Image taken from *Janeway's Immunobiology,* 3rd edition, Garland Science 1997.

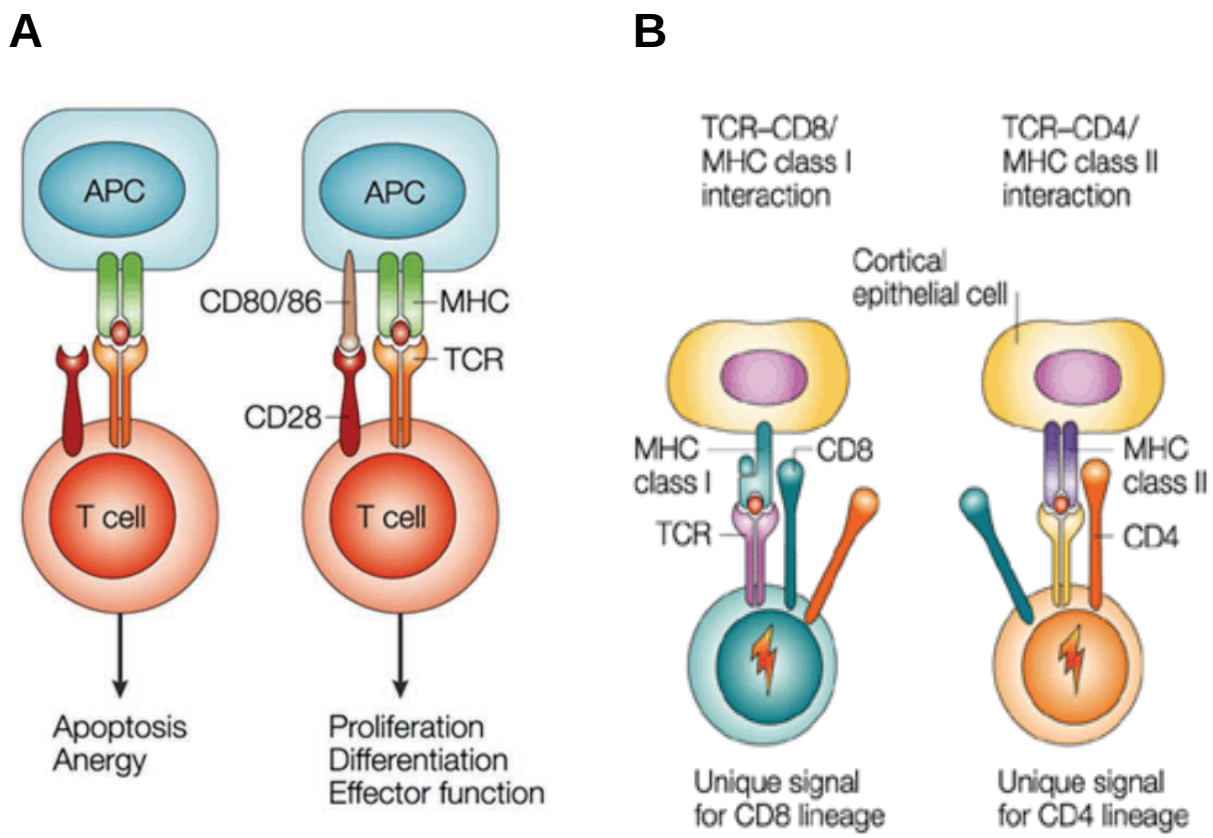


Figure 1.9 Signals required for activation of naïve T cells
(A) Simultaneous interaction of the T cell receptor (TCR) with the antigen-MHC complex and co-stimulation of CD28 by B7 molecules (CD80/CD86) on antigen presenting cells (APCs) resulting in the proliferation and differentiation of the T cell. If CD28 co-receptors are not engaged with their ligands T cells undergo apoptosis or become anergic. Image adapted from (203). (B) $CD8^+$ and $CD4^+$ T cells recognise antigenic peptides presented on APCs by MHC class I and MHC class II molecules, respectively. Figure taken from (204).

1.7.1.1 T cell effector subsets

T cells differentiate into two different lineages: $CD4^+$ and $CD8^+$ T cells (204). Antigen presentation via MHC class I (expressed in all nucleated cells) activates $CD8^+$ T cells whereas MHC class II (expressed in B cells, DCs, and macrophages) activates $CD4^+$ T cells (Fig. 1.9B)(205). While $CD4^+$ T cells are important for stimulation of B cells for isotype class switching, $CD8^+$ T cells are cytotoxic (204). Upon activation, $CD4^+$ T cells differentiate into different subsets including T helper (Th) 1, Th2, Th17, T follicular helper (Tfh), or T regulatory (Treg) cells (Fig. 1.10)(198, 206, 207).

T-bet (T-box expressed in T cells) and IL-12 initiate Th1 cell development resulting in the production of IFNg and switching to IgG2a/c in mice (208). Th2 subsets express GATA-binding protein 3 (GATA3) and are associated with the production of IL-4 and promoting immunoglobulin class switching to IgG1 (209). Th1 cells are normally associated with the clearance of intracellular bacterial (e.g. *Salmonella*) and protozoal infections, such as helminthes, and Th2 responses with the resolution of extracellular pathogens and other humoral responses.

Th17 cells induce production of IL-17 by expression of RORγτ (210) and are important for inflammatory and autoimmune responses. Moreover, these cells play an important role in the immunity against extracellular bacteria and fungi, such as *Streptococcus pneumoniae* and *Candida albicans* (211). Th17 cells have pro-inflammatory functions and are associated to diseases such as rheumatoid arthritis, psoriasis, inflammatory bowel disease (IBD), multiple sclerosis, etc. (198, 212). Th17 cells also contribute during infection by generating an acute innate immune response thanks to the induction of β-defensins in skin cells and IL-22 production (213).

Tfh cells are important in the formation of antibody (Ab) responses through their expression of B cell lymphoma-6 (Bcl-6)(activated B cells), CD40L, IL-21, and inducible T cell co-stimulator (ICOS) (214). Treg cells regulate cell homeostasis and suppress autoimmunity by expression of the transcription factor forkhead box P3 (FOXP3) and IL-10 (215). Treg cells can be further subdivided into naturally arising Treg (nTreg) cells, derived in the thymus, and inducible Treg (iTreg) cells, transformed into Treg cells once activated in the periphery (216).

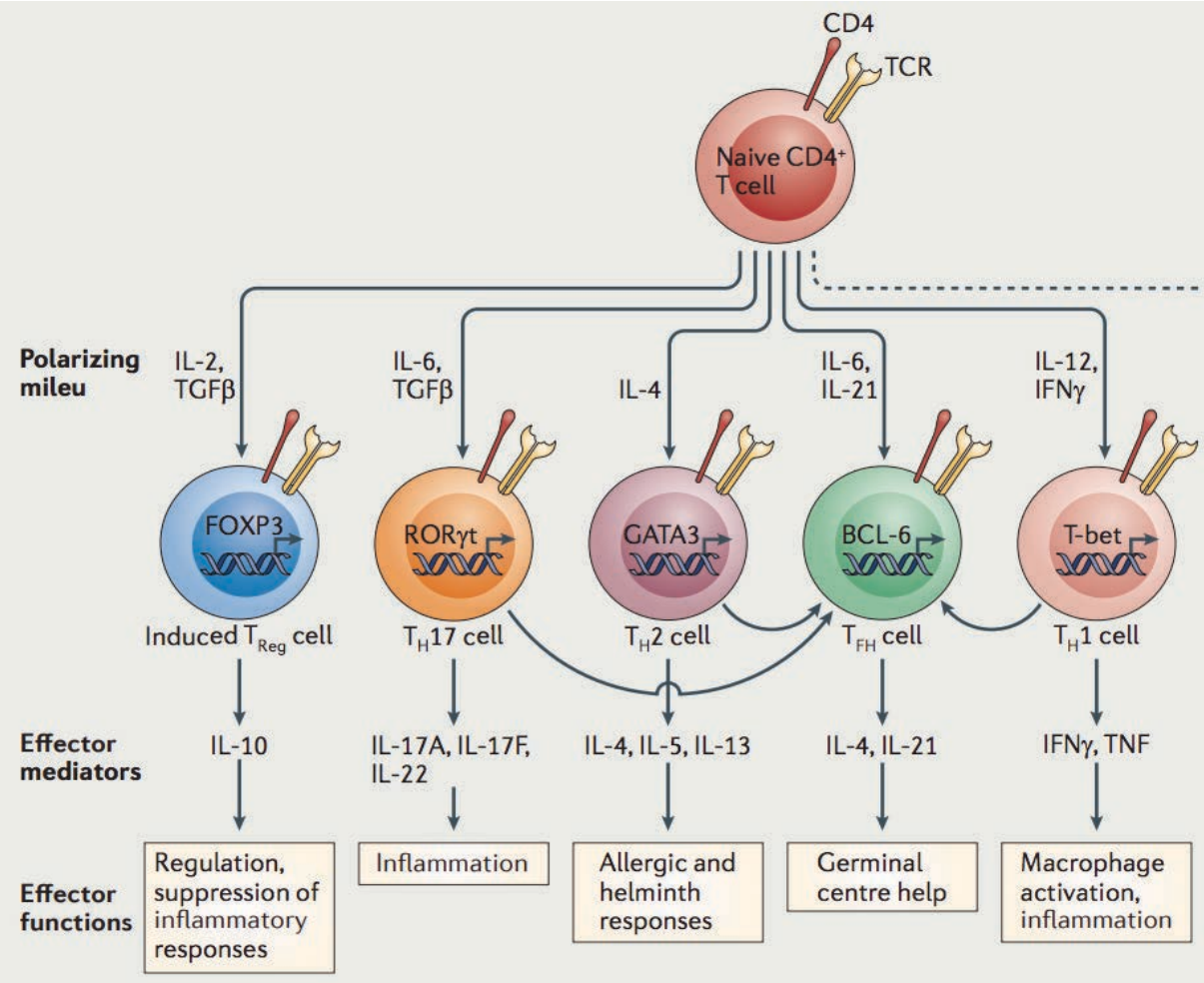


Figure 1.10 CD4$^+$ T cells can differentiate into a different range of subsets
Following Ag recognition presented by APCs, CD4$^+$ T cells become polarised into different T helper (Th) subsets, each of them characterised by a specific effector immune response controlled by transcription factors and expression of different cytokines. T helper cell lineages include: Th1, Th2, Th17, Tfh, and Treg cells. Figure taken from (217).

1.7.2 T cell mediated immunity to *Salmonella* infection

Clearance of *Salmonella* requires T cells particularly $CD4^+$ T cells. Since *Salmonella* are intracellular bacteria and reside within macrophages, induction of IFNg by Th1 cells is required for optimal bacterial killing (218, 219) Moreover, athymic mice and mice lacking TCR α-β, MHC class II or T-bet fail to resolve primary STm infection (220-223). In contrast, mice that lack B cells or TCR γ-δ are able to clear a primary infection with attenuated *Salmonella* (224-226). In acquired immunity, IL-12, IFNg, and TNF are important to resolve *Salmonella* secondary infections in mice infected with attenuated *Salmonella* strains (227, 228). These studies correlate with reports in humans where susceptibility is increased in patients with defects on the IL-12/IL-23/IFNg axis (108), thus indicating the importance of $CD4^+$ T cells and Th1 immunity in protection to *Salmonella.* Additionally, Th17 cells may contribute to protection against *Salmonella* as both IL-17 and IL-22 are produced in the intestine after infection and bacterial dissemination is increased in mice that lack IL-17A (229-231).

$CD8^+$ T cells are also induced after *Salmonella* infection in mice but their role is less clear. Cytotoxic activity from $CD8^+$ T cells has been characterised (227, 232, 233), but studies in mice where $CD8^+$ T cells were depleted showed only a minimum effect in the protective response to *Salmonella* during a secondary infection (227, 228, 233, 234). Furthermore, mice lacking MHC class I granules suggest that MHC class I-restricted $CD8^+$ T cells are not required during a secondary infection but participate in the protective response during *Salmonella* primary infections (235).

1.7.3 B cells and antibody-mediated responses

1.7.3.1 B cells

B cell development in the BM consists of a series of steps where transition of progenitor (pro)-B cell to precursor (pre)-B cells, and immature B cells takes place (236). Characterisation of the B cell development is determined by rearrangements of the immunoglobulin (Ig) gene segments: V_H, D_H, and J_H in the heavy (H) chain and V_L and J_H in the light (L) chain encoding a distinct B cell receptor (BCR)(237). In contrast to TCRs, Ag recognition by BCRs is not dependent on Ag presentation via MHC (238). The region of the Ag that binds to a specific Ab or B cell is called the B cell epitope. Unlike T cell epitopes, B cell epitopes can be continuous or discontinuous, non-linear, and reflect three dimensional structures (239). Ab-epitope interactions are crucial for subunit vaccine development (240-242). During development, B cells are screened for self-reactivity before migrating to secondary lymphoid tissues (243). B cells differentiate into plasma cells or into memory B cells. The main function of B cells is the synthesis and production of antibodies. However, other functions of B cells include cytokine production (such as TNF and IL-17), regulation of immune responses by B regulatory (Breg) cells via IL-10 secretion, and Ag presentation to T cells (244). In mice, B cells are divided into B1 (B1a and B1b) and B2 (follicular and marginal zone B cells) subsets. The surface markers of these B cells vary according to their location, function, and site of migration (Fig. 1.11)(245).

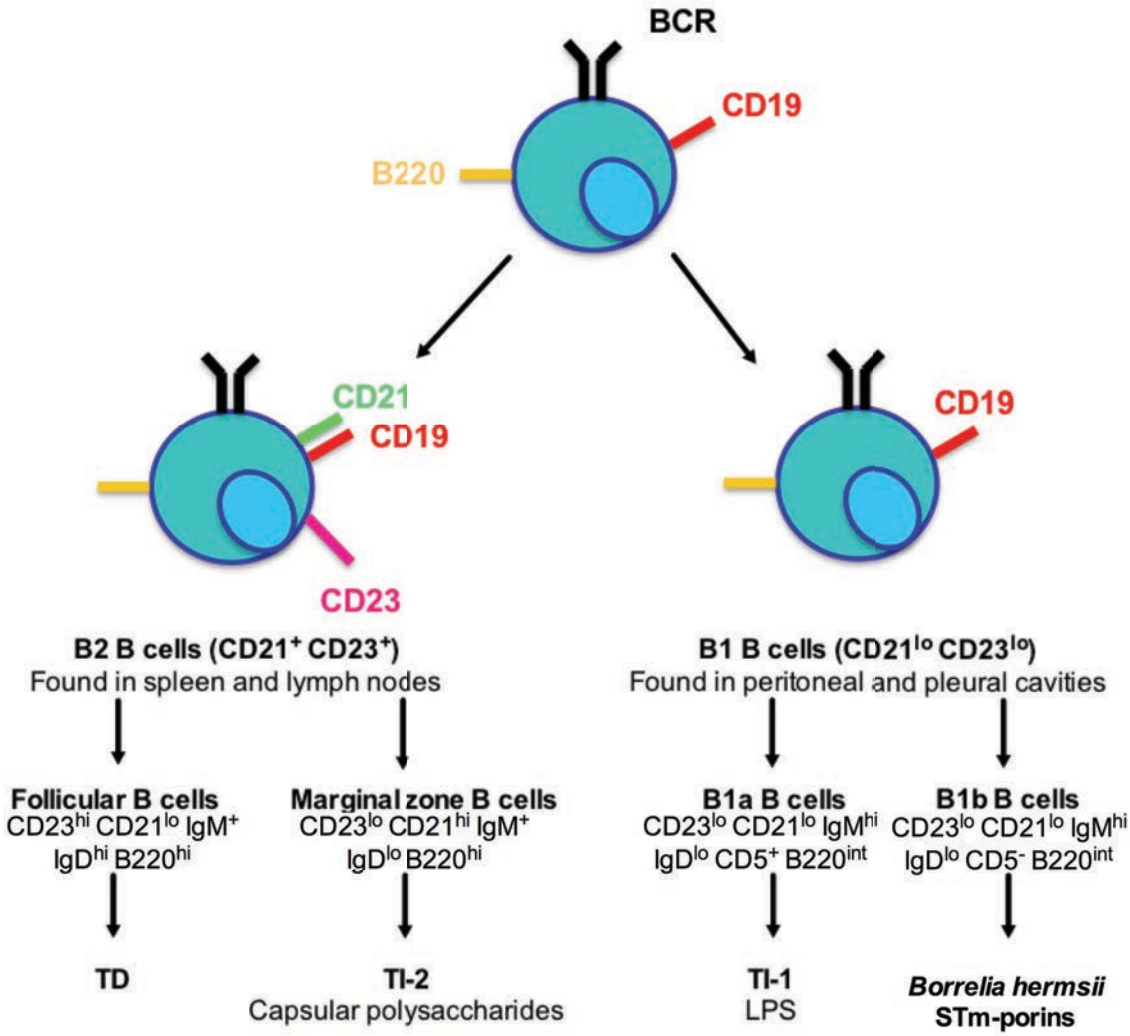


Figure 1.11 Different B cell lineages interact with T-dependent (TD) and T-independent (TI) antigens

B cells are subdivided into two different subsets: B2 B cells and B1 B cells. These subsets are classified based on their location, migration site, surface marker expression, and their response towards TD or TI antigens. B2 B cells are found in the spleen and lymph nodes (LN). They differentiate into follicular (FO) and marginal zone (MZ) B cells, which produce antibodies via TD and TI-2 responses, respectively. B1 B cells, localised in peritoneal and pleural cavities, further differentiate into B1a and B1b B cells. B1a cells respond to TI-1 antigens such as LPS whereas B1b seem to be important in the TI response to *Borrelia hermsii* and STm-porins. Figure modified from Bobat, S. (2011). *Characterising the Immune Response to Salmonella and Salmonella Surface Antigens During a Systemic Infection.* (Doctoral Thesis, University of Birmingham, Birmingham, UK) original.

1.7.3.2 Antibodies (Abs)

Antibodies, also known as immunoglobulins (Ig), are proteins that are produced by mature B cells in response to a specific Ag, although in mice natural Abs are produced by B cells in the absence of a specific stimulation. The specificity of the Ab is determined by the fragment antigen-binding (Fab) region, which consists of two heavy (H) and two light (L) chains, each with one constant and one variable region. Heavy chains determine the subclass and isotype of the Ab. This effector function is mediated by the binding of the fragment crystallisable (Fc) region to the Fc receptor, found in the surface of effector cells, which influences effector mechanisms such as complement activation and opsonisation, (Fig. 1.12)(246). Antibodies are classified in different isotypes: IgA, IgE, IgD, IgM, and IgG. In mice, IgG is further divided in IgG1, IgG2a, IgG2b, and IgG3 (247). In C57BL/6 mice there is no expression of IgG2a but IgG2c (248). Ab isotype specificity in mice is dependent on germ-line transcription and deletional recombination of the H chain of the Ab. The latter leads to splicing of the VDJ gene segments in the H chain, and consequently, the Ab undergoes class-switching recombination (CSR)(249). In mice, Ab class switching occurs in response to a specific antigenic stimulus regulated by IL-4, IFNg, or transforming growth factor beta (TGFβ). IL-4 stimulates production of IgG1 and IgE; IFNg promotes switching to IgG2a/c and IgG3; and TGFβ regulates IgG2b and IgA production (249, 250). Humans express IgA1, IgA2, IgG1, IgG2, IgG3, and IgG4. Nevertheless, these isotypes are not direct homologous of the mice IgG subclasses but may be similar structurally or in their function (247). In humans, IgG1 and IgG3 are normally induced in response to proteins antigens whereas IgG2 and IgG4 to polysaccharides (246).

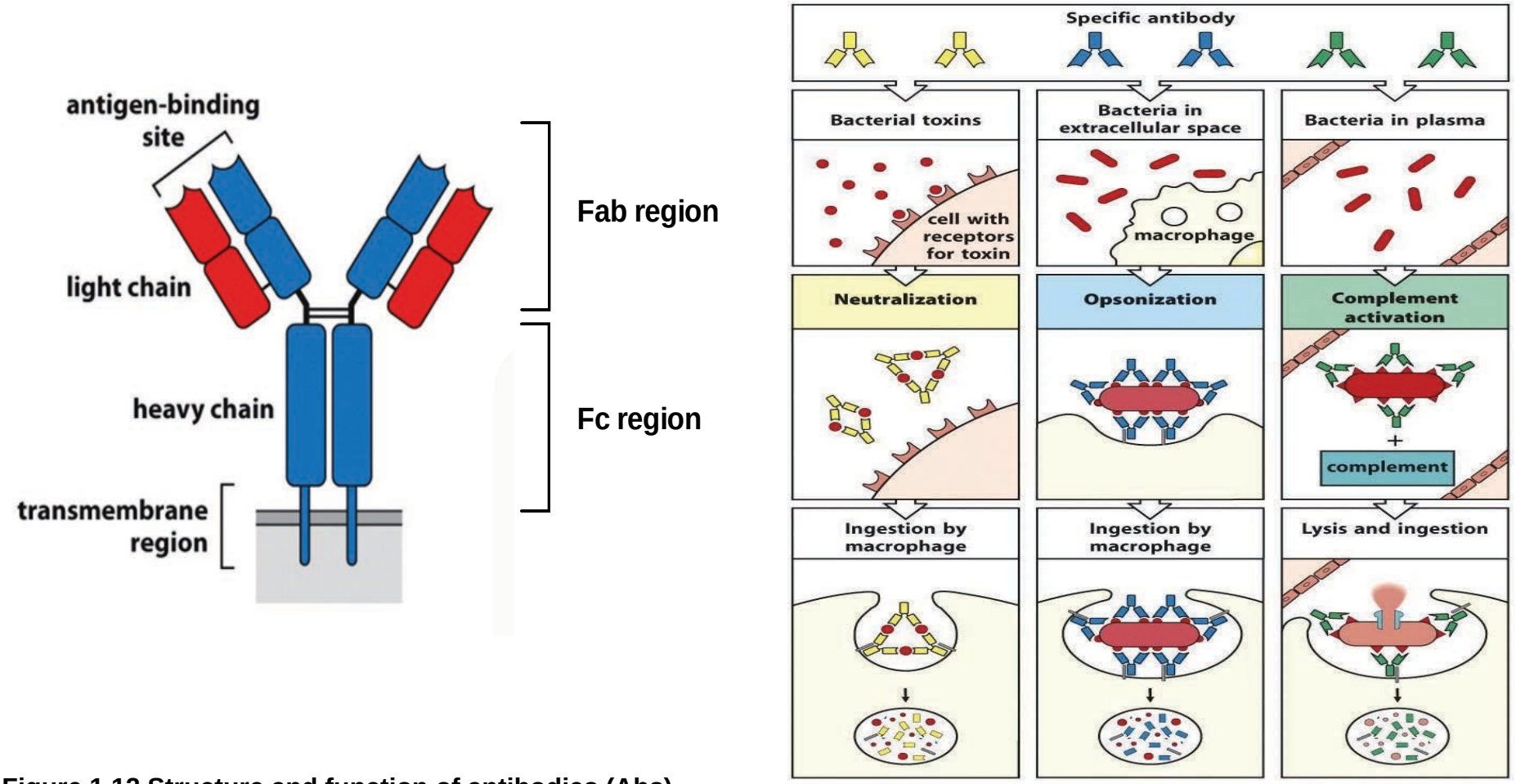


Figure 1.12 Structure and function of antibodies (Abs)
Antibodies are composed by 4 polypeptide chains containing two identical heavy (H) and light (L) chains each. The Fab region determines specificity whereas the Fc region regulates effector mechanisms involved in protection *Janeway's Immunology*, 8th,Garland Science 2009.Antibodies follow three different pathways to prevent pathogens from entering the organism. These include: 1. Neutralisation by binding to the pathogen and blocking bacterial toxins. 2. Opsonisation by coating the surface of extracellular pathogens and facilitating phagocytation. 3. Complement activation via classical pathway. Image taken from *The Immune System*, 3rd edition, Garland Science 2009.

1.7.3.3 T-dependent (TD) antibody responses

TD responses require T and B cell interactions and are typically induced by protein Ags (251). TD responses are normally needed for anti-STm protein Ab responses to proteins such as flagellin. Nevertheless not all proteins require T cells to induce responses, for instance, porins. (72). Follicular (FO) B cells are involved in TD responses. These cells reside in the follicle of secondary lymphoid organs and recirculate via the bloodstream or lymph. Mature FO B cells occupy two niches: 1) B cell follicles, in secondary lymphoid tissues, where they present TD antigens to effector T cells or differentiate into short-lived plasma cells. 2) BM, where they re-circulate around vascular sinusoids to be activated in a TI manner by blood-borne pathogens and differentiate into IgM secreting cells (245, 252). During TD responses antigens are presented to T cells by MHC class II molecules expressed on B cells and other cells such as DCs and macrophages. These interactions result in the production of memory B cells and Ag-specific Ab, which are induced by extrafollicular responses or via formation of germinal centres (GCs)(Fig. 1.13)(253, 254).

1.7.3.3.1 Extrafollicular (EF) responses

Extrafollicular responses develop rapidly after Ag encounter. B cells interact with effector T cells in the T zone of secondary lymphoid tissues to generate plasmablasts. At this stage, B cells express the plasma cell marker syndecan-1 (CD138). These plasmablasts migrate and colonise the red pulp around the external surface of the MZ in the spleen. Here, association of $CD11c^{hi}$ DCs influences differentiation of plasmablasts into plasma cells and markers such as CD19 and B220 are downregulated. Forty eight hours after Ag encounter, expression of the transcription factors, BLIMP-1 and XBP-1, are necessary for differentiation into

plasma cells (255). Additionally, during this stage of plasma cell differentiation, upregulation of the activation-induced deaminase (AID) leads to Ig class-switching recombination. The majority of the plasma cells generated in the EF will die by apoptosis after 3 days. However, some may become long-lived plasma cells (256). Both recirculating and MZ B cells are recruited via the EF pathway (253).

1.7.3.3.2 The germinal centre (GC) response

Germinal centres develop in follicles of secondary lymph tissues in response to a TD Ag (254). B cells found in GCs further differentiate into memory B cells and Ab-secreting plasma cells (257). B cells undergo somatic hypermutation (SHM), which results in the production of high-affinity antibodies in GCs (258). Antigen-specific plasma cells and memory B cells appear during the first 7 days after immunisation with protein/alum antigens (259). GCs are delineated into the dark zone (DZ) and the light zone (LZ)(Fig. 1.12). The DZ, in which centroblasts proliferate, is located near the T cell area and is where SHM takes place (260). The LZ, located closer to the marginal zone (MZ) in the spleen and to the lymph node capsule, is composed of T cells, tingible body macrophages (TBM), and follicular dendritic cells (FDCs). FDCs and Tfh play a role in selection of high-affinity B cells in the LZ and those with lower-affinity encounter apoptosis (261). In a classical response, between day 10 and 14, the GC response peaks upon Ag encounter and then this response diminishes. As a result of this, plasma cells and high-affinity B cells develop. Memory B cells do not produce Ab and respond faster than naïve B cells after re-encounter with the same Ag (262). Differentiation into plasma cells and Ab secretion is driven by B-lymphocyte-induced maturation protein 1 (BLIMP-1), The transcription factor Bcl-6

suppresses BLIMP-1 and therefore allows GC responses occur until differentiation of plasma cells takes place (263).

1.7.3.3.3 Differentiation into long-lived plasma cells

Long-lived plasma cells (LLPCs) can persist for years. LLPCs that are produced after an immune response are home to the BM, however, some of these LLPCs survive in the spleen (264, 265). The majority of the plasma cells that reside in the BM are generated in GC responses and are of high-affinity. These LLPCs are produced as a result of a TD response, in association with B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) expressed by stromal cells with B cell maturation antigen (BCMA)(266). Nevertheless, maturation of GCs is not always required for the generation of long-lived plasma cells as EF responses can contribute to the generation of this plasma cell lineage in spleen and BM (264, 265).

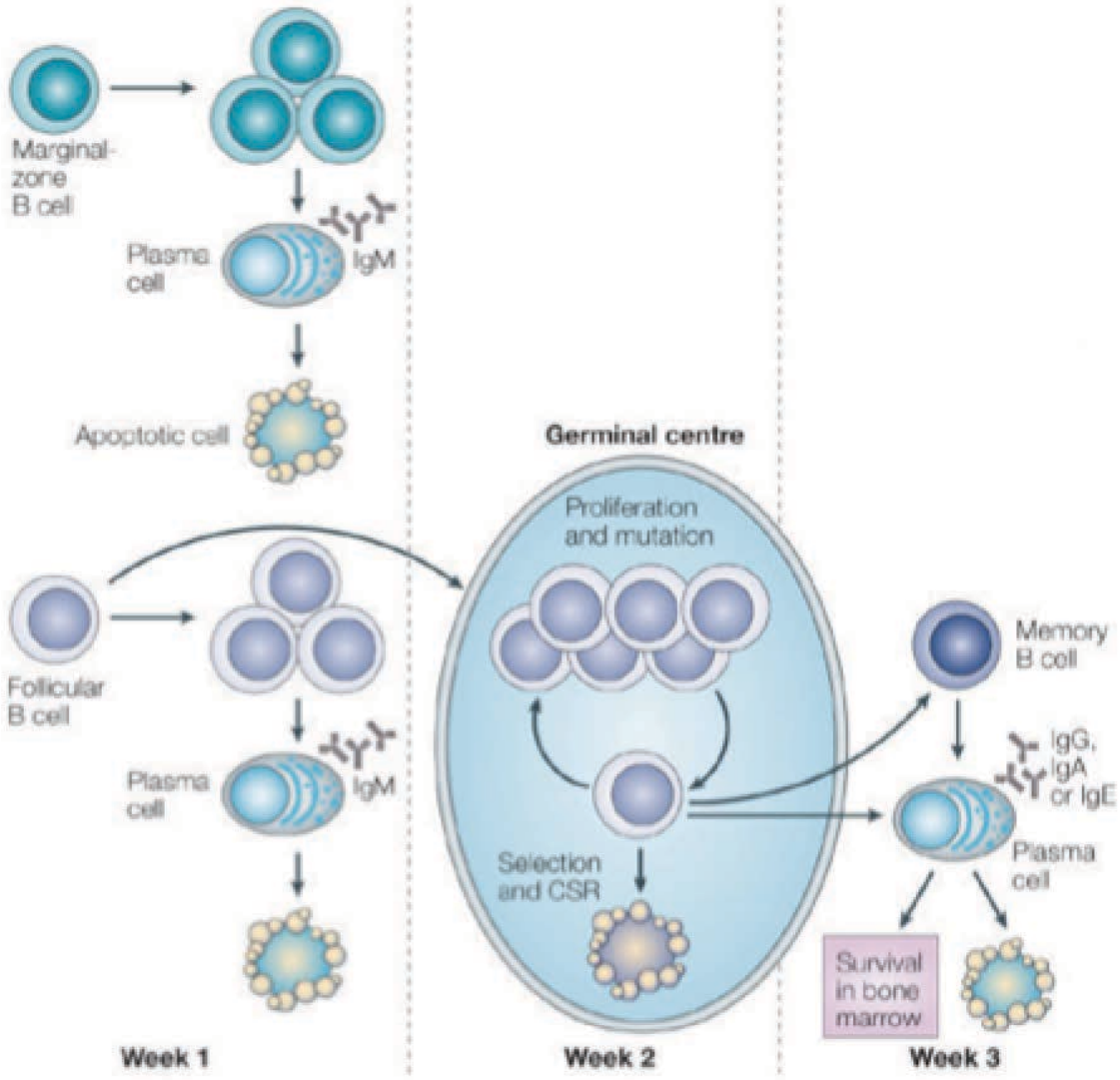


Figure 1.13 Development of plasma cells by TD responses
During early phases, naïve marginal zone (MZ) B cells and follicular (FO) B cells differentiate into plasma cells in the red pulp of the spleen or medulla of the lymph node. The majority of the EF plasma cells induced here are short-lived. Activated FO B cells also induce germinal centre (GC) formation. After GC responses, generation of memory B cells and long-lived plasma cells in the bone marrow (BM) leads to Ab switching. Image adapted from (262).

1.7.3.4 T-independent (TI) antibody responses

TI responses are rapid compared to TD responses and B1 and MZ B cells are the type of cells involved. Marginal zone (MZ) B cells reside in the marginal sinus of the spleen and differentiate into short-lived plasma cells (245). However, there is evidence that under certain circumstances MZ B cells can contribute to the generation of long-lived plasma cells via a TD response and GC formation (267). B1 cells are important in the production of natural Ab and reside mainly in peritoneal and pleural cavities. In mice, these cells can secrete IgM, IgG3 or IgA in the absence of an external Ag (268). B1 cells have a fetal origin and are self-renewing and can be subdivided into B1a and B1b cells. Distinct to B1a cells, B1b cells do not express CD5 (269). In humans there is limited evidence of the existence of these subsets (270-272). TI antigens are classified in type 1 (TI-1) and type 2 (TI-2)(273). TI-1 antigens include LPS while TI-2 antigens include bacterial capsular polysaccharides. These antigens do not need the presence of T cells to respond.

1.7.4 B cell-mediated immunity to *Salmonella* infection

While the role of $CD4^+$ T cells in the clearance of *Salmonella* primary infections has been well established, the role of B cells and antibodies in response to *Salmonella* is less well understood. Studies using mice deficient in B cells showed that B cells are dispensable during a primary infection with attenuated *Salmonella* (224-226). However, B cells play an important role in protection against secondary *Salmonella* infections in mice (224, 226, 274).

Passive transfer of Abs in mice sera indicates the important role of Abs in protection against *Salmonella* (224, 225, 234). While Abs are required to stop bacteria from

spreading via the bloodstream and can facilitate phagocytosis, T cells contribute in the elimination of persisting infections (275). In iNTS infections in humans, T cell-mediated responses control intracellular infections but fail to protect against bacteraemia in the blood. Although patients with immunodeficiencies with a defect in the IL-12/IL-23/IFNg axis are more susceptible to iNTS (108), these patients generate a specific anti-IgG response to STm and do not die after NTS infection. Furthermore, in the presence of complement, IgG and IgM anti-*Salmonella* specific Abs are able to induce killing of the bacteria (38). The latter highlights the importance of Ab and complement in controlling extracellular bacterial growth.

Mice can develop an Ab response when immunised with certain *Salmonella* antigens such as OMPs, LPS, and flagellin (274, 276), However, mouse complement lacks bactericidal activity and is unable to kill bacteria in a cell-independent complement-mediated way (277). Nevertheless, if mice serum is supplemented with exogenous human complement, it is able to promote killing (278).

Moreover, the importance of Ab against *Salmonella* has been demonstrated by the use of the ViCPS vaccine, which provides protection in a TI manner (134). In addition, specific-*Salmonella* Ab is able to induce killing via complement and through opsonisation by phagocytes (38, 279). Nevertheless, when *Salmonellae* are phagocytosed and start replicating inside the cells, Abs are unable to target the bacteria (280). In HIV infected patients, susceptibility to iNTS increases through impairment of Ab and cytokine production, and disruption of the architecture of the intestinal mucosa (109, 281, 282). Thus, both cell-mediated and humoral immunity are required for optimal protection against *Salmonella* infection.

1.7.4.1 Targets of protective immunity in NTS

Activation of humoral responses initiates when Abs encounter protein and non-protein antigens. Mucosal and serological responses are generated after vaccination and recognise these antigens as important targets of protective immunity (274). Antigens found in the OM of *Salmonella* have been studied as possible candidates for vaccines. These immunogenic antigens include the Vi-Ag (present in S. Typhi)(283), the O-Ag (141, 142, 284, 285), flagellin (H-Ag)(75, 286), and porins (61, 72, 287, 288).

In mice, O-Ag conjugated vaccines induce protection after *Salmonella* challenge (141, 142). Furthermore, the importance of protective Ab targeting O-Ag has been shown using monoclonal antibodies (MAbs) and heat-killed invasive *Salmonella* in mice (140, 285). In humans bactericidal Ab to O-Ag is found in both infants and adults but studies have reported lack of protection to iNTS in HIV positive adults (109, 289). Moreover, Ab to O-Ag does not confer cross-protection between STm and SEn suggesting that O-Ag Ab is specific to each *Salmonella* serovar (290). Although the O-Ag acts as an immunogen it also blocks the interaction of protein-specific Abs to exposed epitopes on the cell wall through steric hindrance (291-294).

Flagellin has been reported to be protective in mice when used alone or as a carrier protein for O-Ag conjugated vaccines (75, 141, 286, 295). However, findings like IgG to the O-Ag not being protective against iNTS disease in HIV infected adults (109), variable expression of flagellin, and the fact that both are antigenically diverse between serovars, suggest looking for other effective targets for protection against iNTS.

Other surface proteins such as porins are highly conserved amongst all *Salmonella* serovars. The latter indicates their potential to be used as subunit vaccines. Porins present in *Salmonella* are OmpC, OmpF, and OmpD (this one not present in *S.* Typhi). Studies have shown that immunisation with porins confers protection after *Salmonella* challenge in mice (72, 287) and a study in humans has shown that porins are protective if used as a vaccine against *S.* Typhi (61).

Previous studies in our lab proposed a subunit vaccine consisting on purified OmpD from STm (STm-OmpD). This vaccine is able to protect against STm in a TI response by inducing Ab responses able to control systemic infection in mice (72). Thus, OmpD may be a vaccine candidate against iNTS. However, a better understanding of the immune responses generated after OmpD immunisation still requires further evaluation.

Identifying an antigenic target in both mice and men could offer the possibility of developing a successful vaccine. Moreover, using sera in proteomic assays, a total of 14 common antigenic targets were identified in both species, raising the possibility of using these as subunit or conjugate vaccines. These included OmpA and flagellin (235). Additionally, mapping of Ag-Ab interactions could predict suitable antigens as vaccine components by understanding better the immune response towards them (240). Together, this provides an overview of common targets for Ab that could be exploited as subunit vaccine candidates against invasive salmonellosis.

1.8 Aim of thesis

The main aim of the thesis was to examine the immunological responses elicited after STm-OmpD immunisation against iNTS. The starting hypothesis is that Ab to STm-OmpD offers protection to multiple serovars of salmonellosis. Therefore the work had three objectives:

Objective 1: To identify whether STm-OmpD induces cross-protective Ab.

Objective 2: To identify how Ab to STm-OmpD aids in protection.

Objective 3: To generate a strain of STm for the optimal production of OmpD.

CHAPTER 2: MATERIALS AND METHODS

For a complete list and details of all the media and buffers used throughout this study refer to Appendix A.

2.1 Bacterial strains

For *in vivo* and *in vitro* experiments *Salmonella* enterica serovars Typhimurium (STm) and Enteritidis (SEn) were used. Attenuated and invasive forms of both serovars were used throughout the study as well as rough and LPS chimera mutants. These strains are described in Tables 2.1 and 2.2. The laboratory strain STm SL1344 and the attenuated STm SL3261 were used to generate specific strains, which have been detailed in Table 2.3.

2.2 Mice

Mice typically between 6 to 12 weeks of age were used in all experiments. Wild-type (WT) C57BL6/J mice were sourced from [redacted] and genetically modified mice were obtained from the [redacted] Details and original sources are described in Table 2.4. For studies performed in Cape Town, WT mice were obtained from the [redacted]. All mice were maintained under specific pathogen-free (SPF) conditions. Animal experiments were conducted with approval from both the UK (PPL No. 30/2850 and PIL No. I588DE2B5) and UCT Research Animal Ethics Committees.

Table 2.1 *Salmonella* wild-type strains used

Strain	Characteristics and Source
STm SL3261 *aroA*$^-$	Non-virulent *S.* Typhimurium strain deficient in *aroA*. The attenuation was obtained by deletion of *aroA* from a transposon insertion using *aroA554::Tn10*. Parent strain: STm SL1346 (159).
STm SL1344	Virulent *aroA*$^+$ strain of *S.* Typhimurium. Parent strain: STm S2337 (159).
STm D23580	Virulent strain of *S.* Typhimurium isolated from a child in Blantyre, Malawi. Source: Prof. Calman A. MacLennan (38)
SEn *aroA*$^-$	Attenuated *aroA*$^-$ strain of *S.* Enteritidis. The mutation was introduced by transduction using *aroA554::Tn10* as described in (296). Parent strain: SEn P125109 (PT4). Source: Prof. Paul A. Barrow
SEn D24954	Virulent strain of *S.* Enteritidis isolated from a child in Blantyre, Malawi. Source: Prof. Calman A. MacLennan

Table 2.2 *Salmonella* LPS knockout and LPS chimera strains

Strain	Characteristics and Source
STm D23580 *wbaP*$^-$	Attenuated *S.* Typhimurium strain where the O-antigen (O-Ag) synthesis is inhibited by disruption of the *wbaP (rfbP)* gene. This strain was generated using the λ red-recombination method. Parent strain: STm D23580. Source: Prof. Calman A. MacLennan (140)
SEn P125109 *wbaP*$^-$	Attenuated *S.* Enteritidis strain with disrupted O-Ag. Using the λ red-recombinase method the *wbaP* mutant was generated. Parent strain: SEn P125109 (PT4). Source: Prof. Calman A. MacLennan
STm SL5560 (O:9)	*S.* Typhimurium strain that expresses the O:9 antigen of *S.* Enteritidis. The mutation was obtained through insertion of *hisD8557::Tn10* by transduction from the STm C5, in which the factor 5 of the O-Ag is absent. Parent strain: STm C5 (290)
SEn SL7488 (O:4)	*S.* Enteritidis strain that expresses the O:4 antigen of *S.* Typhimurium. To introduce the mutation transduction via *hisD8557::TnlO* was used. Parent strain: SEn Thirsk (290)

Table 2.3 *Salmonella* Typhimurium OMP knockouts

Strain	Characteristics and Source
RAK82 SL1344	**STm SL1344 *ompR::aph*** Virulent strain of *S.* Typhimurium deficient in OmpR obtained by λ red-recombination. Parent strain: STm SL1344. Source: Dr. Robert A. Kingsley
RAK146 SL3261	**STm SL3261 *ompC::aph ompF::cat*** Attenuated *S.* Typhimurium OmpF and OmpC deficient strain. Parent strain: STm SL3261 *aroA*-. Source: Dr. Robert A. Kingsley
SW515 SL1344	**STm SL1344 *ompA::aph*** Virulent strain of *S.* Typhimurium deficient in OmpA. Parent strain: SL1344. Source: Dr. Robert A. Kingsley
CCD1 SL1344	**STm SL1344 *ompC::aph ompF::cat*** (Described in Chapter 5) Made in conjunction with Dr. Faye C. Morris. Virulent *S.* Typhimurium strain deficient in OmpF and OmpC. Parent strain: STm SL3261 *ompF::cat ompC::aph*
CCD2 SL1344	**STm SL1344 *ΔompC ΔompF wbaP::aph*** (Described in Chapter 5) Made in conjunction with Dr. Faye C. Morris. Virulent *S.* Typhimurium strain deficient in OmpF, OmpC and O-Ag. Parent strain: STm SL1344 *ompF::cat ompC::aph.*

Table 2.4 Genetically modified mice used

Mouse Strain	Characteristics and Source
T-bet$^{-/-}$ (C57B6/J background)	Disruption by the T-bet gene by homologous recombination. Source: Jackson Laboratories (297)
IFNg$^{-/-}$ (C57B6/J background)	Insertion of premature stop codon into the *ifng* gene resulting in no production of interferon gamma. Source: Prof. Richard K. Grencis, University of Manchester (298)
eYFP (GREAT) (C57B6/J background)	Endogenous polyA transcript allele with an IRES-eYFP reporter cassette attached to the *ifng* gene. Source: Jackson Laboratories (299)

2.3 Construction of *Salmonella* mutants

2.3.1 DNA manipulations

2.3.1.1 Preparation of DNA and plasmid isolation

Bacterial genomic DNA was isolated using QIAamp DNA Mini Kit (QIAGEN) in accordance to manufacturer's instructions. Bacterial cell lysates were prepared by boiling the samples for 15 minutes, pelleted, and re-suspended in sterile deionised water (dH_2O). For plasmid isolation, QIAprep Miniprep Spin Kit (QIAGEN) was utilised. A more detailed description of the plasmids used is shown in 2.5.

2.3.1.2 Polymerase chain reaction (PCR)

The PCR primers used are shown in Table 2.6. MyTaq Red Mix x2 (Bioline) was used, according to manufacturer's instructions, with cell lysate template DNA, and 0.4-0.5 µM (final concentration) of each primer. For cloning and mutation purposes Phusion High Fidelity DNA Polymerase (NEB) was used with deoxyribonucleotide triphosphate (dNTP) at a final concentration of 200 µM each. The PCR machine was programmed according to polymerase specifications. For all reactions, a standard annealing temperature of 56 ºC was used. Extension time was determined by the size of the fragment that was being generated (1 minute per kilobase (kb) for MyTaq Red Mix and 30 seconds per kb for Phusion).

Table 2.5 Plasmids used in gene doctoring

Plasmid	Description
pCP20	Temperature sensitive plasmid for the synthesis of Flp. (Ampicillin resistance) (300)
pDOC-C	Cloning plasmid containing I-SceI recognition sites. (Ampicillin resistance) (301)
pDOC-K	Template plasmid for recombination by Flp recognition sites using a Kanamycin resistance cassette. (Kanamycin resistance) (301)
pACBSCE	Recombinant plasmid expressing I-SceI and λ-Red genes upon induction by an arabinose promoter. (Chloramphenicol resistance) (301)

Table 2.6 Primers used in PCR

Primer	Sequence (5'-3')	Use
Gene disruption		
Fwd pDOC-C	TATGCTTCCGGCTCG	For sequencing pDOC-C vector to confirm the cloning of the insert in the corrected direction
Rvs pDOC-C	GGATGTGCAGCAAGG	For sequencing pDOC-C vector to confirm the cloning of the insert in the corrected direction
Fwd pDOC *wbaP*	GAATTCGGATCCTTATTTACATTATGCACGGTCAGAGGGTGAGGATTAAATGGACCGGTCAATTGGCTGGAG	To amplify the kanamycin resistance cassette with 5' homology to the upstream *wbaP* flanking region and an I-SceI site
Fwd pDOC *wbaP*	CATATGGCTAGCCAGTACTTCTCGGTAAGCTTAATACGCACCATCTCGCCGAATATCCTCCTTAGTTCCTATTCC	To amplify the kanamycin resistance cassette with 3' homology to the downstream *wbaP* flanking region and an I-SceI site
Check primers		
Fwd *wbaP* Check flank	GGTTTGGAGAATGAAGGTGC	Flanking (approx. 200 bp upstream) of the *wbaP* gene to confirm the mutation
Rvs *wbaP* Check flank	TCACCGTGTAATAAGGCACC	Flanking (approx. 200 bp downstream) of the *wbaP* gene to confirm the mutation

Fwd *ompC* Check flank	TTTGCGGAGAATGGACTTGC	Flanking (approx. 200 bp upstream) of the *ompC* gene to confirm the mutation
Rvs *ompC* Check flank	ATCCTCATTCGAATGGACGC	Flanking (approx. 200 bp downstream) of the *ompC* gene to confirm the mutation
Fwd *ompF* Check flank	TGTAGCACTTTCACGGTAGC	Flanking (approx. 200 bp upstream) of the *ompF* gene to confirm the mutation
Rvs *ompF* Check flank	CTCAAACATGACGAGGTTCC	Flanking (approx. 200 bp downstream) of the *ompF* gene to confirm the mutation
Fwd Kanamycin	CCTGCAAAGTAAACTGGATG	Internal to the kanamycin cassette, approx. 400 bp inside from the 5' end
Rvs Kanamycin	CATGCTCTTCGTGCAGATCA	Internal to the kanamycin cassette, approx. 400 bp inside from the 3' end
Fwd Chloramphenicol	GTAGAAACTGCCGGAAATCG	Internal to the chloramphenicol cassette, approx. 400 bp inside from the 5' end
Rvs Chloramphenicol	TGTGACGGAAGATCACTTCG	Internal to the chloramphenicol cassette, approx. 400 bp inside from the 3' end

2.3.1.3 Cloning

Purification of DNA was done using a QIAGEN Gel Extraction Kit in accordance to manufacturer's instructions. Prior to this, plasmids and template DNA were digested using Fermentas Fast Digest Enzymes (Thermo Fischer Scientific) and digested plasmids were then treated with alkaline phosphatase. Quantification of both template and plasmid DNA were performed by comparison against known standards prior ligation using T4 DNA Ligase (Invitrogen).

Samples were left at 4 ºC overnight and transformed the next day. Transformants were screened by PCR and/or restriction digests prior to sequence confirmation at the Function Genomics Facility at the University of Birmingham.

2.3.2 Preparation and transformation of electro-competent cells

To prepare competent cells overnight cultures were grown in Luria-Bertani (LB) broth (Sigma-Aldrich) to an OD_{600} of 0.6-1.0 and incubated for 15 minutes at 42 ºC. Cultures were placed on ice for 10 minutes with continuous agitation and centrifuged at 2,880 x g for 30 minutes at 4 ºC. Pellets were re-suspended in cold 20% v/v glycerol with 1mM MOPS and washed three times. Samples were then aliquoted and stored at -80 ºC or used immediately.

For electroporation, electro-competent cells were transferred to a 2 mm chilled electroporation cuvette. Cells were electroporated at 2200 V and 1 ml of LB media was added immediately. Samples were recovered for 1 hour with aeration at the appropriate temperature. The solutions were then centrifuged and plated onto selective LB agar (Sigma-Aldrich).

2.3.3 Gene deletions

2.3.3.1 Gene doctoring

This method was used to generate gene disruption mutants (301). Amplification of the kanamycin resistance cassette from the pDOC-K plasmid was performed using primers with distal I-SceI restriction sites and 5’ and 3’ 40 bases of homology to the flanked DNA sequence to the *wbaP* gene (to be disrupted) and 20 bases of homology to the kanamycin cassette. The enzyme I-SceI was used to digest the

resultant fragments that were previously purified and quantified to be cloned later into the pDOC-C plasmid. Prior to the cloning of the fragment, the pDOC-C plasmid was also digested with I-SceI and alkaline phosphatase treated. The pDOC-C *wbaP* plasmid was generated and the sequence was verified via plasmid to profile sequencing. Both, the pACBSCE and pDOC-C *wbaP* vectors were transformed into electro-competent STm and selected for kanamycin, carbenicillin, and chloramphenicol resistance. Resultant colonies were incubated in LB broth with 0.5% L-arabinose for 5 hours at 37 ºC. Samples were plated on LB agar plates supplemented with 5% sucrose and kanamycin and left overnight at 30 ºC. To confirm the loss of both plasmids resultant colonies were patch plated onto LB agar plates containing carbenicillin and chloramphenicol. Recombination of the resistance cassette was verified by PCR.

2.3.3.2 P22 phage transduction

To transfer mutations between strains and construct multiple gene disruption mutants in the same background P22 transduction was utilised. An squematic overview of the P22 transduction technique used to generate the mutants in Chapter 5 is shown in Figure 5.1. Briefly, overnight cultures (selected with the appropriate antibiotics) harbouring the mutations to be transferred, were inoculated with varying concentrations of the phage (courtesy of the Henderson lab) for 20 hours. The following day, phage lysates were harvested by the addition of chloroform prior to centrifuging at 3,000 x g for 15 minutes at 4 ºC. Recipient bacterial strains were incubated with varying amounts of phage lysate for 15 minutes, prior to the addition of 1 M sodium citrate and LB before incubating for a further 45 minutes. Bacteria

were then pelleted and washed with 1 x Phosphate Buffered Saline (PBS) three times and plated on agar with the appropriate antibiotics.

2.3.3.3 Removal of antibiotic resistance cassette

The removal of the kanamycin resistance cassette was performed using pCP20 and flippase (Flp) recombinase system (300). Electro-competent cells were prepared (as mentioned elsewhere) and transformed with pCP20. Resultant colonies were selected and grown overnight on LB agar plates containing carbenicillin at 30 ºC. The next day, resultant colonies were incubated in LB broth for 6 hours at 42 ºC and plated onto LB agar plates containing no antibiotics. The following day resultant colonies were patch plated onto LB agar with the corresponding antibiotics to confirm loss of the resistance cassette and pCP20. Colonies were confirmed by PCR.

2.3.4 Detection of Lipopolysaccharide (LPS) O-antigen (O-Ag)

The method described by Browning et al. (302) was followed to detect LPS O-Ag in the *Salmonella* strains. Briefly, overnight inoculums were centrifuged at 14,000 x g for 10 minutes and pellets re-suspended in 2x Laemmli buffer. Cells were lysed by repeated freeze thaw cycles prior to centrifugation at 14,000 x g for 2 minutes and treated with 5 mg/ml of Proteinase K for 1 hour. Samples were analysed on a 4-12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen) and visualised with a Silver Staining Kit (Invitrogen) following the manufacturer's instructions.

2.4 Antigens (Ags)

2.4.1 Antigens used for immunisation

All immunisations were given through the intraperitional (i.p.) route. Protocols of immunisation and concentrations used throughout each experiment are specified in the corresponding results section. Antigens used for immunisation or challenge studies were:

1. Purified OmpD from *S.* Typhimurium (STm). The STm-OmpD was kindly provided by Dr. López-Macías (Medical Research Unit on Immunochemistry, Specialties Hospital, National Medical Centre "Siglo XXI" IMSS, Mexico City, Mexico). The STm-OmpD was purified from the STm strain SL3261 *ompC::aph ompF::cat* (Table 2.3). Before immunisation, the STm-OmpD was diluted in sterile PBS (Sigma-Aldrich) according to the stated concentration. A complete protocol of the methodology used to purify STm-OmpD is in Appendix B.

2. Bacterial strains (Table 2.1 and 2.2). The O:9 and O:4 mutant strains were utilised in challenge studies done in conjunction with the UCT in Cape Town.

2.4.2 Antigens used for *in vitro* experiments

Antigens used for analysis *in vitro* in this study were:

1. Outer membrane proteins (OMPs). Purification of OMPs was performed by Ms. Charlotte N. Cook at the University of Birmingham. Preparations were made by 2% Triton X-100 extraction (303).

2. Purified porins of STm were kindly given by Dr. López-Macías. Preparations were obtained from STm strain ATCC 14028.

3. Bacterial strains (Table 2.1 and 2.2). Cultures from *Salmonella* were set up overnight in a loosely capped universal tube with 10 ml of LB broth. Whole antigen preparations were done as previously described (287). Whole organisms were homogenised in sterile PBS containing 0.05% sodium azide (NaN_3). The bicinchoninic acid assay (BCA) was used to quantify the antigen concentration (Appendix C).

For the complete methodology of extraction and purification of OMPs and STm-porins refer to Appendix B.

2.5 Preparation of bacteria for immunisation

A single colony of *Salmonella* was incubated at 37 ºC in 10 ml of LB broth, in a universal tube with a loosened cap overnight. When the culture reached an OD_{600} of 0.8-1.0 (SL3261) or OD_{600} of 0.6-0.7 for the other strains, 1 ml of bacteria was harvested by centrifugation at 6,000 x g for 5 minutes at 4 ºC. Cells were washed and re-suspended in sterile PBS at 6,000 x g for 5 minutes at 4 ºC twice and re-suspended in a final volume of 1 ml of PBS. For an infectious dose, bacteria were diluted in a final volume of 200 µl of PBS per mouse. A dose of $1x10^5$ or $5x10^5$ bacteria/ml was given i.p. per animal.

2.6 Time-course and experiments end-point

Mice were anaesthetised and exsanguinated by cardiac puncture and Schedule 1 method at the indicated end points according to the Home Office Regulation (Chapters 3 and 4).

2.7 Separation of serum from blood

Blood from mice was obtained by cardiac puncture and left at 37 ºC for one hour to clot. Blood was centrifuged at 6,000 x g for 10 minutes and serum was alliquoted and stored at -20 ºC. Sera were used for Enzyme-linked Immunosorbent Assays (ELISAs) and Serum Bactericidal Assays (SBAs).

2.8 Preparation of sera for opsonisation assay

Sera from non-immunised (NI) or STm-OmpD immunised mice were heated at 56 ºC for 30 minutes to denature complement. The STm SL3261 was prepared as described in section 2.5 and mixed in a 1:100 dilution with the heat-inactivated mice sera. Samples were then mixed at room temperature for 1 hour before infection. Bacteria were plated onto agar plates to assess clumping and bacterial viability.

2.9 Enzyme-linked immunosorbent assay (ELISA)

This assay was performed to identify antigen-specific antibodies (Abs) after immunisation with STm-OmpD and STm post-infection. The specificity of the Abs was tested against *Salmonella* outer membrane proteins (OMPs), STm porins and whole bacteria.

Flat-bottomed 96 well NUNC plates (Thermo Fisher Scientific) were coated in carbonate coating buffer (Appendix A) at 5 µg/ml or 10 µg/ml, with proteins or bacteria respectively, and left at 4 ºC overnight. Bovine Serum Albumin (BSA)(Sigma-Aldrich) 1% with 1x PBS was used to block the plates. Plates were blocked at 37 ºC for 1 hour and washed with 0.05% Tween 20 in 1x PBS. Sera were

serially diluted in blocking buffer, the starting concentration was 1:50 or 1:100, and incubated as before. Plates were washed and alkaline phosphate (AP)-conjugated goat anti-mouse secondary Abs were added: IgG or IgG1 (1:1000) and IgG2b, IgG2c, IgG3 or IgM (1:2000)(Southern Biotech). Plates were incubated and washed again. Following this, *P*-nitrophenyl phosphate (pNPP) tablets (Sigma Fast) were used for colour development and plates were read on an Emax microplate reader (Molecular Devices) at λ405 nm using SoftMax pro software version 5.4.5.

2.10 Serum bactericidal assay (SBA)

2.10.1 Growth of bacteria to stationary and log phases

One colony of *Salmonella* was added to 10 ml of LB broth and left incubating overnight at 37 ºC in a universal tube with loose cap to reach stationary phase next day. Logarithmic phase was prepared by diluting 100 µl of the stationary phase culture to 10 ml of LB broth that was previously warmed. The inoculum was incubated at 37 ºC, in a tightly capped universal tube for 2 hours on a rocker plate at 20 rpm. A predetermined volume of the culture was centrifuged for 5 minutes at 3,300 x g. The cell pellet was washed and re-suspended with PBS twice. Finally, the pellet was re-suspended in PBS to reach the concentration desired. The method of Miles and Misra was used to determine the final concentration of the inoculum (304).

2.10.2 Preparation of sera used in SBAs

2.10.2.1 Heat-inactivation of human serum

To inhibit complement function, human serum was inactivated at 56 ºC in a water bath for 1 hour and stored at -80 ºC prior to use (38).

2.10.2.2 Depletion of *Salmonella*-specific antibodies in human serum

A culture of the serovar of *Salmonella* required for the assay was set overnight at 37 ºC in the appropriate volume of LB broth. The following day, bacteria were washed thrice and re-suspended in PBS until the final desired volume of 1ml was acquired. For the depletion, 100 µl of the bacteria were mixed with 900 µl of human serum and incubated at 750-1000 rpm for 1 hour at 4^{o}C. Serum was centrifuged at 3,300 x g for 5 minutes and this first cycle was repeated for other 2-5 times depending on the *Salmonella* serovar used. At the end of the last cycle, serum was filtered using a 0.22 µm syringe filter and stored at -80 ºC.

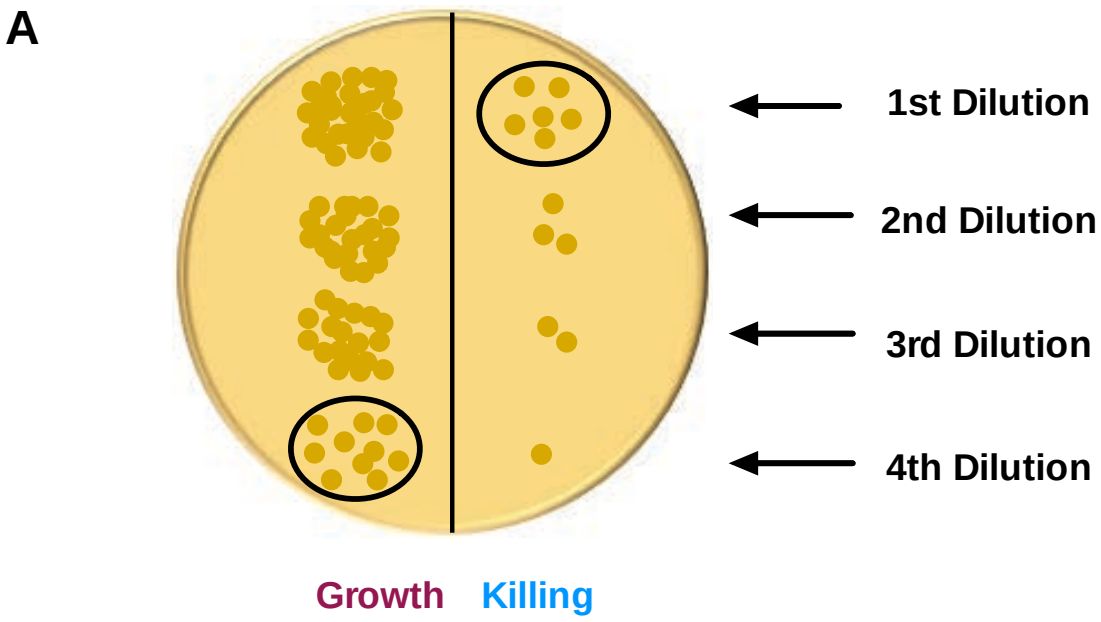
2.10.3 Analysis of mice sera in SBAs

Serum bactericidal assays were performed as described (38). Human sera described in section 2.9.2 were used as controls for these assays because mice complement is not effective for bactericidal killing and needs to be supplemented with human complement to induce killing (278).

Sera from NI or STm-OmpD immunised mice were added and supplemented with antibody-depleted human serum as a complement source (1:2-1:18 ratio to give a final volume of 45 µl). Sera from NI mice were used as controls and supplemented with the human complement source to demonstrate that anti-STm-OmpD Abs were responsible for inducing killing after immunisation and not human complement.

A volume of 5 µl of *Salmonella* in logarithmic (log) growth phase (1x10^{7} CFU/ml), that was prepared as shown in section 2.10.1, was added to 45 µl of complete human serum and the other samples in a round-bottomed 96 wells plate (Sarstedt). Heat-inactivated human serum (HiS) and antibody-depleted human serum were added in a

1:1 proportion with PBS. Plate was incubated at 37 ºC at 20 rpm on a rocker plate and 10 µl of each sample were serially diluted and plated in triplicate on LB agar plates at different time points (45, 90, and 180 minutes). At the end of the assay, agar plates were incubated at 37 ºC overnight and colonies were counted the following day. Calculations were made by subtracting the starting inoculum concentration of the bacteria from the concentration established at the different time points. Growth or killing of the bacteria was represented graphically by transforming the data into $\log_{10}$. Bacterial growth was determined by the increase of bacterial numbers from the starting concentration whereas a decrease in the quantity of the bacteria indicated killing of the pathogen (Figure 2.1).



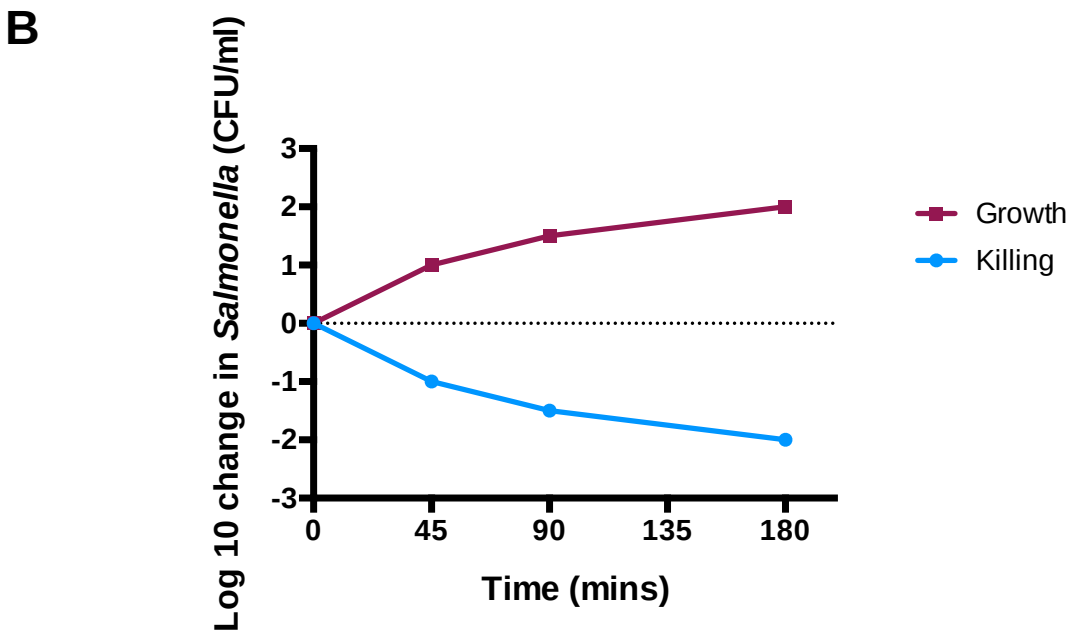


Figure 2.1 Illustrative representation of the SBA analysis
(A) Representative figure of how serially diluted samples are plated onto agar plates. This is done by triplicate. The dilution resulting in approximately 10 or more colonies should be counted and calculations are obtained by making a subtraction of the starting inoculum minus the concentration of the tested sample established at the different time points. Data is transformed into $\log_{10}$ to be represented in a graph. (B) SBA analysis showing bacterial growth (burgundy) and bacterial killing (blue) from 0 to 180 minutes.

2.11 Isolation of single cell suspensions from the spleen and peritoneal exudate cells (PEC)

Spleens were disrupted and filtered through a 70 µm nylon cell strainer (BD Biosciences) in 5 ml of normal RPMI-1640 media (Sigma-Aldrich) or RPMI supplemented with 10% heat-inactivated foetal bovine serum (HiFBS) and 5% penicillin/streptomycin (R-10 media) Cells were then centrifuged at 375 x g for 4 minutes at 4 ºC and red blood cells lysed for 1-3 minutes at room temperature using ammonium chloride potassium (ACK) lysing buffer (Gibco, Life Technologies). Cells were then washed as before and re-suspended to $1x10^7$ cells/ml in RPMI or R-10 media. Cell density was determined for live cells using trypan blue and quantified using fast read counting slides (Immune Systems). The total number of cells per sample was obtained.

Cells were washed and volumes were adjusted to the desired cell density according to the calculations obtained. Samples were kept on ice until needed.

Cells from the peritoneal cavity were collected from mice by injecting 5 ml of PBS into the cavity using a 21 g syringe and extracting the fluid carefully back onto the syringe. The suspension was then transferred to a falcon tube and kept on ice. Cells were then harvested in PBS fluorescence-activated cell sorting (FACS) buffer (Appendix A) at 375 x g for 4 minutes at 4 ºC. When needed, ACK lysing buffer was used.

2.12 Enzyme-linked immunoSpot (ELISPOT)

To assess the antibody specific response to specific Ags, B cell ELISPOT analysis was followed as detailed (305). ELISPOT plates (Merck Millipore) were coated with 5 µg of STm-porins, STm-OmpD or LPS in PBS at 4 ºC overnight. The following day, plates were washed with PBS and blocked with R-10 media. Spleens were processed as described in section 2.11. Spleen cells were plated in triplicate at a concentration of $5x10^5$ per well. Cells were incubated for 6 hours at 37 ºC without agitation. Plates were then washed with 0.05% PBS Tween 20. For detection Abs, AP-conjugated anti-IgG and anti-IgM were used (1:1000)(Southern Biotech). The AID ELISPOT Reader System and AID Software version 3.5 (Autoimmune Diagnostika) were used to count the spots. Cell counts were expressed as spot-forming units (SFUs) per $1x10^6$ cells.

2.13 Flow cytometry

2.13.1 Digestion of spleens with collagenase D

For detection of myeloid and dendritic cells (DCs) spleens were digested using Collagenase D (Roche) prepared at a concentration of 100 µg/ml in normal RPMI medium. Spleens were digested with 1 ml of the enzyme and incubated for 20 minutes at 37 ºC. The collagenase activity was stopped with 0.5 mM EDTA (200 µl) and tissues were processed as mentioned in section 2.11.

2.13.2 Extracellular staining

Cells were re-suspended in FACS buffer (Appendix A) prior to staining at a density of 10^6 cells/sample in a 96 v-bottom well plate and centrifuged at 375 x g for 4 minutes

at 4 ºC. Anti-CD16:CD32 (eBioscience) was used to block cell CD32/FcγIII and CD16/FcγII receptors for 20 minutes on ice before starting the staining. All Abs were prepared in FACS buffer at the stated concentrations in Table 2.7. Cells were pelleted and washed in FACS buffer prior to the addition of 50 µl of the antibody cocktail. Where necessary, biotinylated Abs were incubated and washed twice prior to incubation with secondary and streptavidin-conjugated Abs. Samples for compensations were treated separately and PBS was added to the unstained control. Cells were incubated on ice for 20 minutes and then washed twice at 375 x g for 4 minutes at 4 ºC. When needed, cells were fixed in FACS buffer with 0.1% v/v paraformaldehyde and stored at 4 ºC. Samples were re-suspended in 200 µl of FACS buffer and transferred into FACS tubes prior acquisition. Samples were acquired in a Cyan FACS Analyser (Dako) and analysed in FlowJo 8.7 (Treestar).

Table 2.7 Antibodies used for FACS

Specificity and Clone	Fluorochrome	Concentration	Dilution	Source
Block FcγII and FcγIII receptors				
CD16:CD32 93	Purified	0.5 mg/ml	1:150	eBioscience
Extracellular Staining				
B220 (CD45R) RA3-6B2	PE	0.2 mg/ml	1:200	Pharmingen
B220 (CD45R) RA3-6B2	PE Texas Red (PE-CF594)	0.2 mg/ml	1:250	BD Horizon
B220 (CD45R) RA3-6B2	Brilliant Violet 510	100 µg/ml	1:200	BioLegend
CD3e 145-2C11	FITC	0.5 mg/ml	1:100	eBioscience
CD3e 145-2C11	PerCP Cy5.5	0.2 mg/ml	1:300	BD Pharmingen
CD3e 17A2	Brilliant Violet 510	100 µg/ml	1:100	BioLegend
CD4 RM4-5	PE	0.2 mg/ml	1:200	eBioscience
CD4 RM4-5	Pacific Blue (eFluor 450)	0.2 mg/ml	1:100	eBioscience
CD5 53-7.3	PE Cy5	0.2 mg/ml	1:100	BD Pharmingen
CD8 53-6.7	PE	0.2 mg/ml	1:200	eBioscience
CD8 53-6.7	APC	0.5 mg/ml	1:300	eBioscience
CD11b M1/70	Biotin	0.5 mg/ml	1:300	eBioscience
CD11b M1/70	PE	0.2 mg/ml	1:200	eBioscience
CD11b M1/70	Pacific Blue (eFluor 450)	0.2 mg/ml	1:200	eBioscience
CD11b M1/70	APC	0.2 mg/ml	1:1000	eBioscience
CD11c HL3	PE Cy7	0.2 mg/ml	1:300	BD Pharmingen
CD19 1D3	APC Cy7	0.2 mg/ml	1:100	BD Pharmingen

CD21/CD35 7G6	FITC	0.5 mg/ml	1:100	BD Pharmingen
CD21/CD35 7G6	APC	0.2 mg/ml	1:1000	BD Pharmingen
CD23 B3B4	PE	0.2 mg/ml	1:200	BD Pharmingen
CD44 IM7	PerCP Cy5.5	0.2 mg/ml	1:250	eBioscience
CD62L MEL-14	PE	0.2 mg/ml	1:200	eBioscience
CD64 X54-5/7.1	PE	0.2 mg/ml	1:200	BioLegend
CD138 281-2	APC	0.2 mg/ml	1:200	BD Pharmingen
F480 BM8	APC	0.2 mg/ml	1:300	eBioscience
FAS (CD95) Jo2	PE Cy7	0.2 mg/ml	1:300	BD Pharmingen
GL7	Pacific Blue	0.5 mg/ml	1:100	BioLegend
IgD 11-26c	Pacific Blue (eFluor 450)	0.2 mg/ml	1:200	eBioscience
IgM II/41	PE Cy7	0.2 mg/ml	1:200	eBioscience
Ly6C AL-21	PerCP Cy5.5	0.2 mg/ml	1:300	BD Pharmingen
Ly6G (GR1) RB6-8C5	PE	0.2 mg/ml	1:200	eBioscience
MHC II M5/114.15.2	APC	0.2 mg/ml	1:1000	eBioscience
NK1.1 PK136	PE Cy7	0.2 mg/ml	1:200	BD Pharmingen
Streptavidin	PE Texas Red	0.2 mg/ml	1:300	BD Pharmingen
TCR beta H57-597	PerCP Cy5.5	0.2 mg/ml	1:300	eBioscience

2.14 Bacterial culture from tissues

To determine the numbers of bacteria present after *Salmonella* infection, tissues were disrupted in 1 ml of sterile RPMI medium using 70 µm cell-strainers (BD Biosciences). The suspensions were serially diluted in RPMI and plated on LB agar. Plates were incubated at 37 ºC overnight. Bacterial colonies were counted the next day and the colony-forming unit (CFU) per organ was determined.

For bacterial culture of the blood and peritoneal cavity bacterial numbers were obtained according to the volume of sample acquired and dilution plated.

2.15 Clodronate-coated liposomes treatment

Liposomes can be used vehicles to deliver clodronate into the phagocytic cells causing irreversible damage to the cell, which then dies by apoptosis (306-308). This method was used to deplete macrophages from the spleen and the liver. Mice were given 200 µl i.p. of either PBS control liposomes or clodronate liposomes 24 hours before STm infection.

2.16 Depletion of GR1+ cells

To inhibit expression of GR1+ cells, 500 µg of purified anti-mouse GR1 rat IgG2b (Clone RB6-8C5)(BioXcell). Due to rapid cell turnover, the antibody was administered i.p to mice 48 and 24 hours before infection with STm to maintain cell depletion. The control group was treated with the same dose of IgG from rat serum (Sigma). All mice were infected for 24 hours.

2.17 Statistical Analysis

Analysis of the data was performed using GraphPad Prism Version 6.0. The Mann-Whitney U test was applied to determine differences in non-parametric data where mean and standard deviation were used. One-way ANOVA test was used for comparison between multiple groups.

CHAPTER 3: THE ROLE OF LPS IN OCCLUDING ACCESS OF ANTIBODY TO THE BACTERIAL SURFACE

3.1 Introduction

Effective vaccines typically require of pre-existing antibody (Ab) that prevents bacteria from establishing infection. For intracellular pathogens, such as *Salmonella,* clearance is attributed to cell-mediated responses (223, 309, 310). Nevertheless, before they can infect the cells, Ab can control the infection by stopping the bacteria from entering other cells and making the pathogen more susceptible to killing by phagocytes (275).

Antibody-mediated immunity requires protective antigens (Ags) to be exposed on the surface of the bacteria. Protein Ags such as flagellin, Vi-Ag, O-Ag, and porins have been assessed as subunit vaccines candidates against the different *Salmonella* serovars (75, 130, 141, 142). While flagellin and O-Ag are antigenically variable between the *Salmonella* serovars, porins are highly conserved (10).

In mice, immunisation with purified porins from STm impairs infection in the spleen and blood after STm challenge, and OmpD is a major protective target amongst the porins (72). The protection afforded after immunisation with porins is B cell and Ab mediated, while T cells play a complementary role by promoting switching to IgG for optimal protection (72).

Antibodies to different OMPs show some cross-reactivity with homologous proteins from the cell wall of different *Enterobacteriaceae,* which indicates the possibility of

using conserved shared antigens amongst different bacterial serovars as potential vaccines (311-313). Nevertheless, it was shown previously that immunisation with porins from *S.* Typhi did not cross-protect against STm in mice and anti-*S.* Typhi antibodies from mice and humans sera did not cross-react with STm (60, 61). Furthermore, impaired killing of anti-sera to *Escherichia* coli was observed against heterologous enteric bacteria with the same shared Ag but not against homologous organisms where bactericidal activity was restored (314, 315). The latter suggests the importance of other molecules, such as LPS, for limiting exposure of epitopes of common Ags in different enterobacterial strains.

Porins, including OmpD, are highly thermostable, immunogenic, and contain numerous cell surface exposed epitopes (10, 64). Porins interact closely with the LPS on the outer membrane of the bacteria. LPS has been identified as a potentiator of the immune response to these proteins when used as an adjuvant (288, 316-318). Nevertheless, it has been observed that the immune response to conserved antigens is augmented when some LPS components are lost (311).

The aim of this chapter was to determine how Ab to a known protective Ag, OmpD binds to the surface of Gram-negative bacteria and how LPS influences this binding. This was examined against STm and the closely related serovars SEn.

3.2 Results

For all experiments in Chapter 3, otherwise stated, WT (C57BL6/J) mice were immunised with 20 µg of STm-OmpD as pictured below:

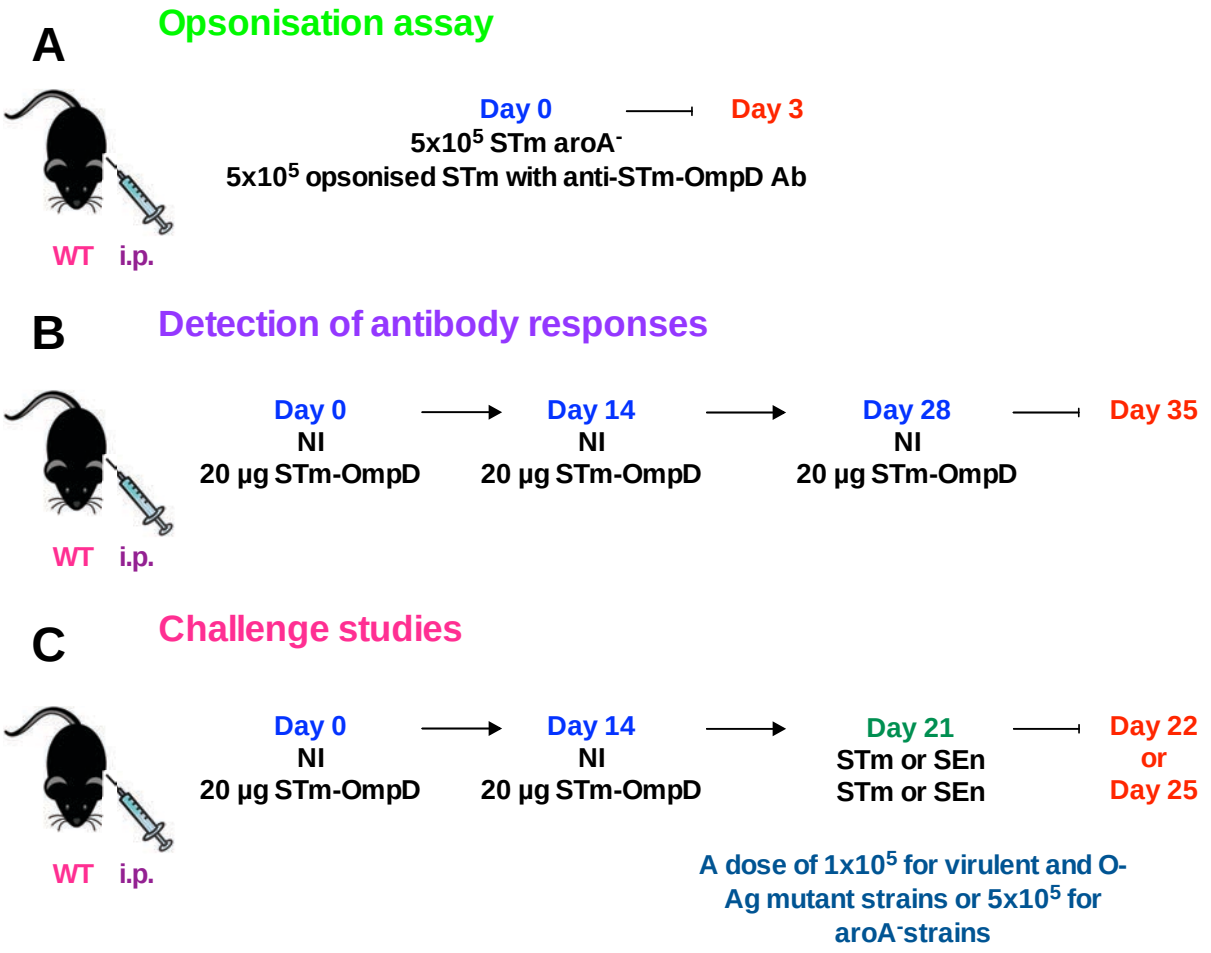


Figure 3.1 Protocols used to immunise mice with STm-OmpD throughout Chapter 3
(A) For opsonisation assays mice were immunised via i.p. either with $5x10^5$ of SL3261 or bacteria previously opsonised with STm-OmpD antibodies (Abs); bacterial numbers were quantified at day 3. (B) To obtain STm-OmpD hyperimmune sera WT mice were primed at day 0 and boosted at day 14 and 28 with 20 µg of STm-OmpD i.p. (C) For challenge studies WT mice were primed at day 0 and boosted at day 14 with 20 µg of STm-OmpD i.p. and challenged at day 21 for 1 day (virulent and O-Ag mutant strains) or 4 days (attenuated strains).

3.2.1 Immunisation with STm-OmpD induces Ab that can impair infection against STm

We have previously shown that immunisation with porins confers protection against iNTS in mice and that OmpD is the porin responsible for the majority of this protection (72). In order to examine the response to OmpD alone (≈ 34-40 KDa), the protein was purified from a STm strain in which the other two major porins, OmpC (36 KDa) and OmpF (35 KDa), were absent (Fig. 3.2A). STm-OmpD was kindly provided by Dr. López-Macías (IMSS, Mexico). To assess the protective capacity of antibody (Ab) generated after immunisation with STm-OmpD we conducted an opsonisation assay using sera from mice that were previously immunised with STm-OmpD. Mice infected with bacteria that were opsonised with anti-STm-OmpD Ab showed a 100-fold reduction in bacteria in the spleen and liver compared to those mice, which were only infected with intact bacteria for 3 days (Fig. 3.2B and C). Therefore, Ab to OmpD is sufficient to protect against STm infection.

3.2.2 Ab to STm-OmpD kills STm lacking other porins

To test if STm-OmpD Ab could directly promote killing of STm, including STm strains lacking other porins, SBAs were performed using sera from STm-OmpD immunised and NI mice against the new SL1344 mutant lacking OmpC and OmpF (described in Chapter 5) and different other mutants lacking other proteins such as OmpA and OmpR (Fig. 3.3). In all mutants tested killing was observed in this complement dependent assay. Sera to STm-OmpD but not naive sera promoted killing of all strains tested. Therefore, Ab to STm-OmpD is able to kill STm in multiple assays.

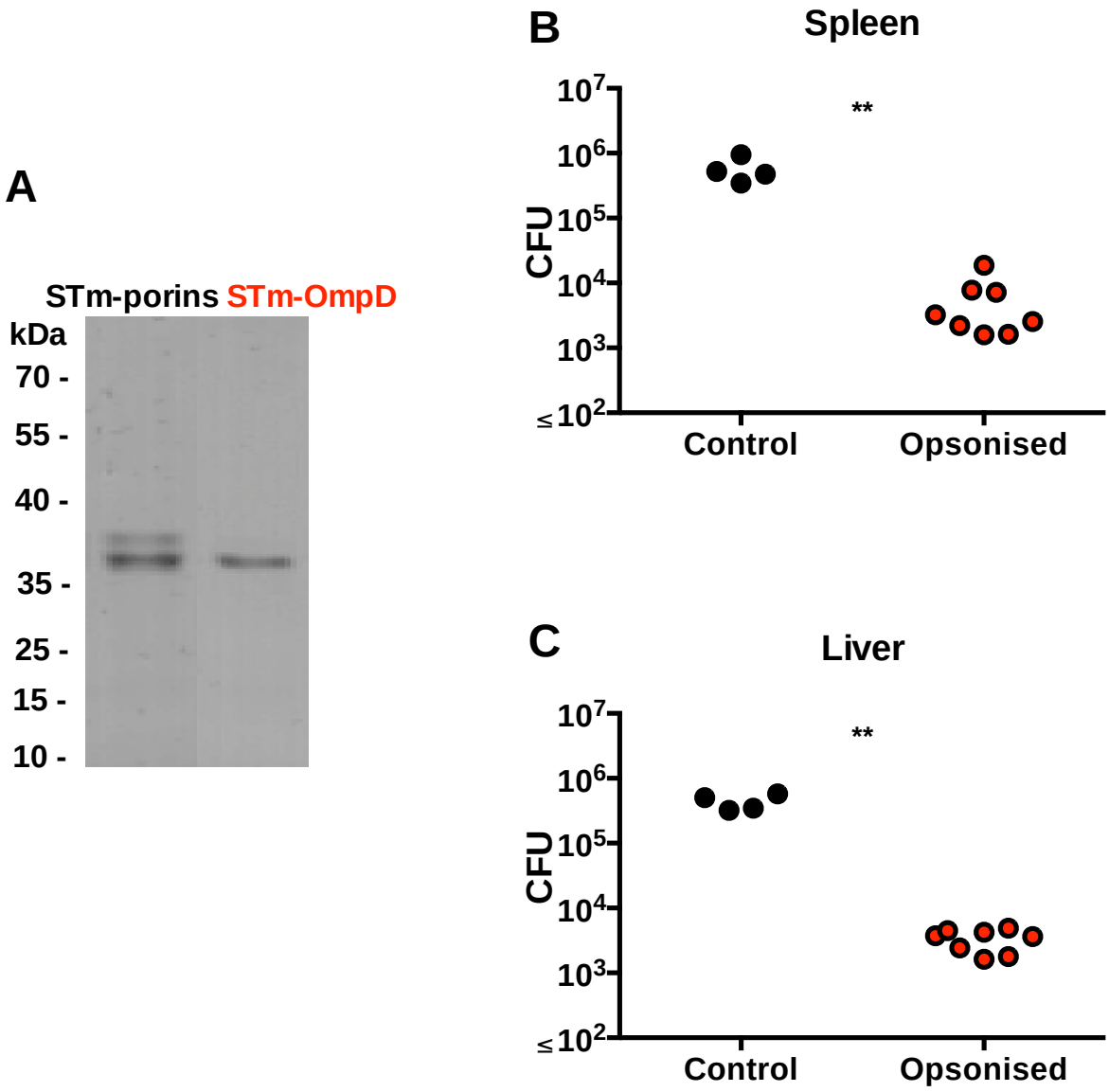


Figure 3.2 Ab to STm-OmpD plays an important role in conferring protection after STm-OmpD immunisation in mice
(A) Purified preparations of STm-porins (left) and STm-OmpD (right)(≈ 34-40 KDa) (kindly given by Dr. López-Macías) stained with Coomassie blue on a 15% SDS-PAGE. Samples were adjusted to a concentration of 1.1 µg. (B) Mice were infected i.p. with $5x10^5$ of either STm SL3261 or bacteria that was previously opsonised with STm-OmpD antibodies and bacteria numbers were quantified at day 3 from spleen and liver. Data is from one experiment and each dot represents one mouse. ** = $P \leq 0.01$.

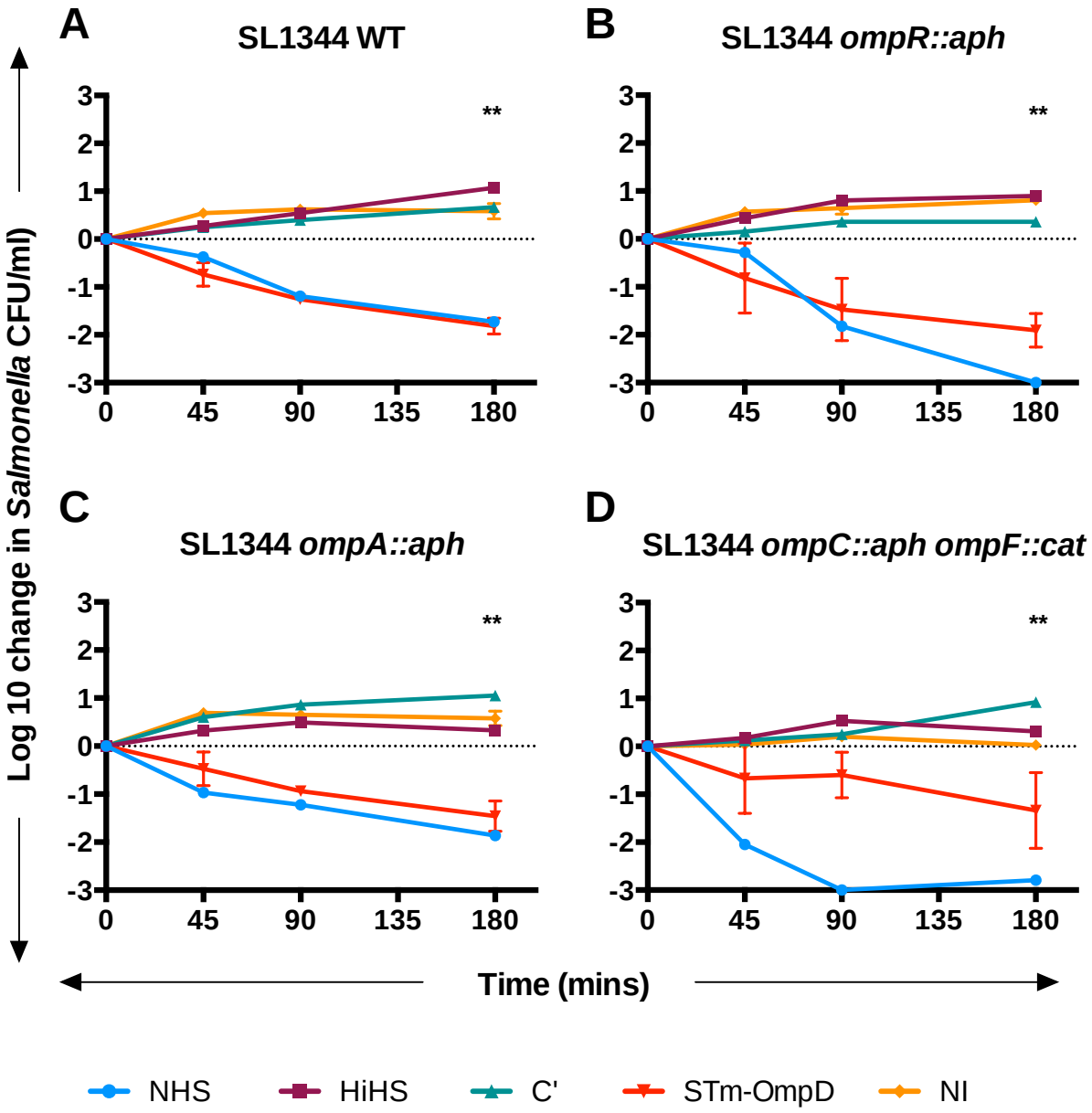


Figure 3.3 Loss of other porins in STm does not affect killing of STm SL1344 via complement-mediated killing

Serum bactericidal assays of (A) SL1344 WT, (B) SL1344 *ompR* KO, (C) SL1344 *ompA* KO, and (D) SL1344 *ompC ompF* KO performed at 45, 90, and 180 minutes where a concentration of 10^6 CFU/ml was used. Growth or killing of the bacteria by non-immune (yellow) and STm-OmpD immune (red) mice sera is shown in the graphs. Mice sera were supplemented with human complement. Normal human serum (NHS)(blue), pre-adsorbed human serum (C')(green) and heat-inactivated human serum (HiHS)(green) were used as positive and negative controls. Data are representative of 2 different experiments and represent the mean of 5-6 mice sera used. Difference in growth at 180 minutes between immune and non-immune mice sera was determined by Mann-Whitney U test. ** = $P \leq 0.01$. *aph* = kanamycin resistance gene, *cat* = chloramphenicol resistance gene.

3.2.3 Immunisation with STm-OmpD induces a rapid IgG response

We then assessed the immune response to STm-OmpD by examining the response induced to itself after immunisation and to LPS as a control Ag. To do this mice were immunised with either PBS, 20 µg STm-OmpD or infected with STm SL3261. We determined the number of IgM^+ and IgG^+ antibody secreting cells (ASCs) to STm-porins and STm-OmpD 7 days following immunisation/infection. LPS-specific ASCs were determined in parallel (Fig. 3.4). Both IgM^+ and IgG^+ ASCs were detected to STm-porins and STm-OmpD but no IgG^+ cells were observed to LPS in mice immunised with STm-OmpD. After infection with STm IgM^+ ASCs were detected in mice infected with STm to all Ags, but at levels lower than those observed from STm-OmpD immunised mice (Fig. 3.4) In addition, Ab titres in these mouse sera were assessed against STm-porins. This showed an induction of IgM and IgG in both immunised and infected groups at day 7 (Fig. 3.5A). However all IgG isotypes with the exception of IgG1 were detected at day 7 against STm-porins in both groups of mice, and almost undetectable levels of IgG2c were detected in immunised mice (Fig. 3.5B).

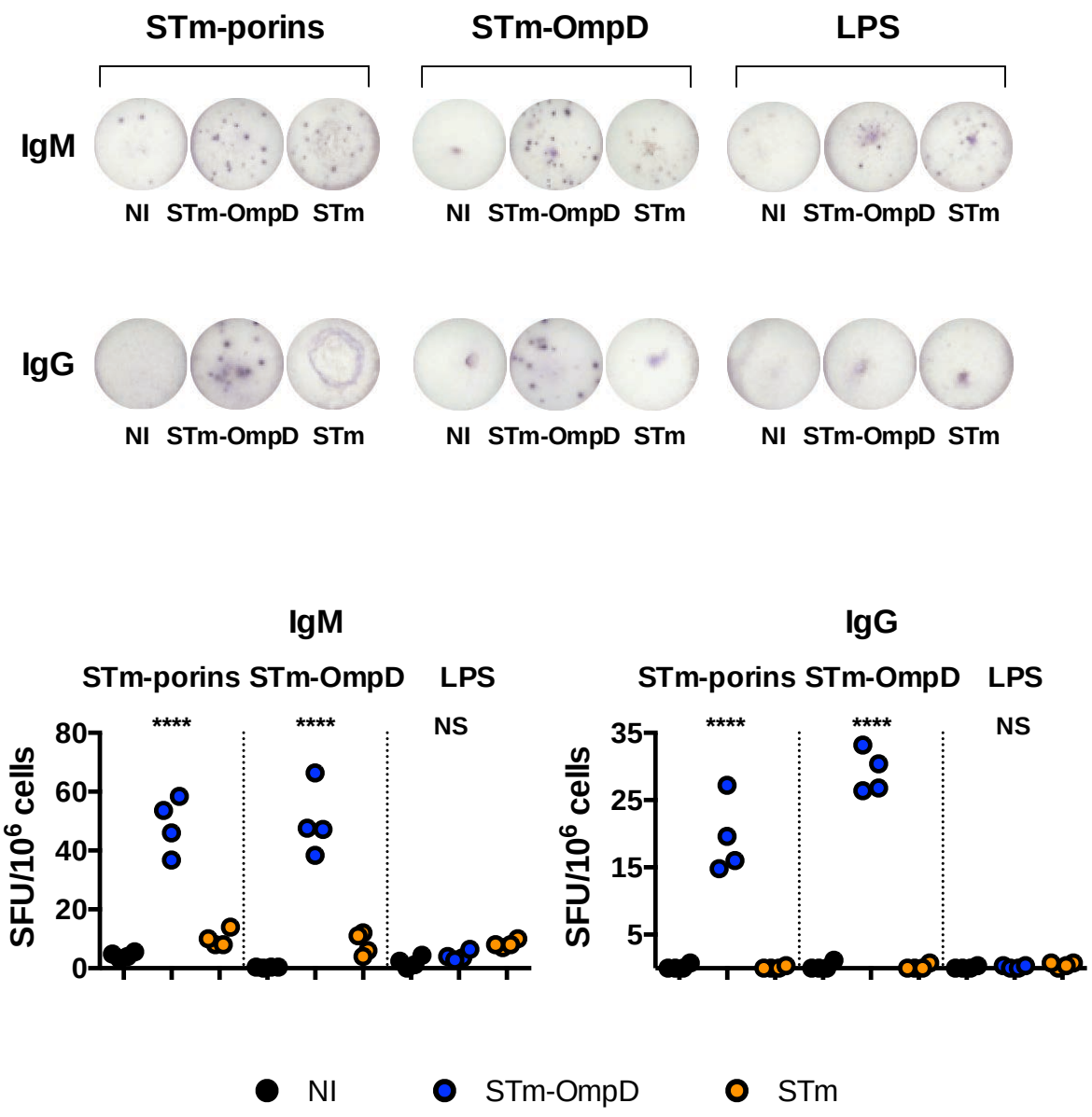


Figure 3.4 Response of splenic antibody secreting cells (ASCs) is specific to STm-porins and STm-OmpD but not to LPS in STm-OmpD immunised mice
ELISPOT analysis of specific IgM (bottom left) and IgG (bottom right) response to STm-porins, STm-OmpD, and LPS in non-immunised (NI) mice (black circles) and mice immunised with 20 µg of STm-OmpD (blue circles) or infected with $5x10^5$ of STm SL3261 (orange circles) for 7 days. Data is representative of 2 independent experiments where 4 mice per group were used. One-way ANOVA test was used to determine statistical difference between the 3 groups. **** = $P \leq 0.0001$, NS = non-significant, SFU = spot-forming units.

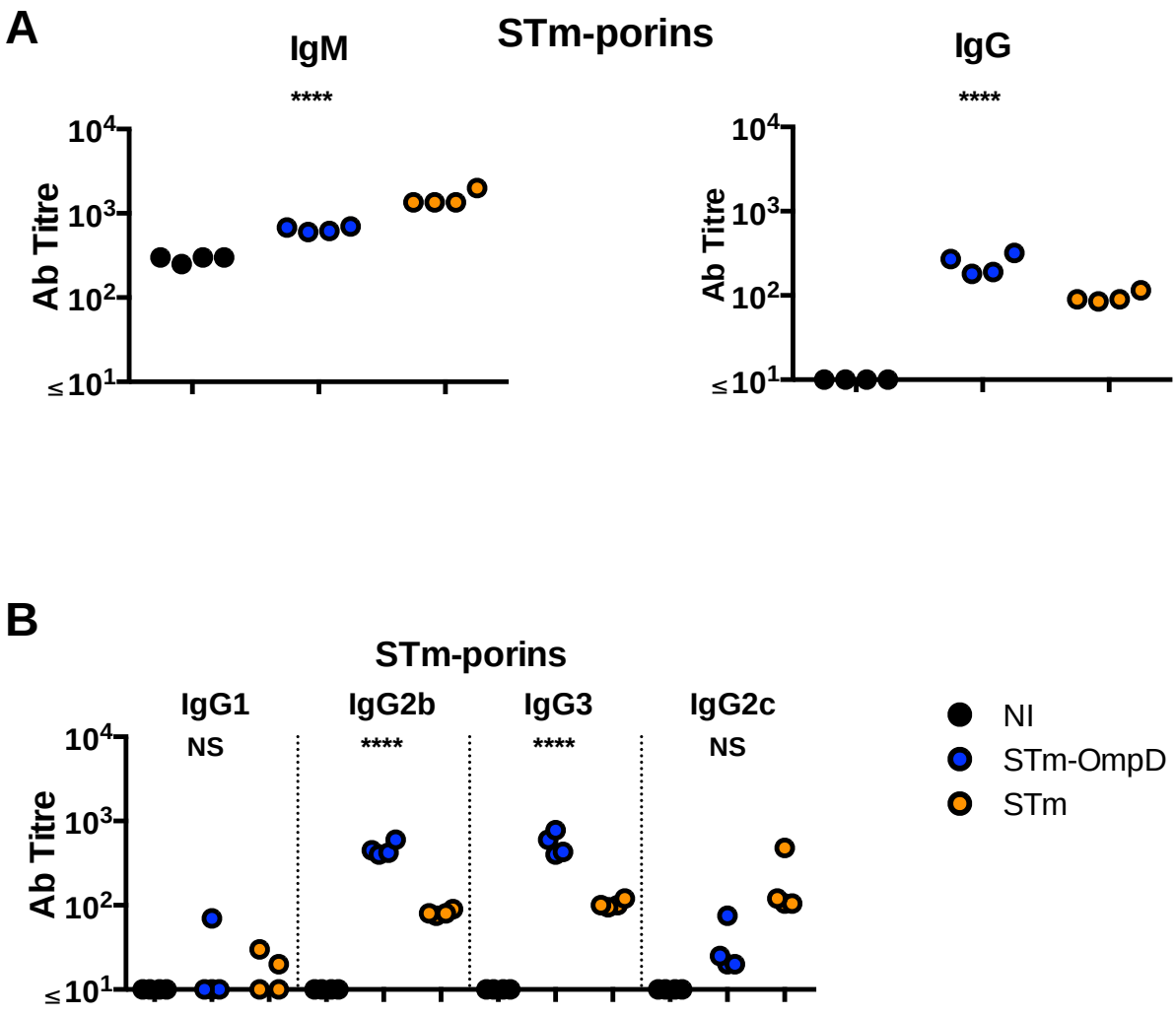


Figure 3.5 Induction of Ab to STm-porins 7 days after immunisation with STm-OmpD includes IgM and different IgG subclasses but not IgG1
Mice were immunised with PBS, 20 µg of STm-OmpD or infected with $5x10^5$ STm SL3261 i.p. for 7 days. Anti-STm porins Ab titres for (A) IgM and total IgG, were assessed by ELISA. (B) IgG subclasses were also detected. Data is representative of 2 different experiments where 4 mice per group were used. Non-immunised (NI) and mice infected with STm were used as control. ONE-Way ANOVA test was used to determine statistical difference between all the groups. **** = $P \leq 0.0001$, NS = non-significant.

3.2.4 Robust Ab responses are induced to porins, OMPs and intact bacteria in mice primed and boosted with STm-OmpD

Our results showed the importance of Ab for the protection afforded by STm-OmpD immunisation and so we examined the response to STm-porins, total OMPs and whole bacteria in mice that had been immunised with STm-OmpD three times. IgG was detected to all Ags, whilst IgM was not increased or only marginally increased above background (Fig. 3.6). Moreover, ELISAs showed that all IgG isotypes were detected to all the three Ags (Fig. 3.7). Therefore, after prime early immunisation IgG2b and IgG3 are induced to STm-OmpD but after multiple immunisations all IgG isotypes are detected.

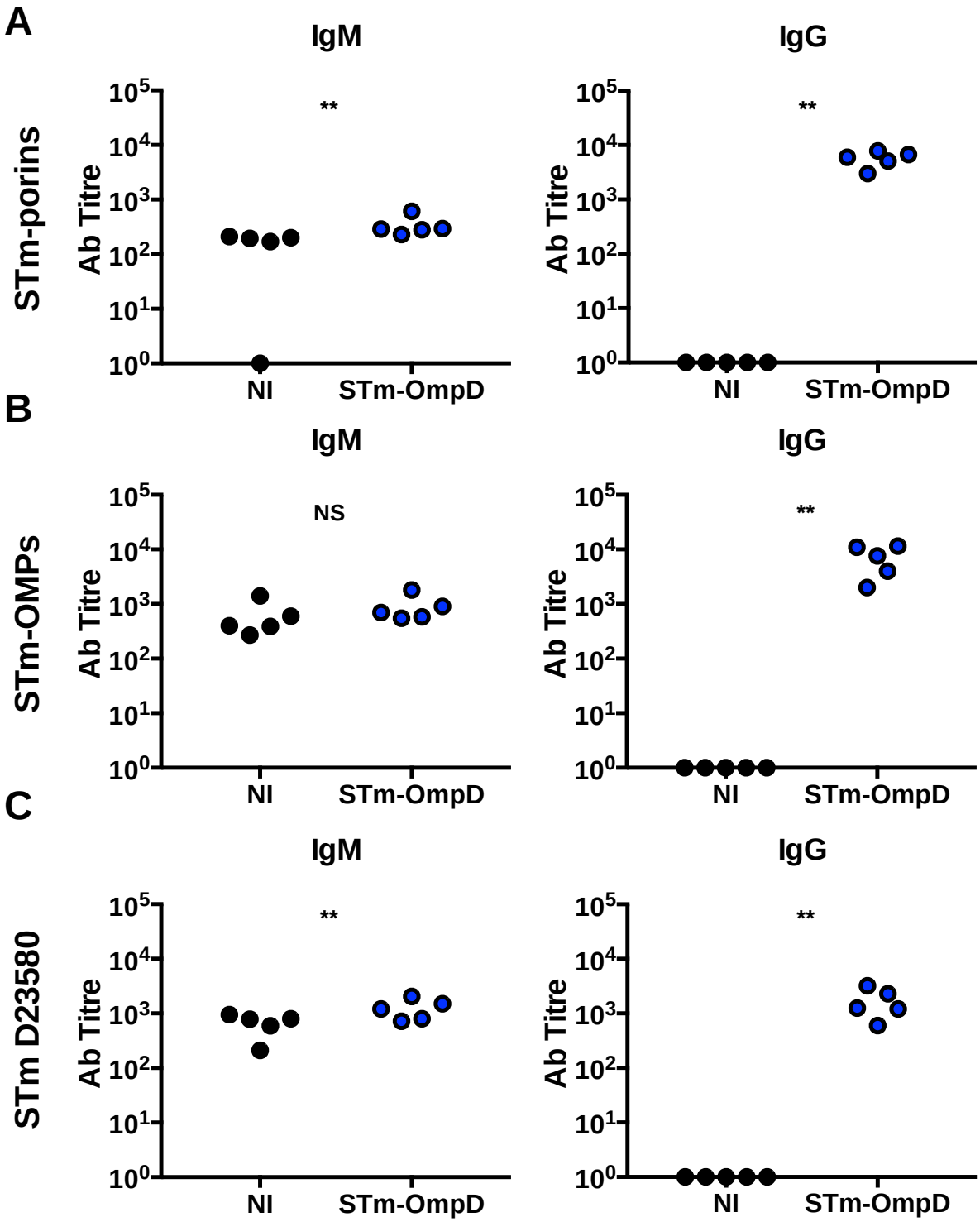


Figure 3.6 STm-OmpD induces IgM and IgG responses against STm-porins, OMPs, and whole bacteria

IgM and IgG Ab titres to (A) STm-porins, (C) STm-OMPs, and (C) STm D23580 were detected from sera of WT mice non-immunised (NI) or immunised with 20 µg of STm-OmpD i.p. at day 0 and boosted at day 14 and 28. Each dot represents one mouse and data is representative of at least 2 different experiments. ** = $P \leq 0.01$, NS = non-significant.

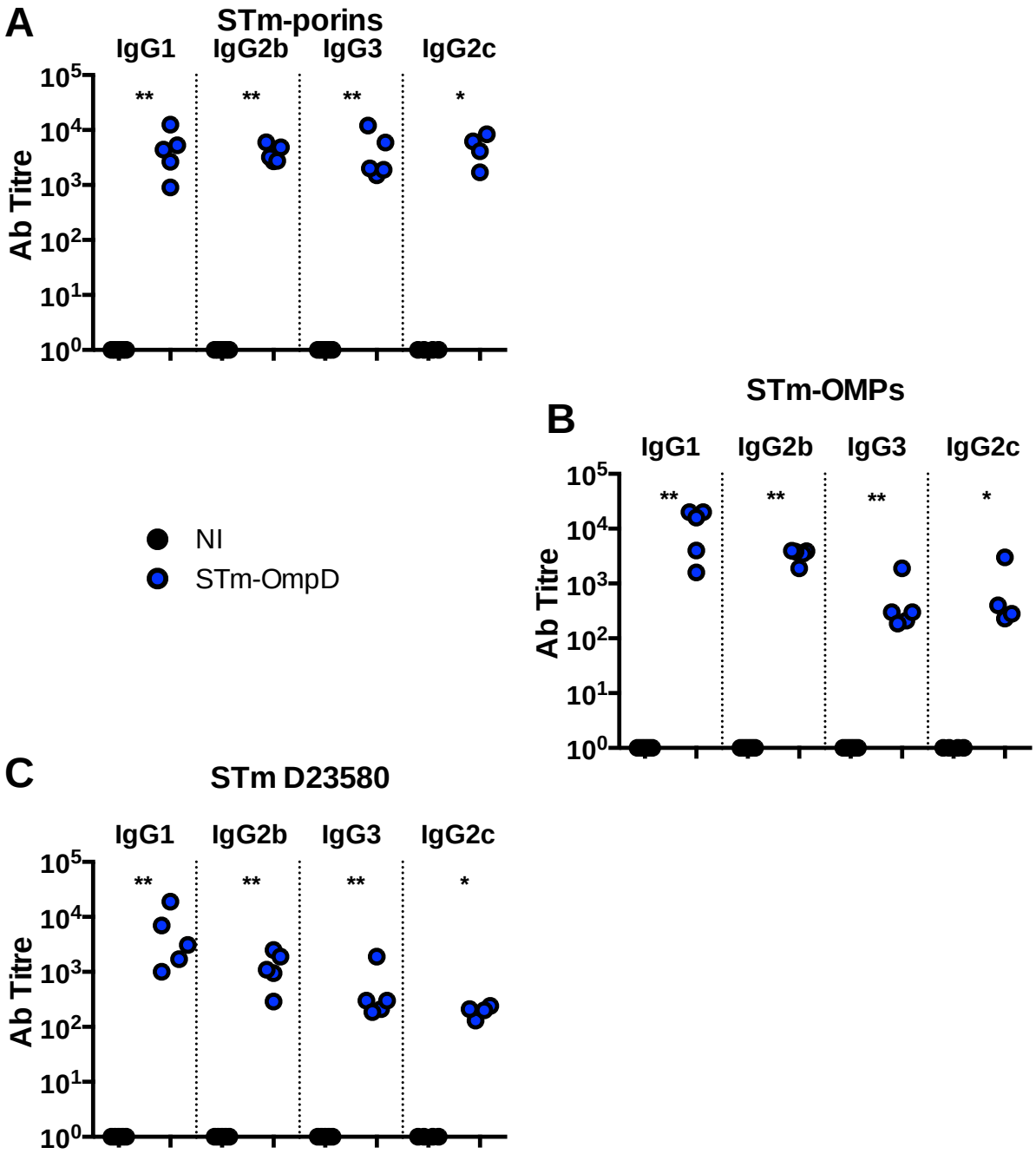


Figure 3.7 STm-OmpD hyperimmune mice sera generates different Ab isotype responses to STm-porins, OMPs, and whole bacteria
Ab titres for IgG subclasses were detected to (A) STm-porins, (B) STm-OMPs, and (C) STm D23580 from non-immune (NI) or STm-OmpD immunised WT mice sera as mentioned before. Each dot represents one mouse and data is representative of at least 2 different experiments. * = $P \leq 0.05$, ** = $P \leq 0.01$.

3.2.5 Immunisation with STm-OmpD does not offer equivalent protection against SEn infection

The majority of the cases of iNTS in sub-Saharan Africa are predominantly caused by STm and SEn isolates (5, 104). We compared the amino acid sequence of the OmpD proteins of both serovars and identified a difference in one amino acid, at position 263, which corresponded to an alanine in STm and a serine in SEn (Fig. 3.8A). Porins are known to be highly conserved in all *Salmonella* serovars (10), so we therefore tested if anti-STm-OmpD Abs bound OMPs from SEn by ELISA (Fig. 3.8B). Both STm and SEn OMPs were recognised by anti-STm-OmpD Abs showing a similar level of IgG binding, suggesting the existence of several cross-reactive epitopes, which is consistent with published studies with OMPs for other Gram-negative bacteria.

We then examined if immunisation with STm-OmpD could confer cross-protection against SEn. Groups of mice were primed and boosted with 20 µg of STm-OmpD and infected with attenuated STm or SEn strains for 4 days. Although a statistical difference was observed in both immunised groups, bacterial numbers in the spleens and livers of immunised mice challenged with STm were considerably lower, corresponding to a 100-fold reduction, compared to the immunised mice infected with SEn, which showed a decrease of less than 5-fold in both tissues (Fig. 3.9A and B). This suggested in the context of the whole organism that the A263 polymorphism is important.

To identify this polymorphism (A263S) within the OmpD protein we used structural models developed with Dr. Vassily N. Bavro (University of Essex). This predicted that

the change of amino acid occurs in the largest and most prominent external loop of the OmpD trimer (Fig. 3.10). This loop is the most prominent and largest in the protein and suggests it could contain an epitope accessible for Ab to bind.

Sequences of OmpD from different *Salmonella* strains, kindly provided by Dr. V.N. Bavro, were analysed. These showed the same amino acid substitution at position 263 (Fig. 3.11A and B). An alanine was found in serovars classified as being in groups A, B, and C (*S.* Paratyphi, *S.* Typhimurium, *S.* Montevideo), according to the Kauffman-White classification (2), and a serine to those strains that belong to group D (*S.* Enteritidis, *S.* Dublin, *S.* Gallinarum). These groups vary in O-Ag usage. Therefore, this suggested there may be a relationship between this polymorphism and the O-Ag used, which may influence the protection afforded by immunisation with STm-OmpD.

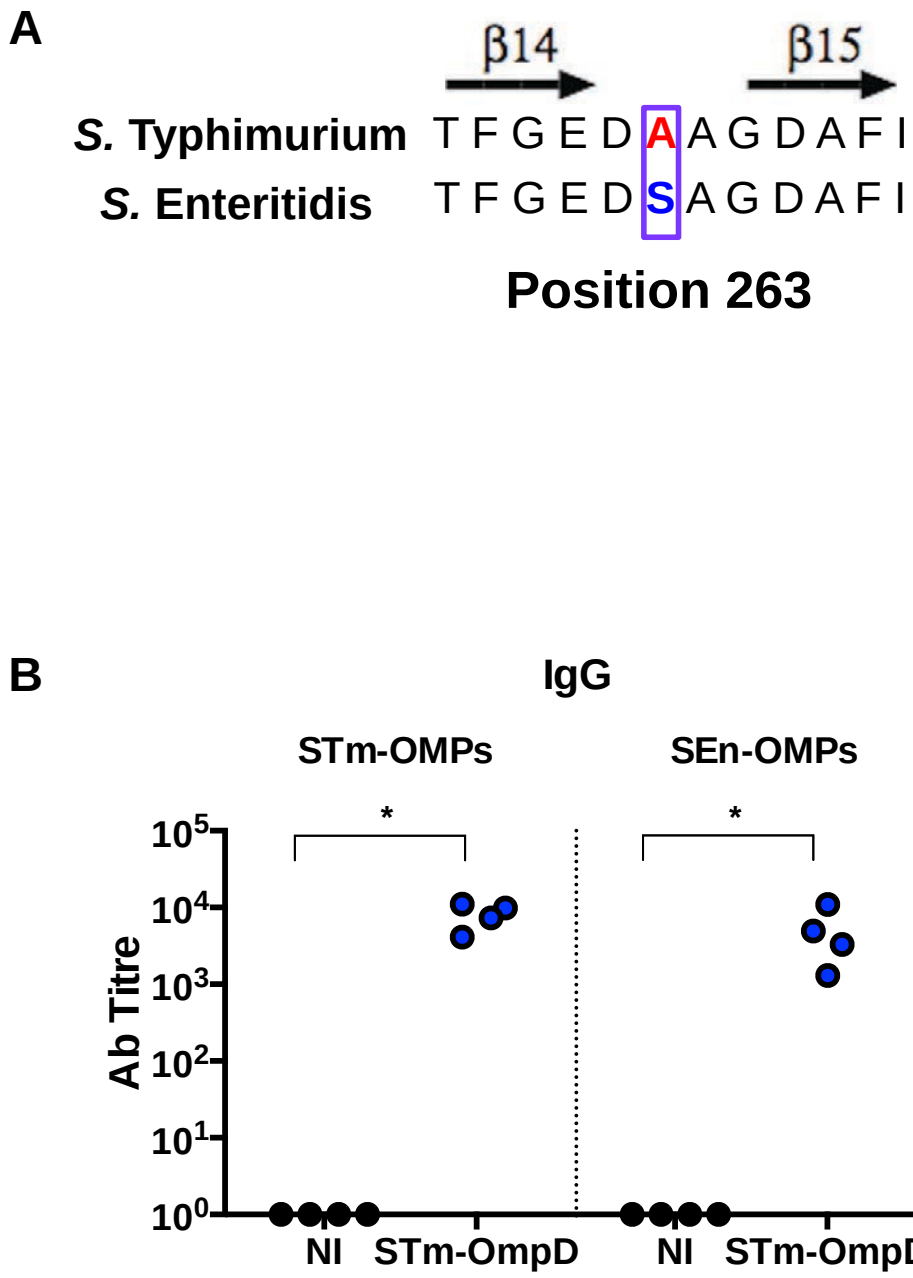


Figure 3.8 OmpD is highly conserved in STm and SEn and Ab to STm-OmpD recognises cross-reactive epitopes in SEn-OMPs
(A) Representative figure of the OmpD sequence in STm and SEn where a difference of an alanine to serine substitution is observed in the position 263 of the whole sequence. (B) IgG titres to OMPs of STm and SEn were detected using sera of NI and STm-OmpD immunised WT mice. * = $P \leq 0.05$.

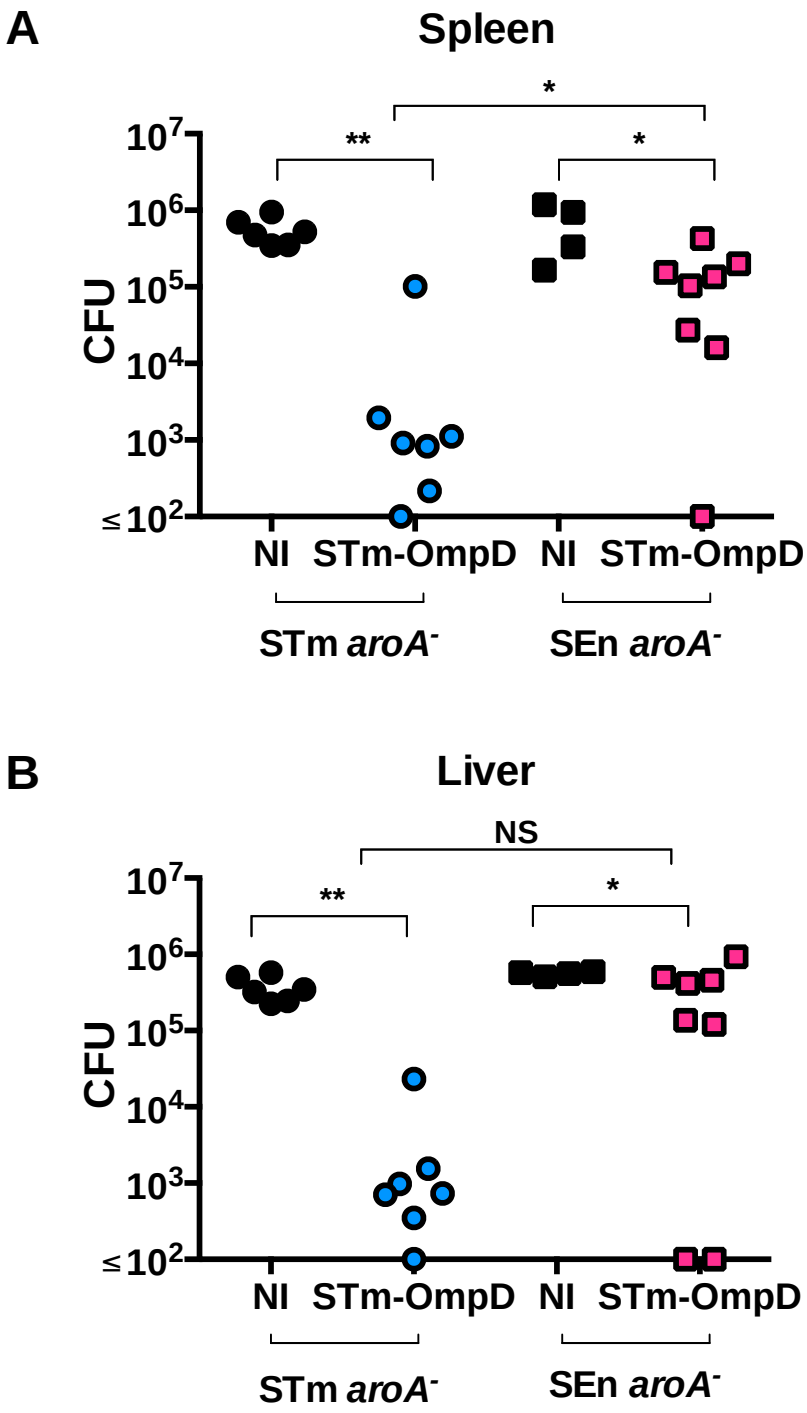


Figure 3.9 Lack of protection after STm-OmpD immunisation in mice infected with SEn is influenced by small differences in the sequence of OmpD between STm and SEn

Bacterial numbers of (A) spleen and (B) liver of non-immunised (black circles and squares) and STm-OmpD immunised (blue circles and pink squares) WT mice challenged with $5x10^5$ STm *aroA*$^-$ or SEn *aroA*$^-$. Groups of 4-8 mice or more were used. Data is representative of more than one experiment. * = $P \leq 0.05$, ** = $P \leq 0.01$, NS = non-significant.

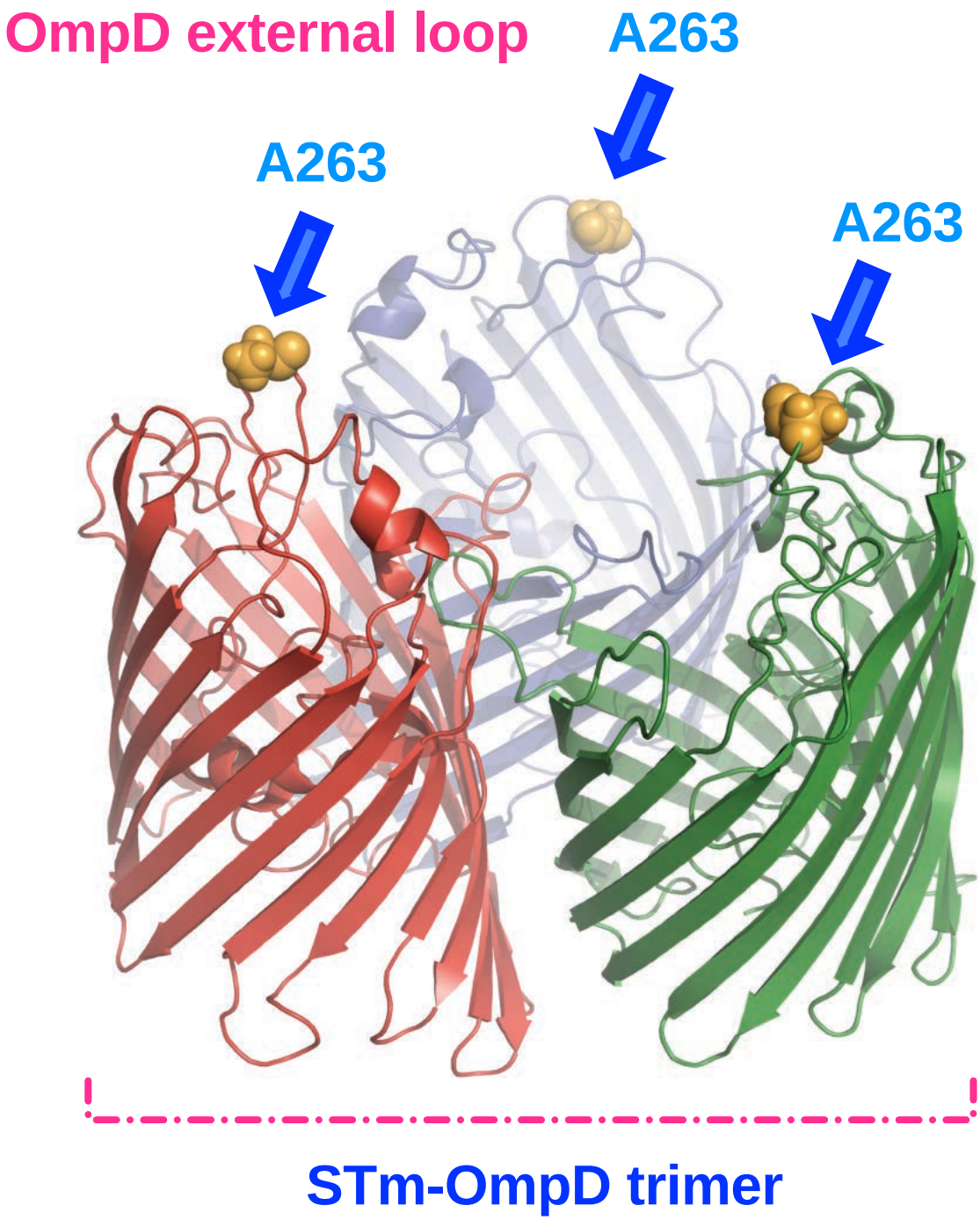


Figure 3.10 The amino acid alanine-serine substitution in position 263 is predicted to be in the most external loop of the STm-OmpD trimer
Structural model of an OmpD barrel designed by Dr. Vassily N. Bavro where the change of one amino acid at position 263 can be observed in the external loop of this porin (yellow spheres).

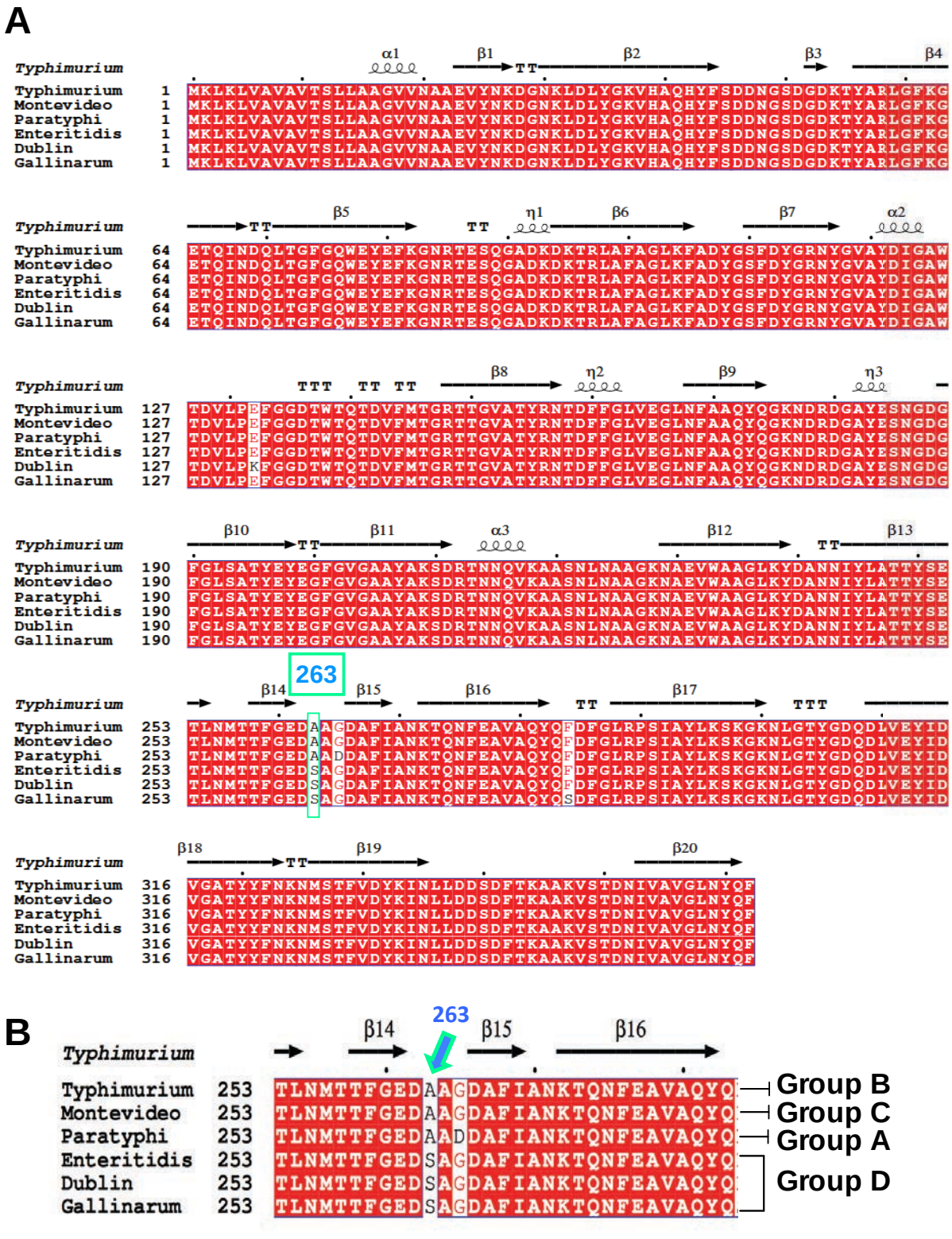


Figure 3.11 OmpD sequence in different *Salmonella* serotypes is highly conserved between them

(A) OmpD sequence of different *Salmonella* serovars. (B) Comparison of OmpD sequence between representative *Salmonella* serovars at position 263 where a change in one amino acid (alanine/serine) is observed.

3.2.6 Loss of O-Ag from SEn enhances anti-STm-OmpD Ab binding and protection

The reduction in protection and binding of anti-STm-OmpD Ab to SEn could be due to several reasons: first is that changes in O-Ag usage may block access to protective conserved epitopes. Second, is that the majority of antibodies target the 263 epitope as it is in the most external loop of the OmpD trimer, making it more accessible. Variability in the sequence may relate to lack of cross-protection by anti-STm-OmpD antibodies. Third is that the combination of O-Ag and OmpD may generate LPS-OmpD epitopes to multiple antigens reducing the access to surface epitopes of OmpD (Fig. 3.12A). To examine these possibilities in more detail, the level of cross-reactivity of anti-STm-OmpD Abs was examined. Intact inactivated bacteria were used as coating Ag for ELISA and binding of anti-STm-OmpD Abs examined. Binding of IgG Ab was considerably higher against STm than SEn (Fig. 3.12B). Loss of O-Ag on STm did not alter binding to a significant level. In contrast, there was a 10 to 100 fold higher binding of anti-STm-OmpD IgG in the absence of O-Ag on SEn (Fig. 3.12B and data not shown). These results suggest that O-Ag occludes access of Ab to the bacterial surface and that the A263 epitope is the primary target of Ab in intact bacteria (Fig. 3.13).

To determine if the lack of Ab binding to SEn was responsible for the limited protection against SEn after immunisation with STm-OmpD we performed SBAs using murine Ab and human Ab-depleted sera as a source of complement. In these experiments we used WT and O-Ag mutants of STm and SEn. Anti-STm-OmpD Abs and not Ab in NI mice sera were able to kill both WT and an O-Ag mutant of STm, but not SEn (Fig. 3.14A-C). However, Ab to STm-OmpD was able to kill the SEn mutant

that lacked O-Ag (Fig. 3.14D). These results indicate that there is an occlusion of conserved epitopes in SEn-OmpD since the equivalent epitope does not exist in SEn, because of the S263 amino acid substitution, resulting in much less efficient bacterial killing.

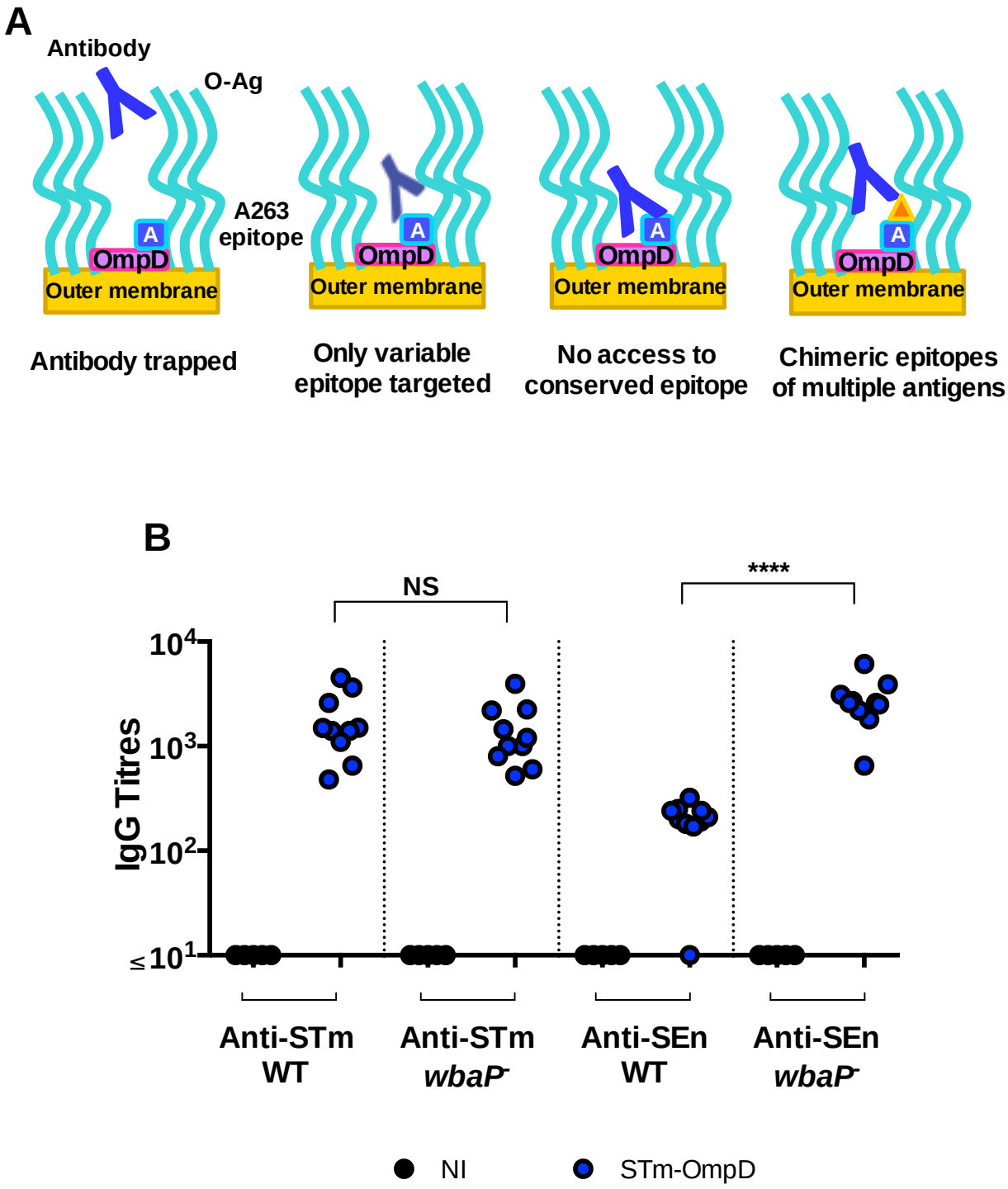


Figure 3.12 Binding of anti-STm-OmpD Abs to the surface of the bacteria is influenced by presence of LPS O-Ag

(A) Illustrative representation of the different hypothesis of why epitope occlusion and lack of protection after STm-OmpD immunisation occurs in SEn. OM = Outer membrane. Note: Figure not drawn to scale. (B) IgG titres to STm and SEn WT and O-chain KO mutants were quantified from non-immune and STm-OmpD immune WT mice sera (described elsewhere). Data is from 2 independent experiments and each dot represents one mouse. **** = $P \leq 0.0001$, NS = non-significant.

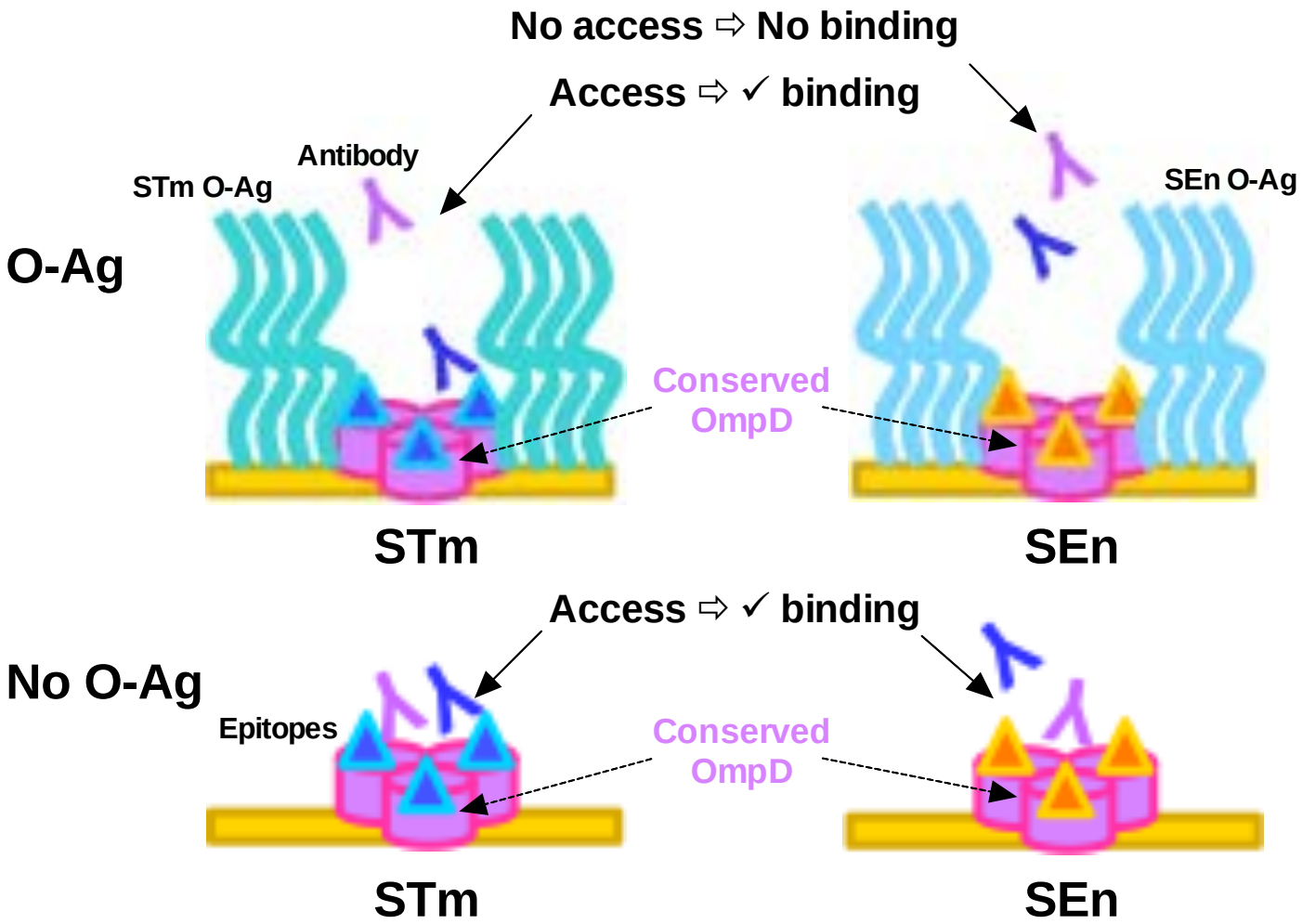


Figure 3.13 The O-Ag of LPS limits access of STm-OmpD Ab to bind SEn-OmpD by occluding access to conserved epitopes in this porin

Schematic of the relation between O-Ag and trimeric proteins, on the outer membrane of STm and SEn, indicating the importance of O-Ag in limiting access to conserved epitopes for Ab to bind. Triangles represent the 263 epitopes in OmpD; A263 (blue) and S263 (orange). Note: Representative figure not drawn to scale.

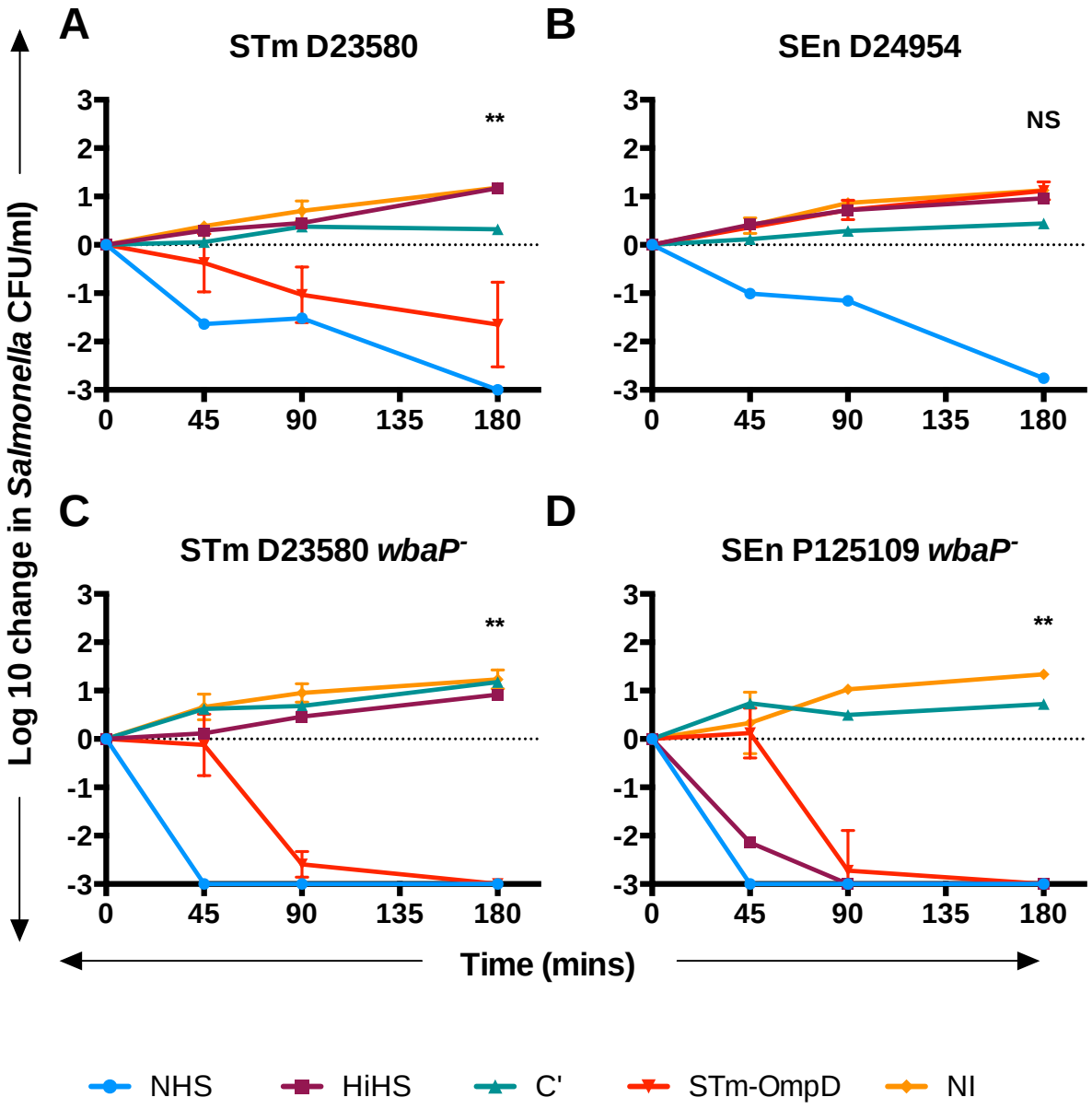


Figure 3.14 Removal of LPS O-Ag enables killing of SEn by STm-OmpD Abs via complement

Serum bactericidal assays of (A) STm D23580, (B) SEn D24954, (C) STm D23580 *wbaP*, and (D) SEn P125109 *wbaP*. Samples were taken at minute 45, 90, and 180 using a starting concentration of 10^6 CFU/ml. Non-immune (NI)(yellow) and STm-OmpD immune (red) WT mice sera were tested and controls were used as described before. Data is representative of more than one experiment and represents the mean of 4-6 mice sera used. U Whitney test was used to determine difference in killing at minute 180 between NI and immunised mice sera. * = $P \leq 0.05$, ** = $P \leq 0.01$, NS = non-significant.

3.2.7 Immunisation with STm-OmpD protects against infection with O-Ag mutants of STm and SEn

In order to determine how the loss of O-Ag would affect protection *in vivo,* we immunised mice with STm-OmpD and challenged with virulent WT and O-Ag deficient STm or SEn for 24 hours. Immunisation with STm-OmpD reduced STm numbers by almost 100-fold in the spleen and liver (Fig. 3.15A and B). Moreover, anti-STm-OmpD Abs were capable of controlling bacteremia in immunised mice infected with WT STm (Fig. 3.15C). STm-OmpD also conferred significant protection in O-Ag deficient STm, confirming that O-Ag Ab did not contribute to protection, although the bacterial numbers in NI mice were lower than after infection with O-Ag sufficient bacteria. In STm-OmpD immunised mice challenged with SEn or the SEn O-Ag mutant, bacterial numbers were lower compared to the NI mice but the SEn mutant did not colonise as well as the parent strain. (Fig. 3.15 and 3.16). Thus, anti-STm-OmpD Ab can provide protection against O-Ag deficient STm and SEn.

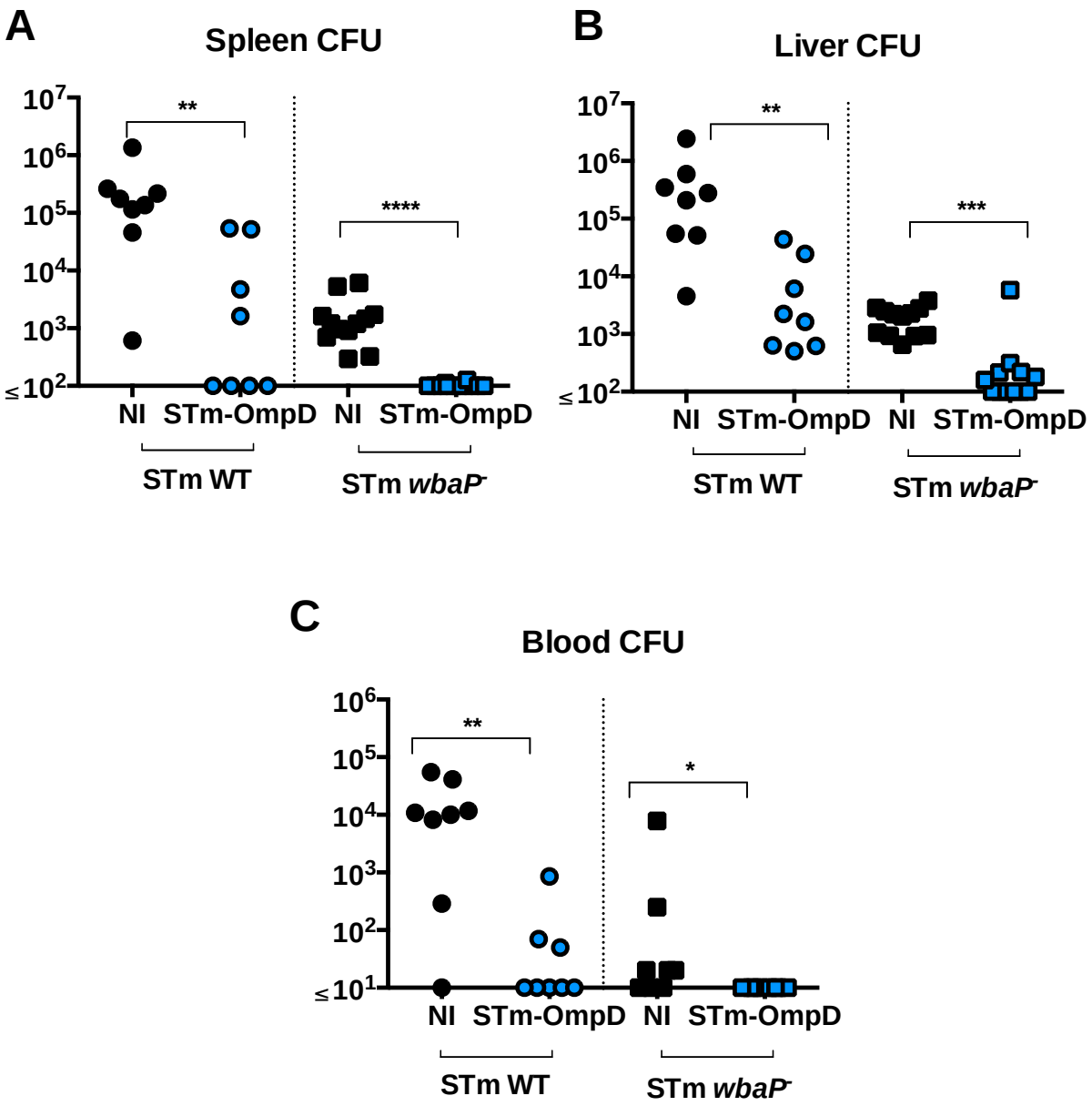


Figure 3.15 Immunisation with STm-OmpD controls bacteraemia and provides protection against invasive STm while loss of O-Ag in STm does not affect protection in mice but colonisation in tissues is reduced during the first hours of infection

Bacterial numbers of non-immunised (NI) and STm-OmpD immunised WT mice (as described) infected with $1x10^5$ STm D23580 or STm D23580 *wbaP*$^-$ were enumerated from (A) spleen, (B) liver and (C) blood. Data is from 3 independent experiments and each dot represents one mouse. * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

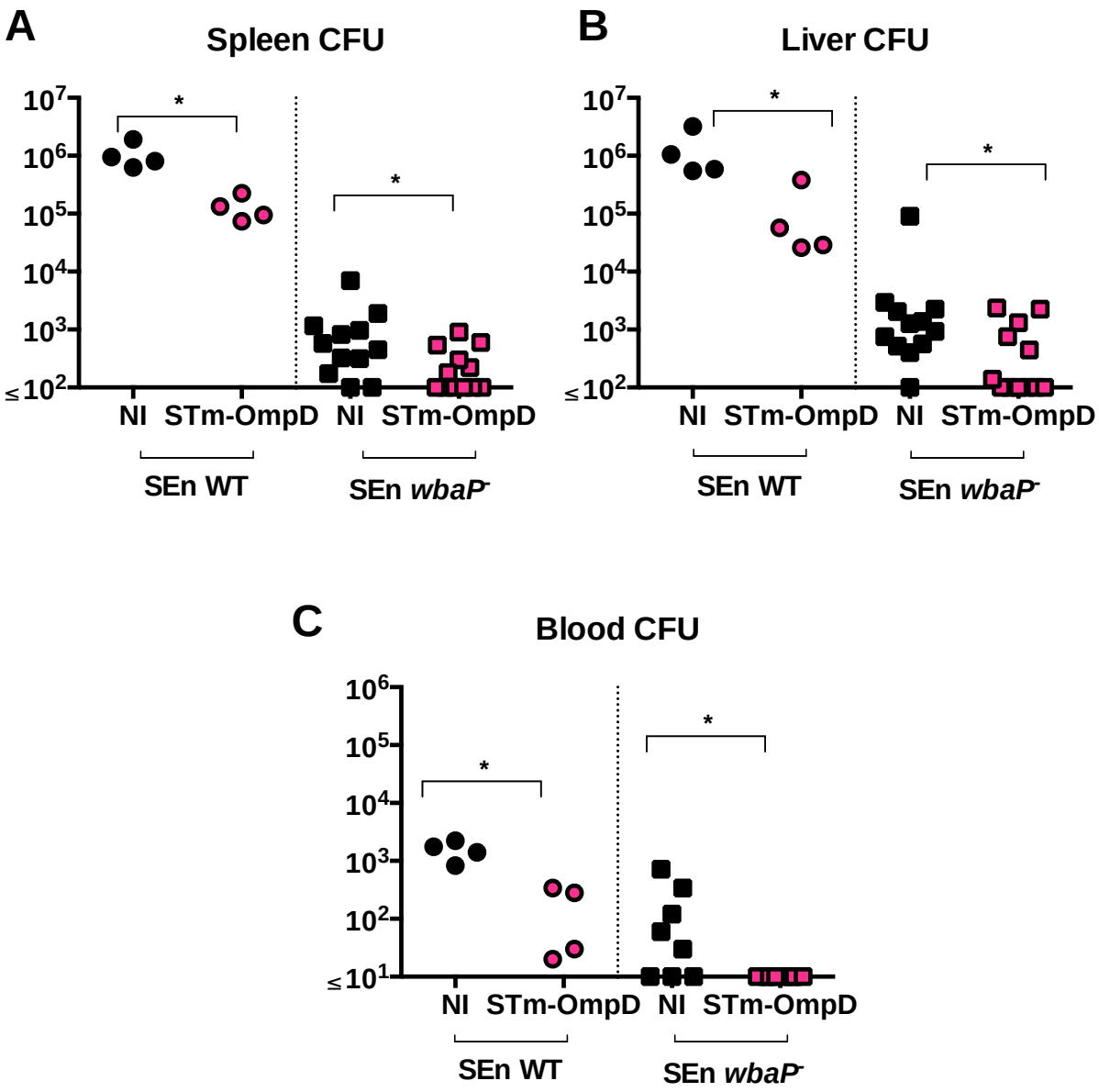


Figure 3.16 Immunisation with STm-OmpD is not able to impair infection in mice infected with SEn and loss of O-Ag does not influence lack of protection after 24 hours of infection

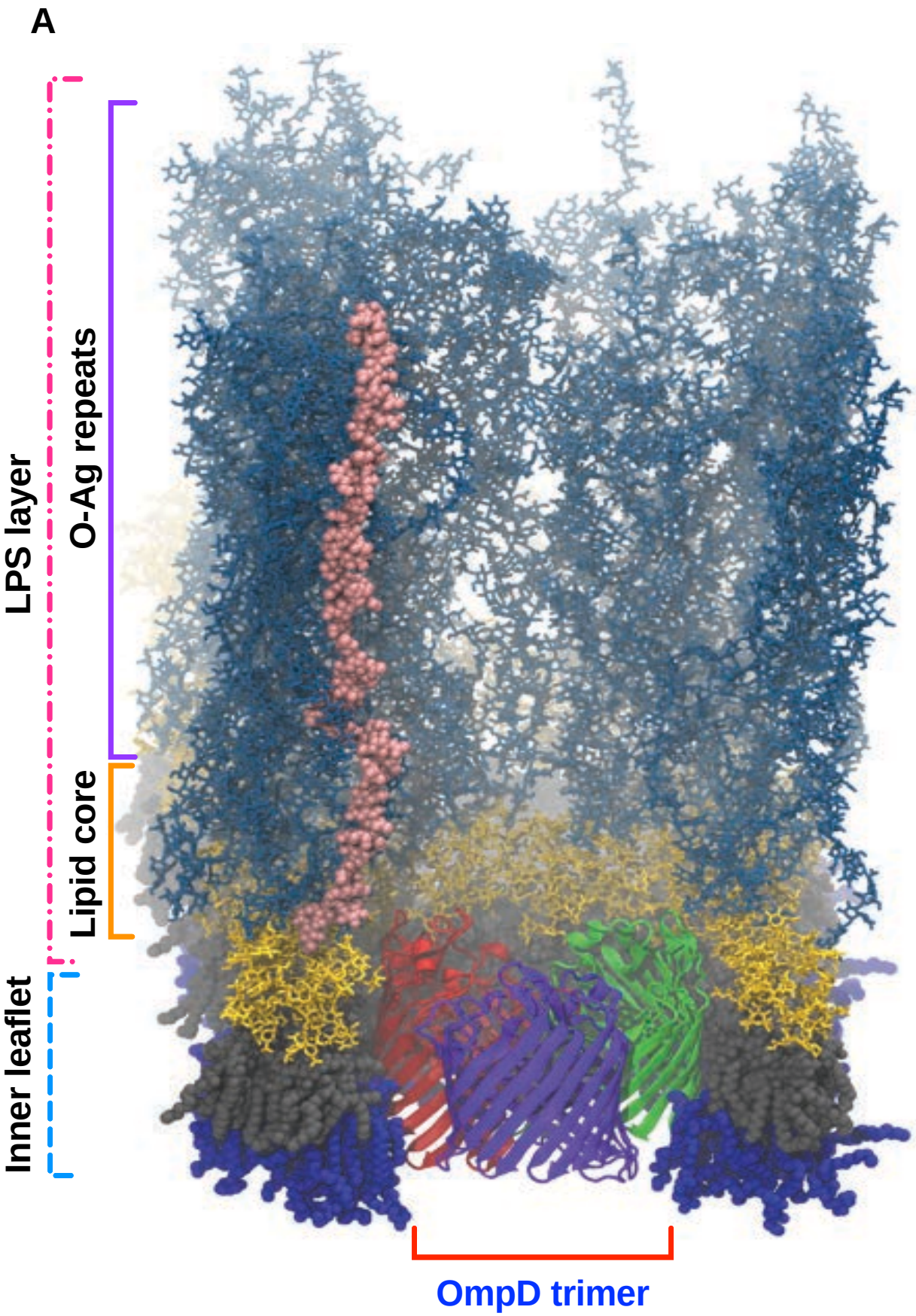
Non-immunised (NI) and STm-OmpD immunised mice were infected with $1x10^5$ SEn D24954 or SEn P125109 *wbaP*$^-$ and bacterial numbers were counted from (A) spleen, (B) liver, and (C) blood. Data is from 3 different experiments and each dot represents one mouse. * = $P \leq 0.05$.

3.2.8 Structural modeling of the OmpD trimer with LPS shows how Ab to STm-OmpD can bind to the bacterial surface

Our previous data established the importance of the immunodominant A263S epitope in intact bacteria and that conserved epitopes in OmpD become accessible when LPS O-Ag is not expressed. To understand better the role of O-Ag and epitope access an analysis of the relationship between OmpD, Ab, and LPS was performed using different models of these components. This was performed with Dr. V.N. Bavro and Dr. J.C. Gumbart (Georgia Tech), based on bioinformatics provided by myself. The outcome of this suggests that trimeric OMPs can generate tunnels within the LPS O-Ag and that proximal epitopes on the membrane are occluded by numerous chains of LPS (Fig. 3.17A). From a top view down, full atomistic models of the LPS from STm reveal that dense chains of LPS that create a barrier that can block access to OmpD on the cell surface (Fig. 3.17B), but this blockage is not complete. However, at sites where the porins are present, the tunnel remains open, leaving clear access for Ab to penetrate but not in areas of the membrane where the porins are absent (Fig. 3.17C). Strikingly, this model of Ab binding to the OmpD trimer suggests that the footprint of the tunnel permits access to one Fab fragment to target the immunodominant epitope in OmpD (A263)(Fig. 3.17D and E).

Differences in the O-Ag structures between STm and SEn are determined by a dideoxyhexose side sugar, abequose and tyvelose respectively, attached to a mannose (29, 33)(Fig. 3.18). Hydrogen bonds between both side-chain sugars and their respective O-Ags are more frequent in SEn than STm (J.C. Gumbart, not published), which may explain the reduced access to SEn-OmpD by STm-OmpD Ab as numerous hydrogen bonds between the O-Ags create a more rigid barrier of SEn

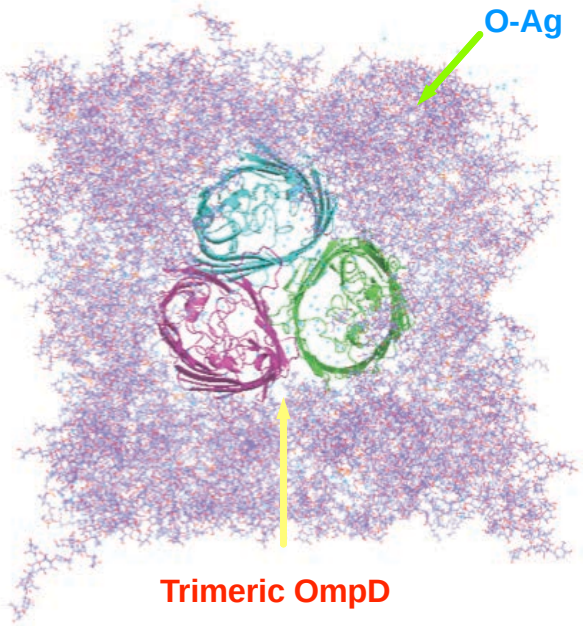
LPS and therefore offer better protection to the outer membrane of the bacterium. The latter proposes that LPS chains of STm may show more flexibility compared to those of SEn but access to the OmpD trimer is still maintained in both organisms. Thus, occlusion of protective epitopes to OmpD is influenced by LPS expression and structure.


A
LPS layer
O-Ag repeats
Lipid core
Inner leaflet
OmpD trimer

B

Top view

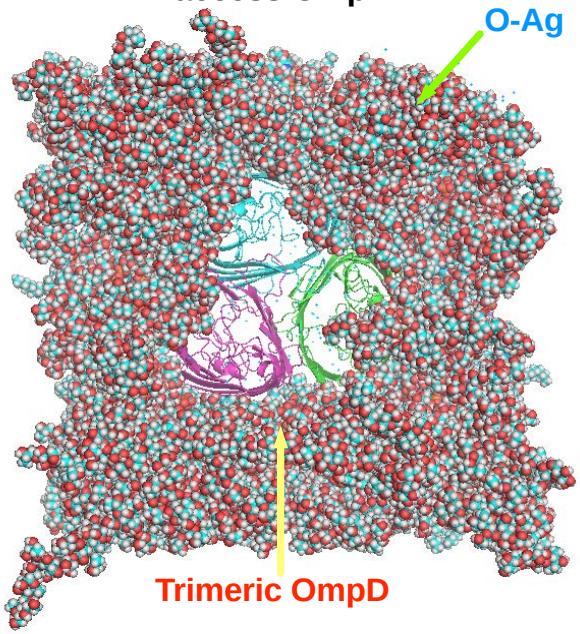
No O-Ag occlussion shown



C

Top view

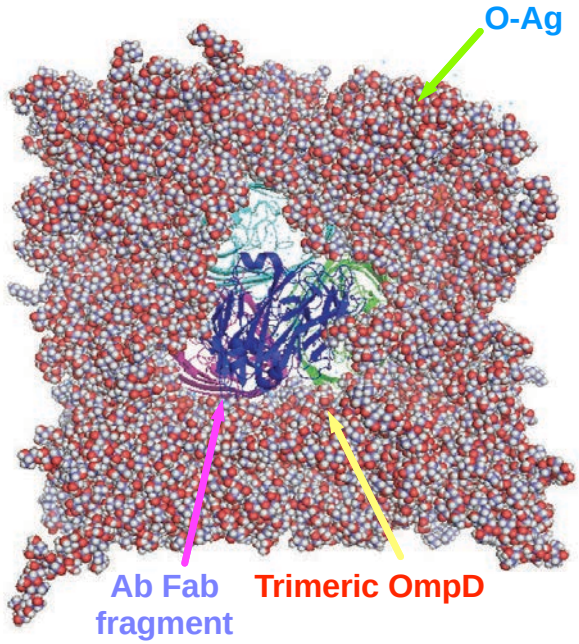
Dense chains of LPS create a tunnel for Ab to access OmpD



D

Top view

O-Ag occlussion (space fill)



E

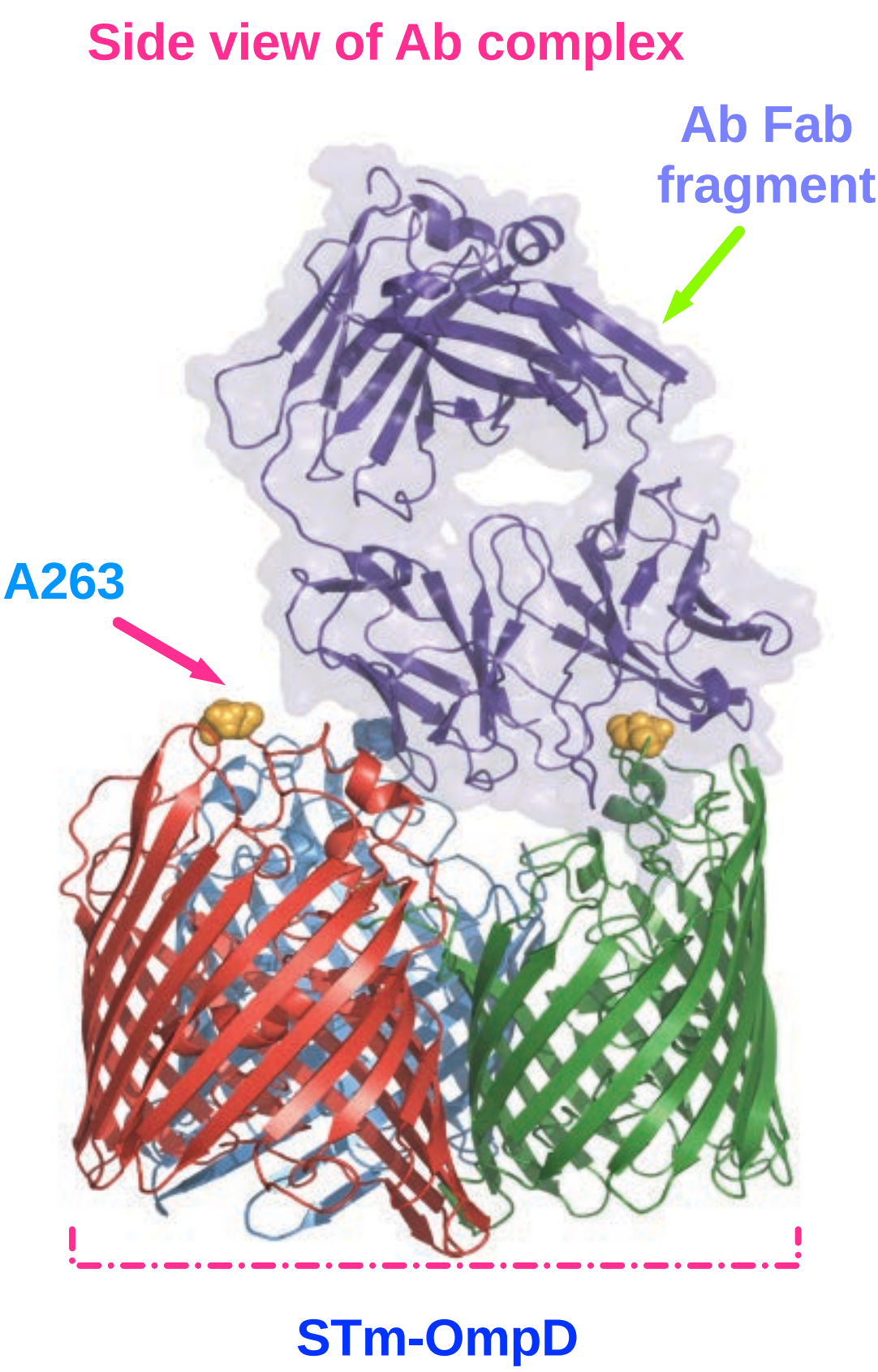


Figure 3.17 O-Ag usage and small changes in OmpD are sufficient to limit access of Ab to the immunodominant epitopes
Molecular model of the outer membrane of the bacteria containing a trimeric porin and LPS molecules in the outer leaflet. (A) A single O-Ag chain (salmon), core LPS sugars (yellow), inner leaflet phospholipids (blue) are observed. The accessibility "tunnel" formed by the OmpD trimer is clearly visible. (B) Top-down view from the outside of the cell looking down the "tunnel" created by OmpD in the LPS layer, the O-Ag is shown in purple. (C) Same as B but with a space-filling model of O-Ag sugars added (D) Top-view of the structural model of dense O-Ag chains and a bound Ab Fab fragment (blue). (E) Side view of the predicted Ab binding to the surface loop epitope of an STm-OmpD trimer. Structural models were provided by Dr. V. N. Bavro.

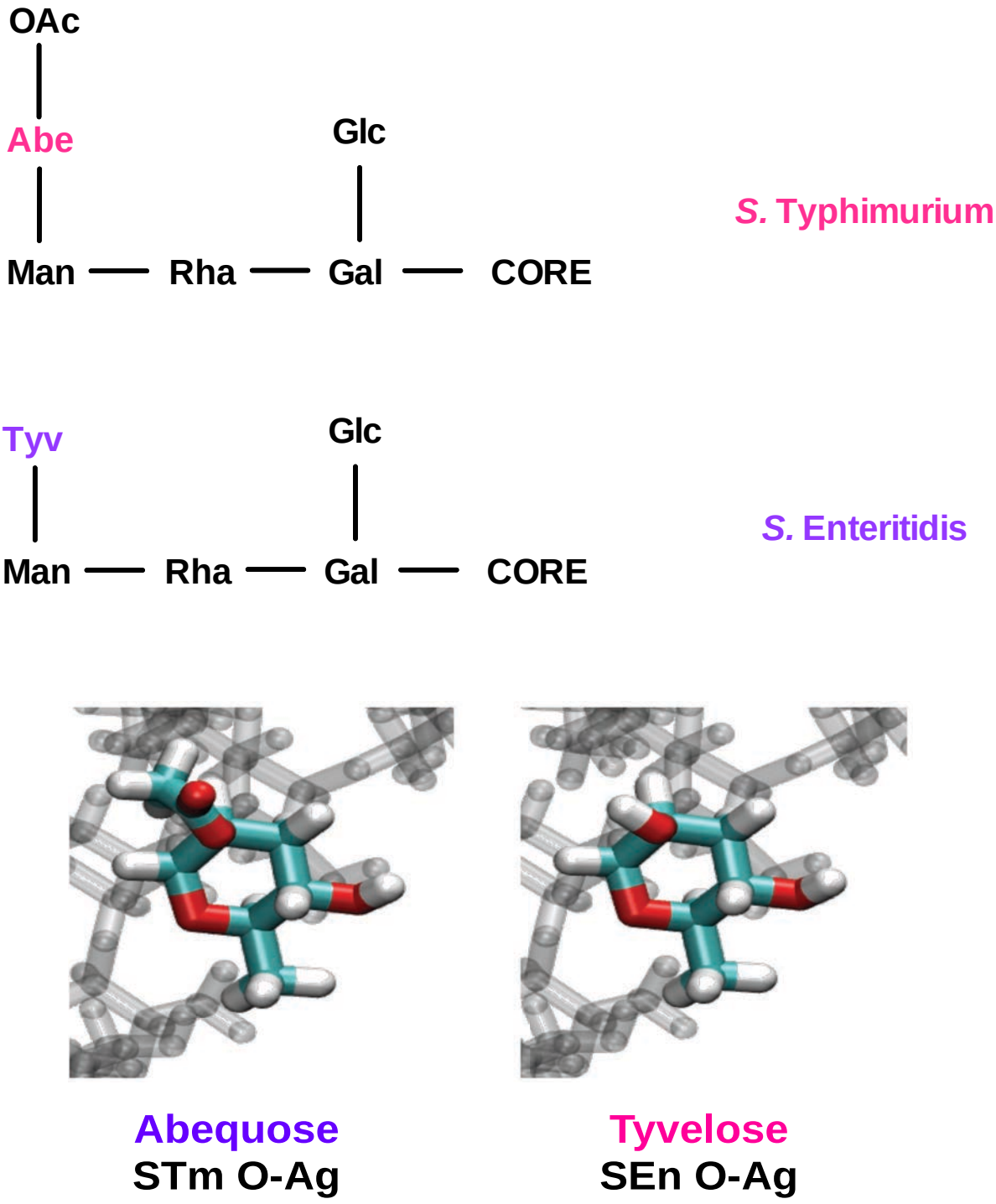


Figure 3.18 Changes in LPS structure correlate with limited access of antibody to OmpD in SEn

Schematic of O-Ag chain repeat units corresponding to: abequose with acetoxy group in the O-Ag of STm and tyvelose in the O-Ag of SEn (top). Structural images of the O-Ag sugar complexes in STm and the analogous SEn (bottom). Structural models were designed by Dr. J.C. Gumbart.

3.2.9 Optimal binding of Ab to OmpD requires matched O-Ag and OmpD usage

Since there is a possibility that O-Ag could create chimeric epitopes in combination with OmpD, we assessed whether altering O-Ag usage could influence Ab binding to access to OmpD (Fig. 3.19). To do this the ability of anti-STm-OmpD Abs to bind and protect against WT STm (O4), SEn (O9), and chimera strains that express O9 and O4 respectively was examined (Fig. 3.20A). Binding of IgG to WT STm was readily observed, but this was dramatically reduced against SEn and the chimera strains. Furthermore, when mice were immunised with STm-OmpD and challenged with WT STm, SEn, and their chimeric counterparts for 24 hours, a significant reduction of 100-fold in bacterial numbers was observed only in the spleens of mice challenged with STm (Fig. 3.20B) whereas in the other immunised groups protection was diminished (Fig. 3.20B and C). To complement these experiments we examined the ability of sera to kill these strains in a SBA using sera to STm-OmpD. These experiments showed that Ab was capable of killing STm but was not able to kill the other strains (Fig. 3.21). Thus, O-Ag usage and structure influence access of Ab to conserved epitopes in OmpD.

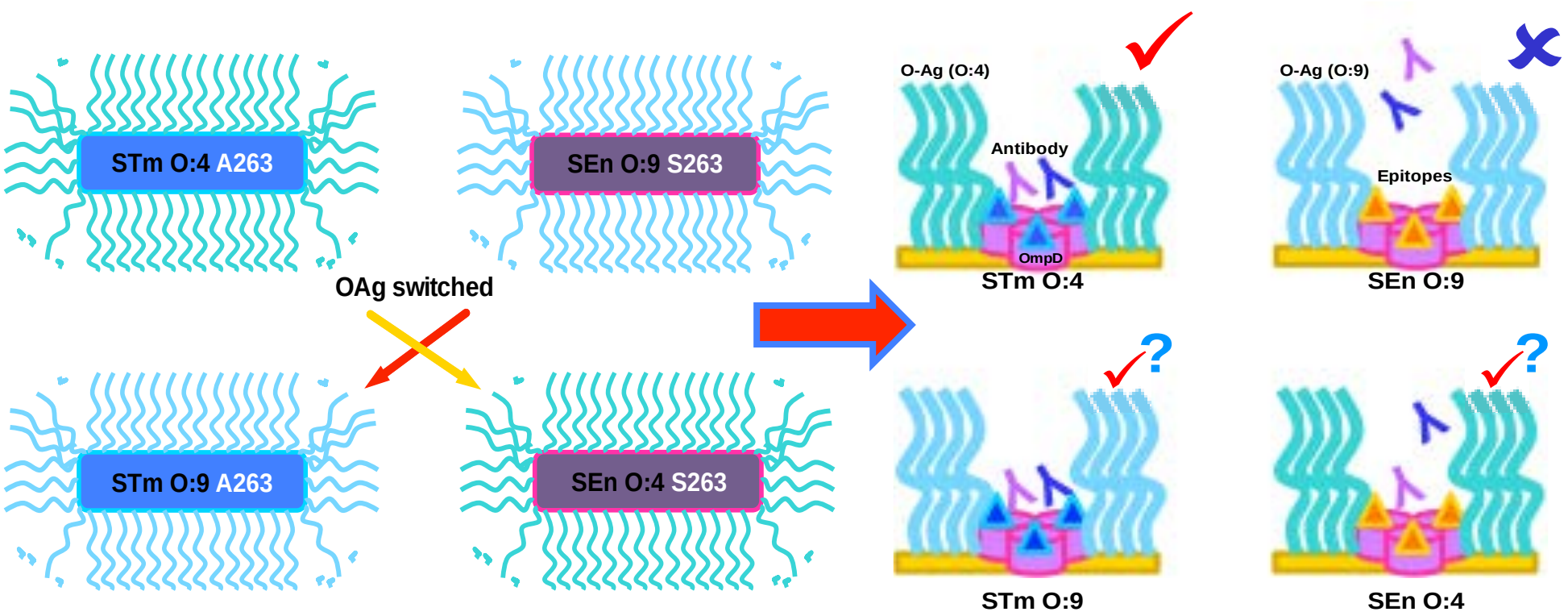


Figure 3.19 Does altering O-Ag but not OmpD in STm and SEn affect protection after immunisation with STm-OmpD? Illustration of switched O-Ags from STm and SEn into their heterologous strain (left). Prediction of protection conferred by STm-OmpD immunisation when the O-Ag is altered (right). Triangles represent the 263 epitopes in OmpD; A263 (blue) and S263 (orange). Note: Figure not drawn to scale.

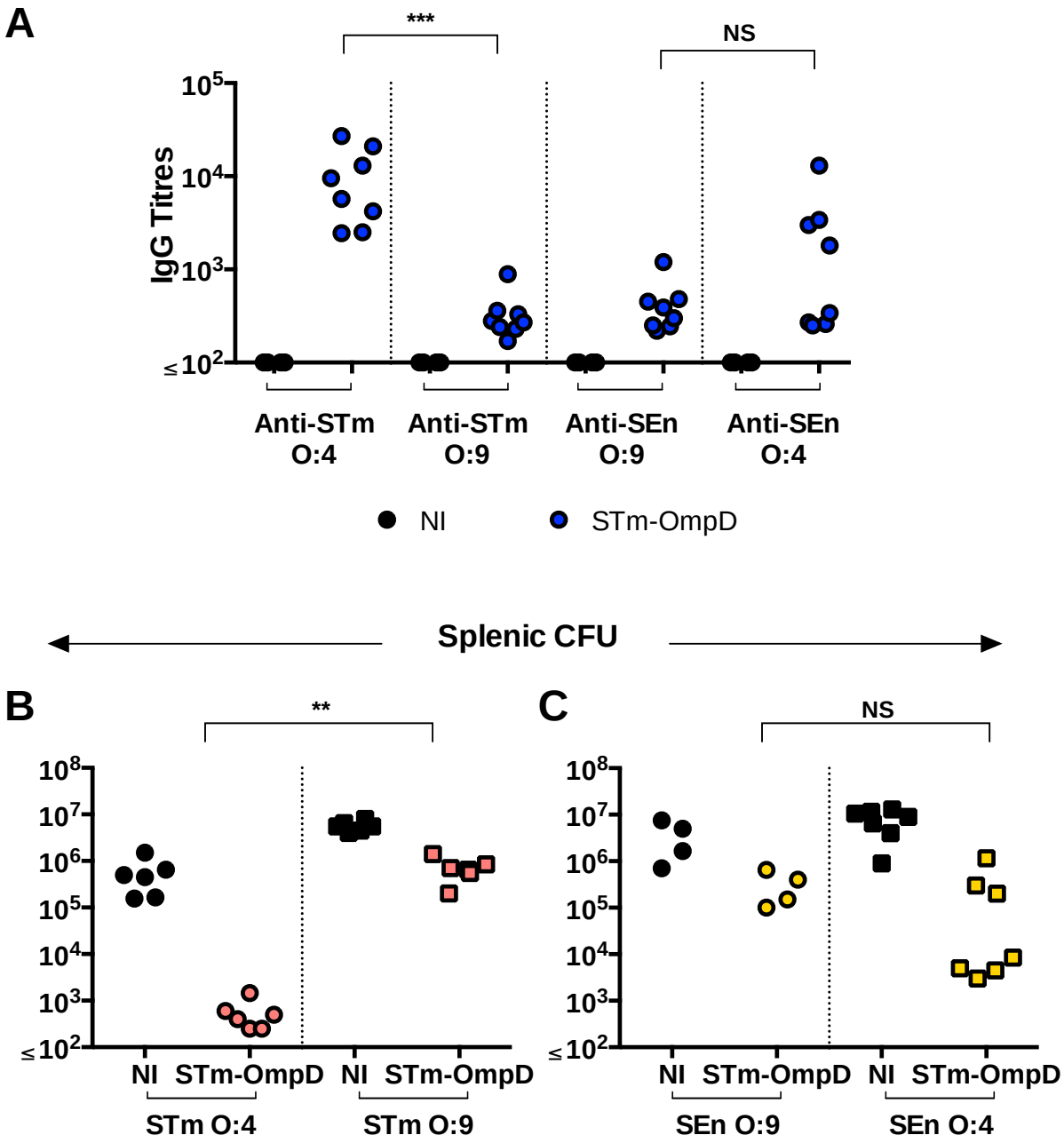


Figure 3.20 Access of Ab to conserved OmpD epitopes is impaired by differences in O-Ag structure and variability between strains.
(A) Ab titres for IgG to STm and SEn WT and LPS chimera strains were quantified using sera from non-immunised (NI) and STm-OmpD immunised WT mice (as mentioned before). Each dot corresponds to one mouse and data is from 2 different experiments. (B and C) Bacterial burden from the spleen of non-immunised (NI) and STm-OmpD immunised WT mice infected with $1x10^5$ (B) STm O:4 or STm O:9 and (C) SEn O:9 or SEn O:4. Data is representative of 2 individual experiments repeated twice where 4-7 mice were used per group. ** = $P \leq 0.01$, *** = $P \leq 0.001$, NS = non-significant.

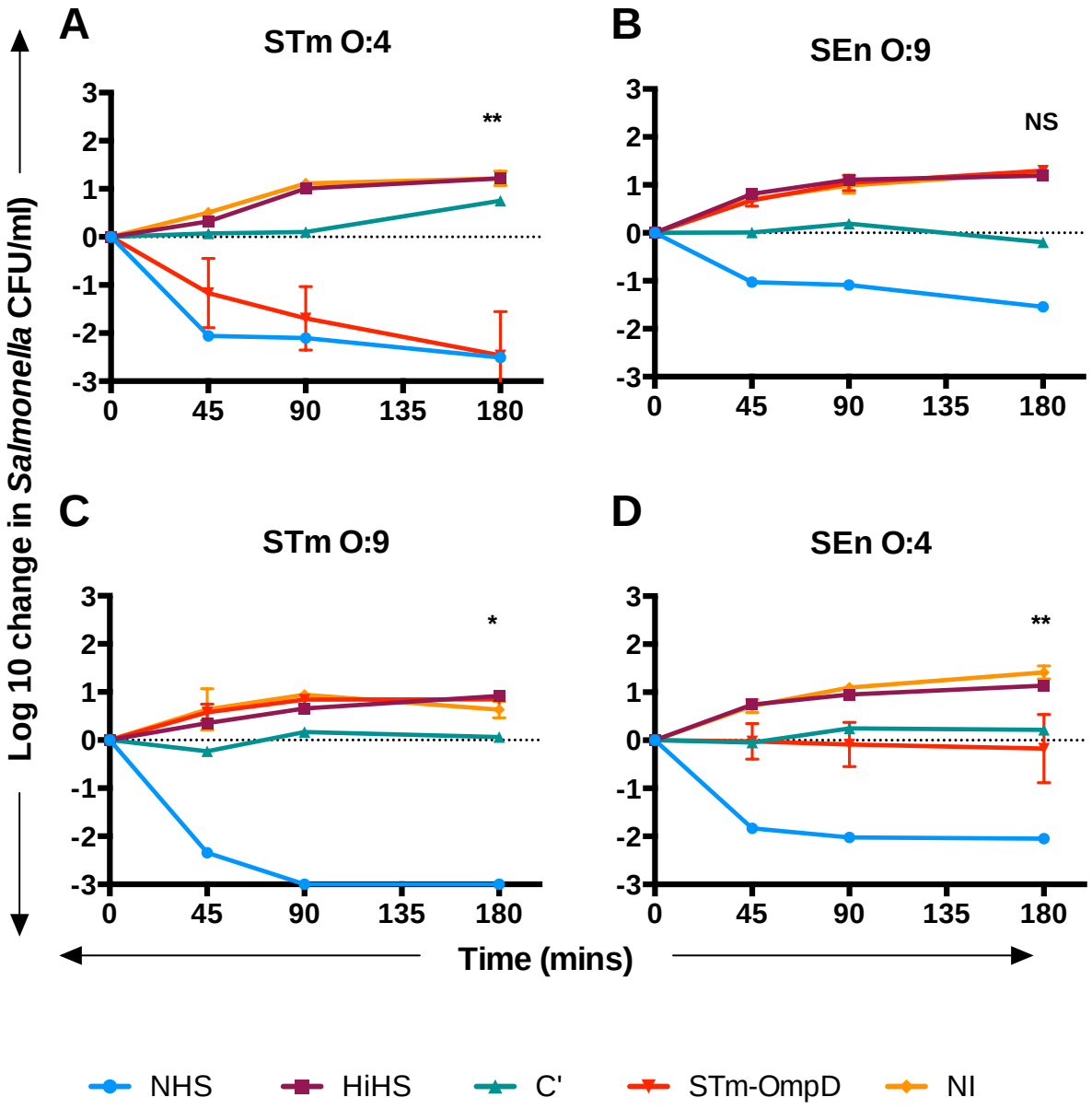


Figure 3.21 Ability of complement and anti-STm-OmpD Abs to kill the bacteria is also affected by O-Ag usage and hidden epitopes to OmpD
Serum bactericidal assays of (A) STm O:4, (B) SEn O:9, (C) STm O:9, and (D) SEn O4 where samples were taken at 45, 90, and 180 minutes of a starting concentration of 10^6 CFU/ml. Non-immune (NI)(yellow) and STm-OmpD immune (red) WT mice sera were tested and controls were used as mentioned elsewhere. Data are representative of more than one experiment and represent the mean of 4-7 mice sera used. Difference in growth at minute 180 between NI and immunised mice sera was determined by Mann-Whitney U test. * = $P \leq 0.05$, ** = $P \leq 0.01$, NS = non-significant.

3.3 Discussion

Multiple studies in mice have examined the use of porins as vaccines against salmonellosis (312, 319-321). Moreover, we previously identified OmpD as the major porin responsible for protection against STm in a B cell dependent manner (72). While clearance of the bacteria is strictly T cell dependent in primary infections, Ab is required to impair secondary virulent infections (225, 226, 322). Here we showed that Ab to STm-OmpD induce cell-free complement-mediated killing against virulent bacteria in the absence of other porins (OmpA, OmpC, OmpF, and OmpR).

When Ab responses to STm and porins were analysed after STm-OmpD immunisation we found that all isotypes were induced at day 35. However, at day 7 IgG1 was not detectable. This means that protection after immunisation with STm-OmpD could be provided by multiple isotypes. This is important as isotypes, like IgG1, are not induced after natural infection with STm. (287, 323).

Analysis and comparison of the OmpD sequence between STm and SEn showed OmpD is a highly conserved porin, with homology of >99% and only a single amino acid substitution at position 263. Furthermore, detection of cross-reactive Abs to STm and SEn-OMPs raised the possibility of using STm-OmpD as a single Ag for protection against other *Salmonella* serovars. However, lack of Ab binding and protection in mice to SEn showed this was not viable. Binding of Ab to SEn-OMPs was observed in ELISAs when present as a purified Ag, but not when intact organism was used to coat plates. Therefore a major proportion of this Ab targets conserved epitopes.

Studies suggest that lack of protection between serovars is due to a change in conformation or structure of protective epitopes in live bacteria rather than differences in the Ag used (324). However, it is likely that LPS may be playing a role in occluding access to the conserved epitopes (294). While porins posses numerous epitopes that vary between 6-25 residues in length, the number of O-Ag chains in live bacteria varies to >80 repeats per LPS molecule, comprising more than 50% of the cell surface in Gram-negative bacteria (293, 325, 326). This helps explain the lack of cross-protection between different serovars that share many conserved Ags, indicating that LPS-porin complexes can restrict Ab access. Using ELISAs and SBAs we showed that removing O-Ag enabled STm-OmpD Ab to bind whole SEn and kill SEn via complement. Furthermore, mice infected with O-Ag mutant bacteria were colonised to a lower degree than those infected with WT bacteria. Therefore Ab to OmpD is protective in the absence of O-Ag and so O-Ag cannot be the target of protective Ab in this scenario. Thus, *in vivo* Ab to the A263 epitope and the conserved epitopes can both protect, but under normal circumstances only the A263 epitope is accessible. This is consistent with there being a polymorphism at this site. Using structural models we showed the generation of a tunnel of sufficient size for a Fab to access OmpD (Fig. 3.17). This revealed that although dense O-Ag on the bacterial surface is widespread there is incomplete coverage.

Not all antibody responses produced after infection are protective. Moreover, simply using IgG titres as a readout may overestimate the success of vaccination, as not all the IgG isotypes generated are protective due to their affinity, specificity or effector function (327, 328). For instance, monoclonal Abs to porins induce binding to exposed epitopes *in vitro* but fail to protect against infection when tested individually

(328). Furthermore, there is a restricted access of monoclonal Abs to surface Ags in WT bacteria but not in rough mutants (294, 329). This reduces the need to introduce variability into functional proteins and therefore emphasizes the importance of O-Ag as a barrier for Ab. Thus, IgG class switching and epitope specificity may contribute to an important role in protective responses to conserved Ags.

In collaboration with Prof. Kai-Michael Toellner (University of Birmingham) we have found that IgG1 is required to provide protection after STm-OmpD immunisation whereas IgG2a/c is not essential. This suggests that IgG switching influences the capacity of Ab to inhibit or create a protective immune response. This could be due to the intrinsic differences in the flexibility of the IgG molecules in the hinge region (330)(Fig. 3.22). Therefore, a polyclonal response, involving multiple IgG isotypes after vaccination may be important.

O-Ag may limit access of Ab to other conserved, protective epitopes in the OM of Gram-negative bacteria, particularly Ags that interact closely with the response to the O-Ag (Fig. 3.23). This correlates with work studying the autotransporter SadA (331) and unpublished studies using OmpA. This showed that immunisation with SadA could protect but OmpA did not. There are polymorphisms found in SadA but not OmpA from STm and SEn indicating that there is one selective pressure on the former but not the latter. Furthermore, many protein Ags have been identified as *Salmonella*-specific Ab targets in mice and humans that could potentially be used as subunit vaccines against different *Salmonella* serovars (235) but whether they are protective or not still needs to be determined.

This chapter indicates the importance of considering the structural relationship between Ags on the bacterial surface when generating effective immune responses; in particular, it identifies the complex relationship between LPS-protein complexes in limiting access of Ab to conserved Ags in the surface of Gram-negative bacteria.

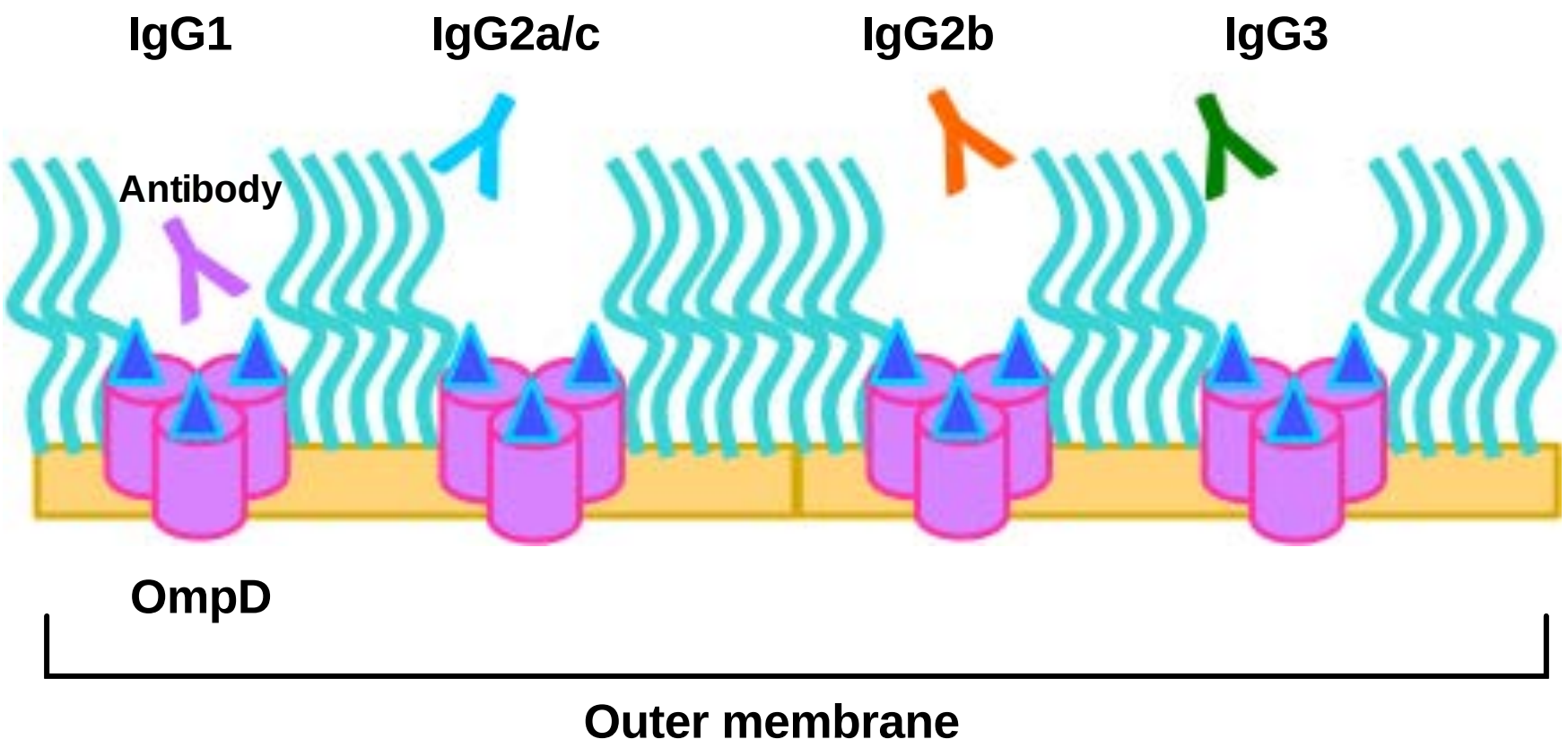


Figure 3.22 Illustration of different IgG isotypes binding OmpD
Model hypothesising why not all IgG isotypes are protective. IgG1 is important for protection after OmpD immunisation but other isotypes may not be protective due to differences in Fab fragments or flexibility in the hinge region of each isotype, which may limit Ab to reach protective epitopes of OmpD. All this with addition of O-Ag usage may interfere in generating protective responses from specific IgG subclasses. Triangles represent the 263 epitopes in OmpD. Note: Figure not drawn to scale.

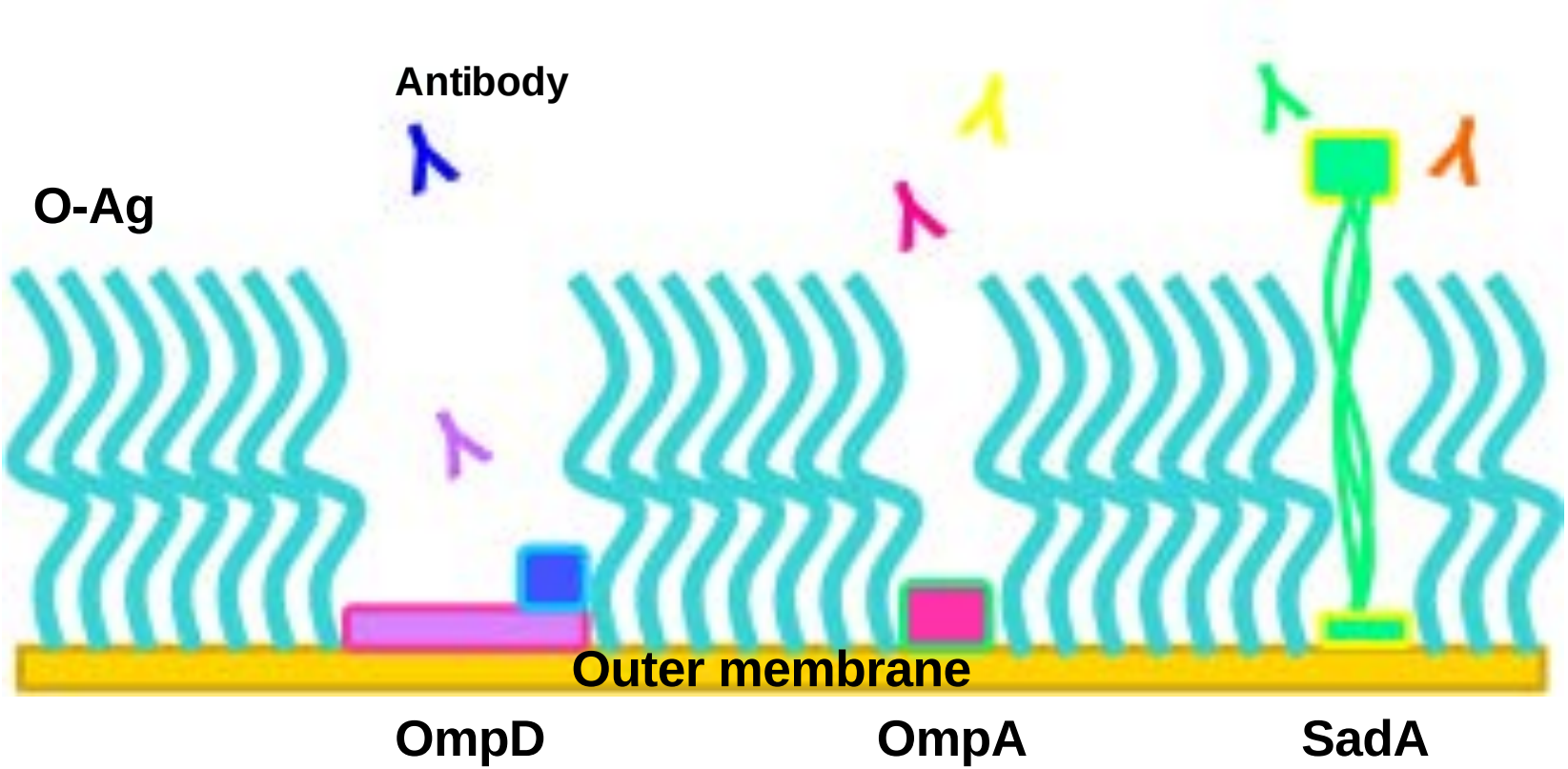


Fig. 3.23 Schematic of interaction between cell surface Ags and O-Ag in the outer membrane of Gram-negative bacteria

Access of Ab to conserved Ags in the cell wall is influenced by O-Ag usage. While some epitopes may be accessible for protective Ab, access to other epitopes may be limited due to O-Ag variability between different serovars. Note: Figure not drawn to scale.

CHAPTER 4: THE ROLE OF CELLS AND CYTOKINES IN PROTECTION AFTER STM-OMPD IMMUNISATION

4.1 Introduction

We have identified the importance of antibody (Ab) after STm-OmpD vaccination. What is less clear is how the host controls the infection in the presence of this Ab.

In mice T helper 1 (Th1) responses are associated with the transcription factor T-bet and induction of IgG2a/c and Th2 responses with IgG1 and IgE class switching (220, 287). Infection with *Salmonella* induces a typical Th1 response (287). Therefore, it is possible that Ab and Th1 responses combine to help control infection. Mice lacking T-bet and IFNg do not induce IgG2a/c after immunisation (343). Moreover, T-bet$^{-/-}$ mice have reduced production of IFNg from T cells (297). Therefore, assessing protection in these mice allows us to examine the role of IFNg from T cells and IgG2a/c in protection to OmpD in this process. Furthermore, not only is the role of individual molecules not fully elucidated but neither is the role of different cell types. The use of clodronate liposomes and administration of anti-GR1 Ab can deplete macrophages and neutrophils, respectively. Together this allows us to “map” the role of innate cells and cytokines.

The aim of this chapter was to understand better the mechanisms involved in how Ab to STm-OmpD protects and how quickly it can provide protection.

4.2 Results

4.2.1 Immunisation with STm-OmpD protects against STm 4 days of infection

To understand better the protective responses and cells involved in STm-OmpD protection we primed and boosted WT mice with 20 µg of purified STm-OmpD and challenged them with STm SL3261 for 4 days as shown in Figure 4.1A. NI mice infected with STm SL3261 were used as controls. Tissues were weighed and bacterial numbers enumerated and showed a reduction in bacterial numbers of approximately 100-fold in the spleen and liver (Fig. 4.1B) after immunisation with STm-OmpD. Spleens and livers were smaller or of equivalent size in immunised mice, respectively (Fig. 4.1C).

4.2.2 Class switching to all isotypes is induced to STm-OmpD

Antibody responses were assessed using sera from the mice immunised as in Figure 4.1A and relative titres to IgM, IgG, and IgG subclasses were determined. IgM and IgG were induced pre- and post-infection in mice immunised with STm-OmpD. While IgM titres remained similar between the NI and immunised groups, IgG levels were clearly increased in STm-OmpD immune sera compared to NI mice sera (Fig. 4.1D and E). All IgG isotypes detected before and after infection in STm-OmpD immunised mice (Fig. 4.1F), and did not rose significantly in the 4 day time frame post-infection.

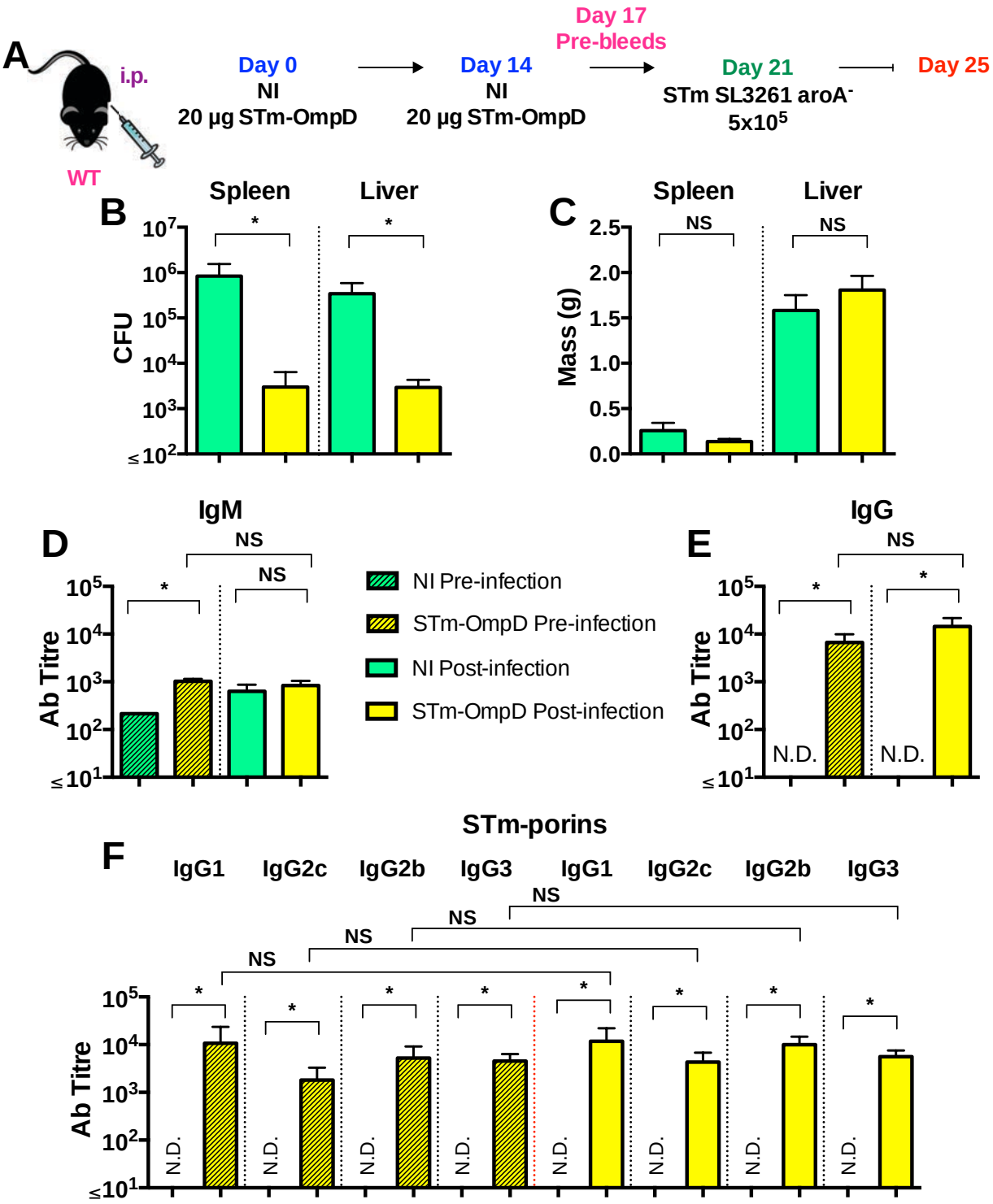


Figure 4.1 Immunisation with STm-OmpD protects against STm after 4 days of infection and induces robust Ab responses to STm-porins

(A) Protocol used for immunisation and challenge in this experiment. Bacterial numbers of (B) spleen and liver of NI (green bars) and STm-OmpD immunised (yellow bars) WT mice challenged with 5x10^5 STm SL3261 *aroA*$^-$. Tissues were weighed and mass for (C) spleens and livers determined. Relative (D) IgM and (E) IgG titres to STm-porins before and after STm infection were quantified. (F) IgG subclasses from sera obtained pre-infection and post-infection were determined. Groups of 4 mice were used. Data is representative of more than one experiment. * = $P \leq 0.05$, NS = non-significant.

4.2.3 Splenic T cells at day 4 after STm infection are similar in immunised and NI mice

As T cells are important in clearance of STm we assessed the phenotype of T cells after immunisation and challenge. T cells from mice that were immunised and infected as described in Figure 4.1A were analysed by flow cytometry. The gating strategy used is outlined in Figure 4.2A and B. The proportion and total numbers of $CD4^+$ and $CD8^+$ T cells were determined. Although there was a slight increase in the proportion of $CD4^+$ T cells in mice immunised with STm-OmpD (Fig. 4.2C), this was not reflected in the total numbers (Fig. 4.2D) or for $CD8^+$ T cells (Fig. 4.2E and F). Assesment of $CD62L^{lo}$ T cells showed there were no changes in the proportion and total number of cells between the NI and STm-OmpD immunised mice (Fig. 4.2G-J).

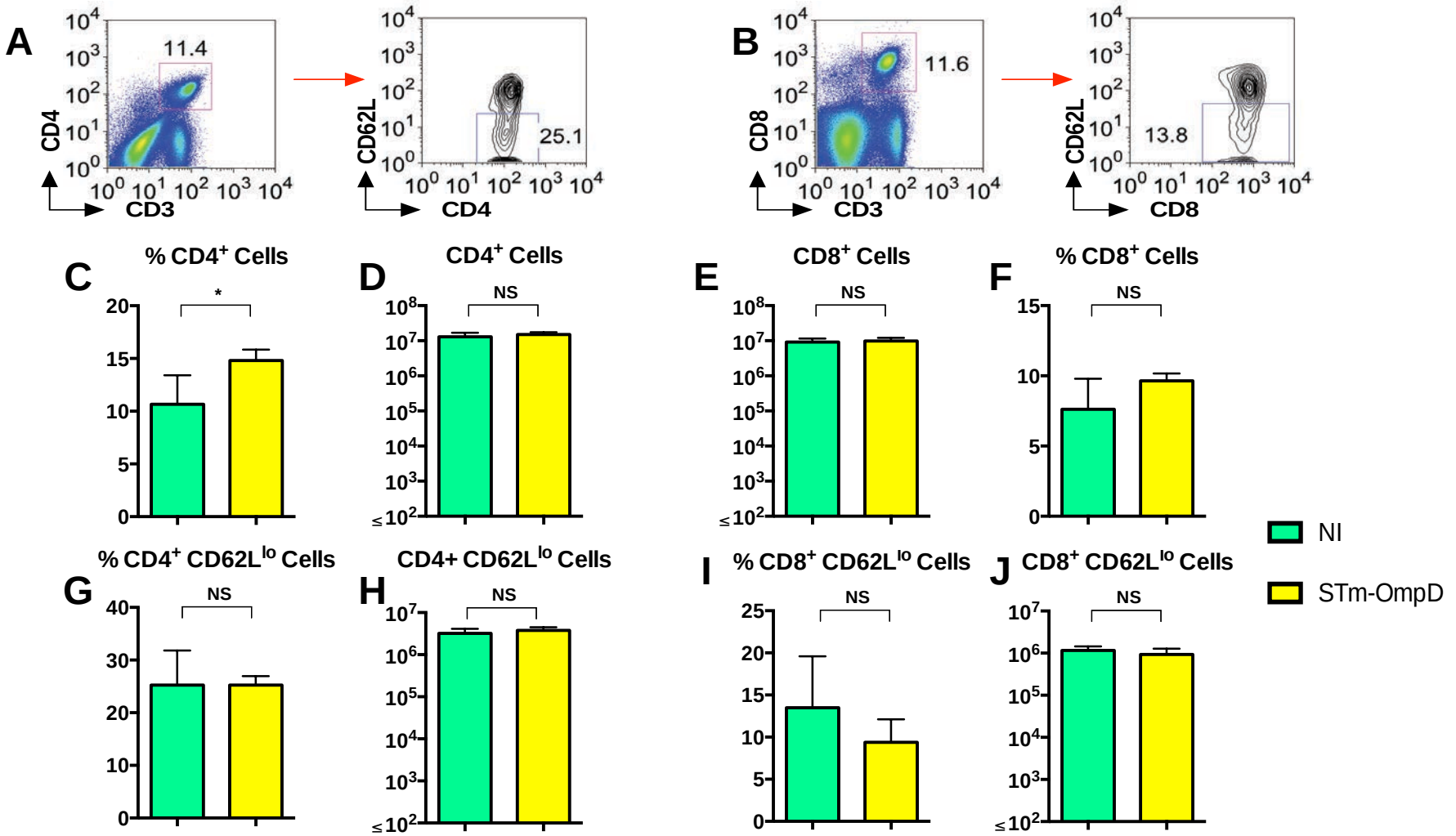


Figure 4.2 CD4$^+$ and CD8$^+$ T cells in the spleen at day 4 post-infection in STm-OmpD immunised mice
Analysis of CD4$^+$ and CD8$^+$ T cells from WT mice immunised as mentioned in Figure 4.1A. Representative FACS plots of (A) CD4$^+$ CD62L^{lo} cells and (B) CD8$^+$ CD62L^{lo} T cells that were previously gated on CD3$^+$ CD4$^+$/CD8$^+$ cells, respectively. Proportion and total number of (C and D) CD4$^+$ and (E and F) CD8$^+$ T cells were determined. Proportion and total cells of (G and H) CD4$^+$ CD62L^{lo} and (I and J) CD8$^+$ CD62L^{lo} T cells are shown. Groups of 4 mice were used. * = $P \leq 0.05$, NS = non-significant.

4.2.4 Splenic B cells populations after STm-OmpD immunisation and challenge

We then analysed the different B cell subsets present after STm-OmpD immunisation and infection. B cells were gated as $CD19^{+}$ $B220^{+}$ cells (Fig. 4.3A). No difference in the proportion or total numbers of $CD19^{+}$ $B220^{+}$ cells was observed between NI and STm-OmpD mice 4 days after infection (Fig. 4.3B and C). Three B cell subsets were gated for: $CD23^{hi}$ $CD21^{lo}$ (follicular B cells), $CD23^{lo}$ $CD21^{hi}$ (marginal zone B cells), and $CD23^{lo}$ $CD21^{lo}$ (transitional B and B1 cells)(Fig. 4.3A). No differences in the total number of cells from the three subsets was observed in any of the groups (Fig. 4.3G-I). However, the proportion of $CD23^{hi}$ $CD21^{lo}$ and $CD23^{lo}$ $CD21^{hi}$ B cells did differ with an increase in the STm-OmpD immunised mice, whereas the proportion of cells in the $CD23^{lo}$ $CD21^{lo}$ subset decreased by approximately 10% compared to the NI mice (Fig. 4.3D-F).

Since B cell responses are important in immunity against STm and Ab to STm-OmpD is required for protection we examined the germinal centre (GC) response produced after immunisation and challenge (Fig. 4.3A). The number and proportion of $GL7^{+}$ FAS^{+} GC B cells were higher in immunised mice than in NI mice (Fig. 4.3J and K).

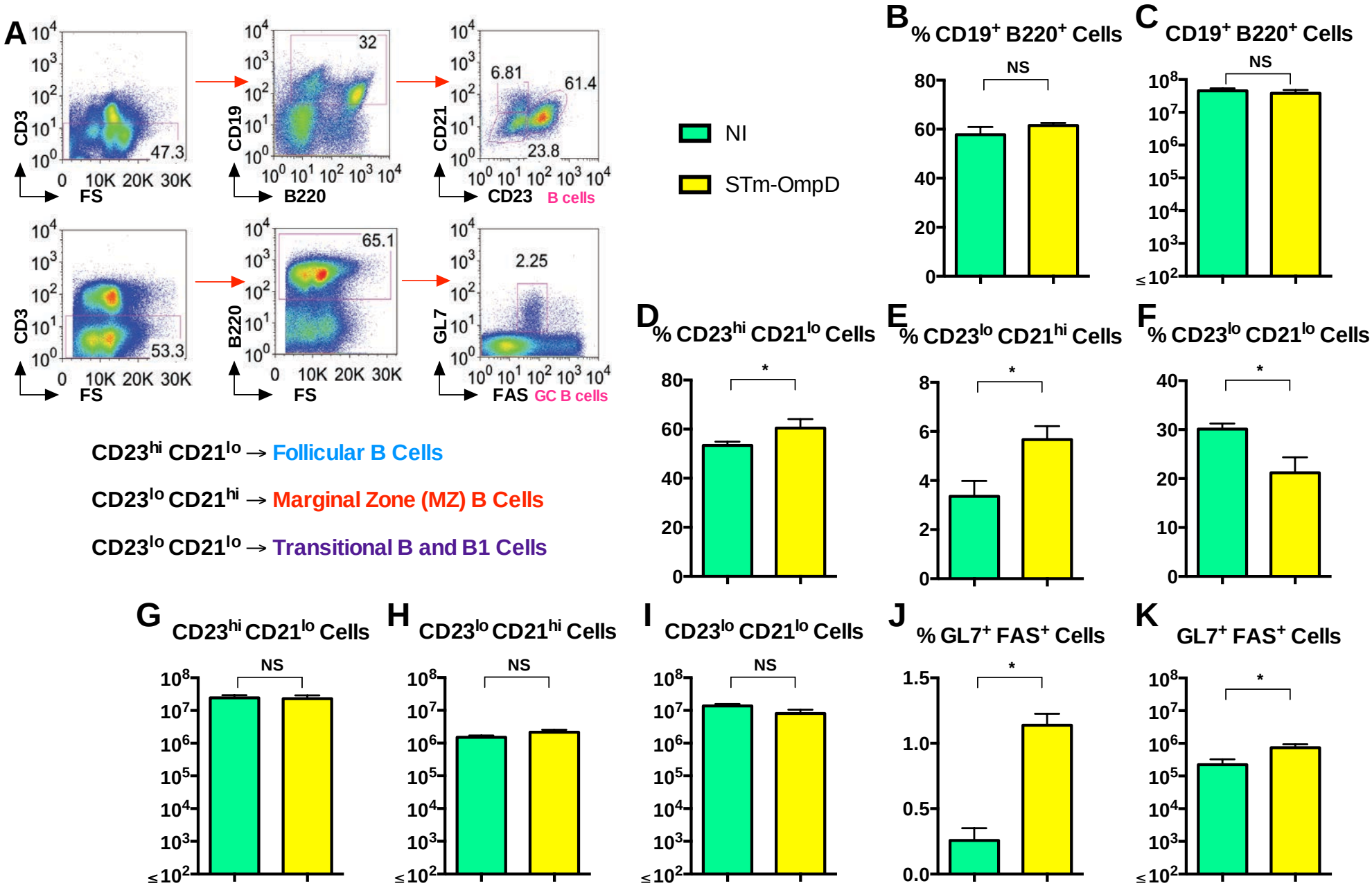

A
CD3
FS
47.3
CD19
B220
32
CD21
CD23
6.81
61.4
23.8
B cells
CD3
FS
53.3
B220
FS
65.1
GL7
FAS
2.25
GC B cells
NI
STm-OmpD
B % CD19+ B220+ Cells
NS
C CD19+ B220+ Cells
NS
D % CD23hi CD21lo Cells
*
E % CD23lo CD21hi Cells
*
F % CD23lo CD21lo Cells
*
CD23hi CD21lo → Follicular B Cells
CD23lo CD21hi → Marginal Zone (MZ) B Cells
CD23lo CD21lo → Transitional B and B1 Cells
G CD23hi CD21lo Cells
NS
H CD23lo CD21hi Cells
NS
I CD23lo CD21lo Cells
NS
J % GL7+ FAS+ Cells
*
K GL7+ FAS+ Cells
*

Figure 4.3 Immunisation with STm-OmpD induces a B cell response and formation of GC B cells after 4 days of infection

Mice were immunised and challenged as described in Figure 4.1A. (A) Representative FACS analysis of the different subsets from the B cell population in the spleen. (B and C) Proportion and total number of $CD19^{+}$ $B220^{+}$ B cells were determined. Frequencies and total cells of (D and G) follicular B cells ($CD23^{hi}$ $CD21^{lo}$), (E and H) marginal zone (MZ) B cells ($CD23^{lo}$ $CD21^{hi}$), and (F and I) transitional B cells ($CD23^{lo}$ $CD21^{lo}$) are shown. (J) Representative FACS gating for $GL7^{+}$ FAS^{+} GC B cells in the spleen. (K) Proportion and (L) total number of $GL7^{+}$ FAS^{+} GC B cells. Groups of 4 mice were used. * = $P \leq 0.05$, NS = non-significant.

4.2.5 T-bet and IFNg are required for optimal control of STm infection after STm-OmpD immunisation

The earlier experiments with STm-OmpD in this Chapter showed that immunised and NI mice had similar numbers of lymphocytes. Therefore, we wanted to examine what influence Th1 associated molecules had on the control of infection. It has been shown that IFNg plays a key role in the control of *Salmonella* during the first week of infection (154, 219) but it is not clear if this is also the cause for Ab-mediated protection and if so is this through the production of IFNg by $CD4^+$ T cells. To examine this, groups of WT, T-$bet^{-/-}$, and $IFNg^{-/-}$mice were immunised i.p. either with PBS or 20µg of STm-OmpD. These mice were boosted at day 14, and 7 days later challenged with STm SL3261 for 4 days (Fig. 4.4A). In all immunised groups a reduction in the bacterial numbers in the spleen was observed compared to the NI groups (Fig. 4.4B). However, while STm numbers were reduced by almost 1000-fold in WT mice, numbers were a 10-fold higher in the T-$bet^{-/-}$ immunised group, and >100 fold higher in $IFNg^{-/-}$ mice (Figure 4.4B). Thus, T-$bet^{-/-}$, and $IFNg^{-/-}$ mice are needed for the optimal control of infection in the presence of anti-STm-OmpD Ab.

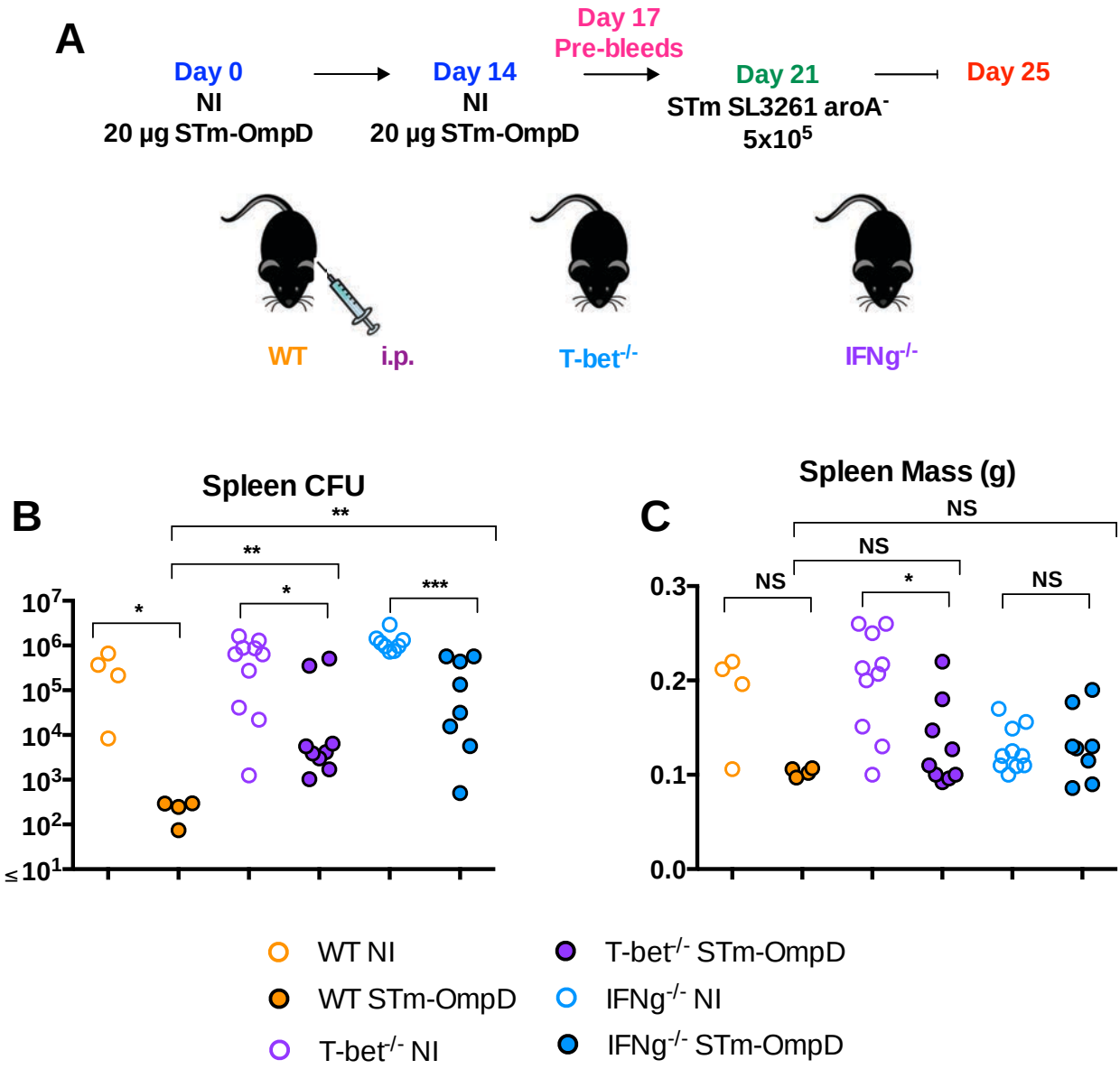


Figure 4.4 T-bet and IFNg are required for optimal control of infection after STm-OmpD immunisation

(A) Diagram showing protocol for immunisation used in this study. (B) Bacterial numbers in the spleen of non-immunised (NI) and STm-OmpD immunised WT (orange circles), T-bet$^{-/-}$ (purple circles), and IFNg$^{-/-}$ (blue circles) mice infected with 5x10^5 STm SL3261. (C) Spleens were weighed and mass determined. Data is from 3 different experiments and each dot represents one mouse. * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, NS = non-significant.

4.2.6 Switching to IgG2a/c is not needed to control STm infection in STm-OmpD immunised mice

While IgG2a/c contributes to protection after natural infection (287) our data showed that lack of T-bet and IFNg have a variable impact on protection after vaccination with STm-OmpD. To determine if this was the cause of deficient IgG2a/c induction between these mice, we analysed the Ab titres to porins in all groups. Relative titres of IgM were similar in all groups after infection with STm (Fig. 4.5A) whereas IgG was only detected in those mice that received STm-OmpD (Fig. 4.5B). Moreover, all IgG isotypes were detected in immune sera from WT mice. No IgG2a/c was detected in the KO mice (Fig. 4.5C and D). Surprisingly IgG3 was only detected in a high level in WT immunised mice. While IgG3 was detected in T-bet$^{-/-}$ and IFNg$^{-/-}$ mice, it was lower than in WT mice (Figure 4.5C and D). Therefore, loss of T-bet and IFNg leads to a loss of IgG2a/c and a reduced IgG3 response to STm-OmpD. Nevertheless, the anti-STmOmpD IgG2a/c and IgG3 titres were similar between the KO strains, suggesting that the difference in the level of control of infection seen in these strains is not due to a difference in Ab titres.

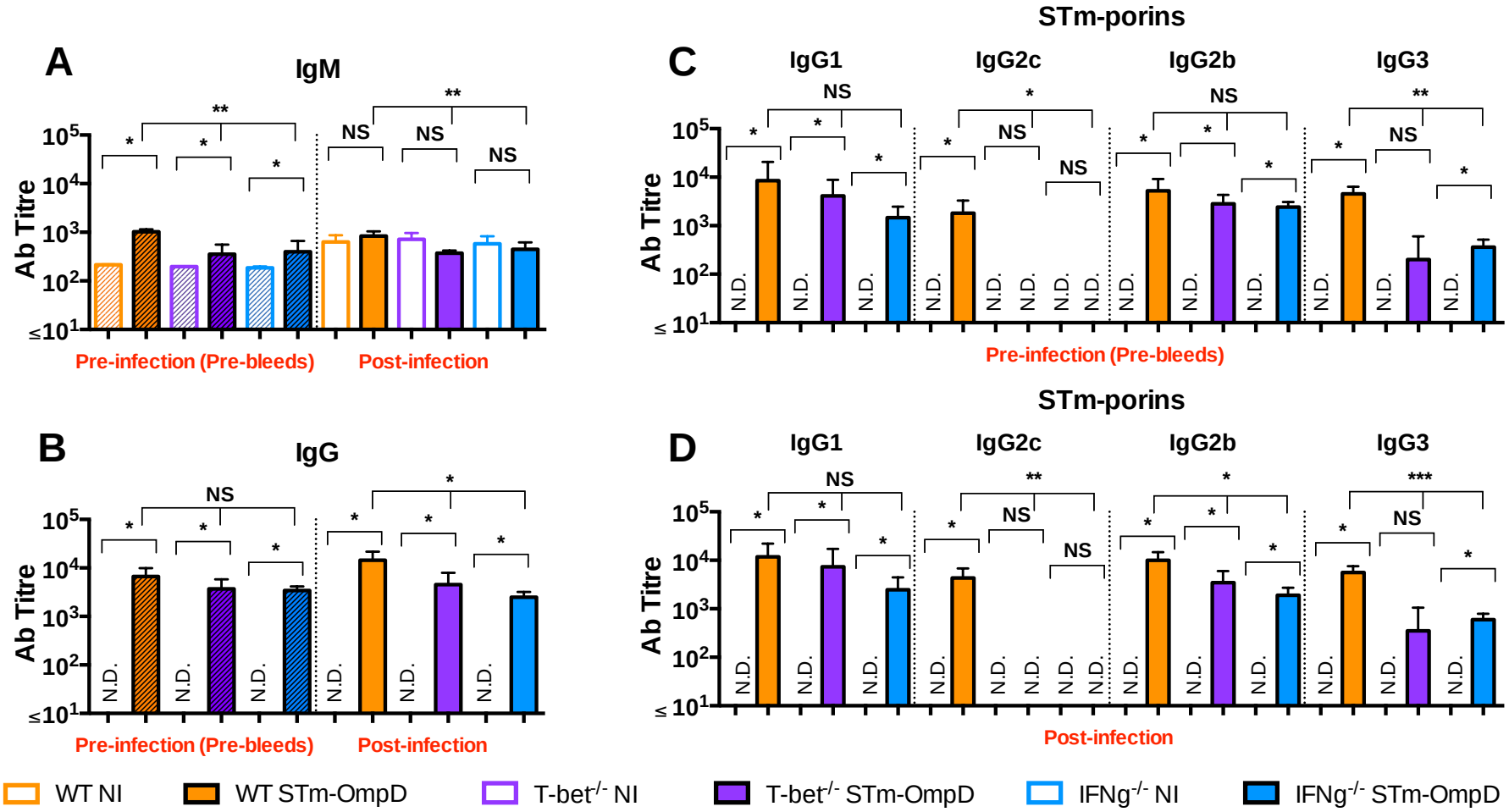


Figure 4.5 Ab responses are induced after immunisation with STm-OmpD in KO mice

ELISAs from murine sera (Fig. 4.1A). Relative (A) IgM and (B) IgG Ab titres to STm-porins before and after infection with STm in WT (orange bars), T-bet$^{-/-}$ (purple bars), and IFNg$^{-/-}$ (blue bars) mice were detected. (C and D) IgG subclasses to STm-porins from sera obtained pre and post-infection, respectively. Data is representative from more than one experiment. Groups of minimum 4 mice per group were used. To determine statistical differences, the Mann-Whitney U test was used to compare 2 groups. The ONE-Way ANOVA test was used to compare the STm-OmpD immunised groups * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, NS = non-significant. N.D. = not detected.

4.2.7 Numbers of activated $CD4^+$ and $CD8^+$ T cell populations are similar in all immunised and infected groups

To examine the T cell response in these mice we examined T cells by flow citometry. Splenocytes were isolated and the proportion and number of $CD4^+$ and $CD8^+$ T cells were assessed as shown in Figure 4.2A and B. The proportion and total numbers of $CD4^+$ activated T cells were gated on $CD4^+$ $CD62L^{lo}$ cells from the $CD3^+$ $CD4^+$ lymphocyte population (Fig. 4.6E and F). Figure 4.6E and F show that T cell activation in the CD4 subset is similar between groups. The same was observed for $CD8^+$ T cells (Fig. 4.6G and H).

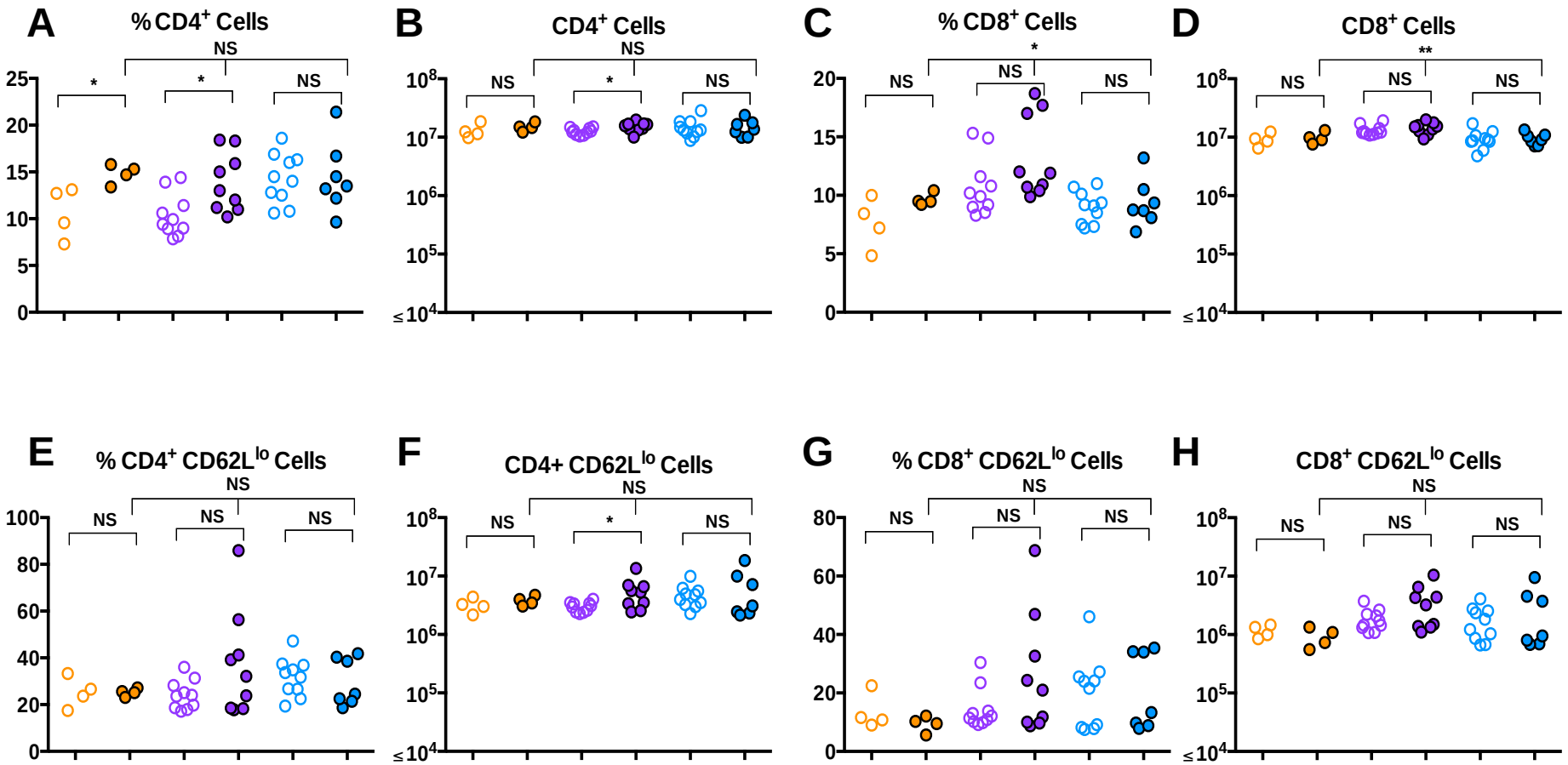


Figure 4.6 CD4$^+$ and CD8$^+$ T cells are not influenced by immunisation with STm-OmpD in WT and KO mice
For FACS analysis used to gate CD4$^+$ and CD8$^+$ T cells refer to Figure 4.2A and B. Proportion and total number of (A and B) CD4$^+$ (C and D) CD8$^+$ (E and F) CD4$^+$ CD62L^{lo} and (G and H) CD8$^+$ CD62L^{lo} T cells from spleens of WT (orange circles), T-bet$^{-/-}$ (purple circles), and IFNg$^{-/-}$ (blue circles) mice were determined. Data is from 3 individual experiments and each dot equals one mouse. The Mann-Whitney U test was used to determine statistical difference between 2 groups and the ONE-Way ANOVA test between all the STm-OmpD immunised groups. * = $P \leq 0.05$, NS = non-significant.

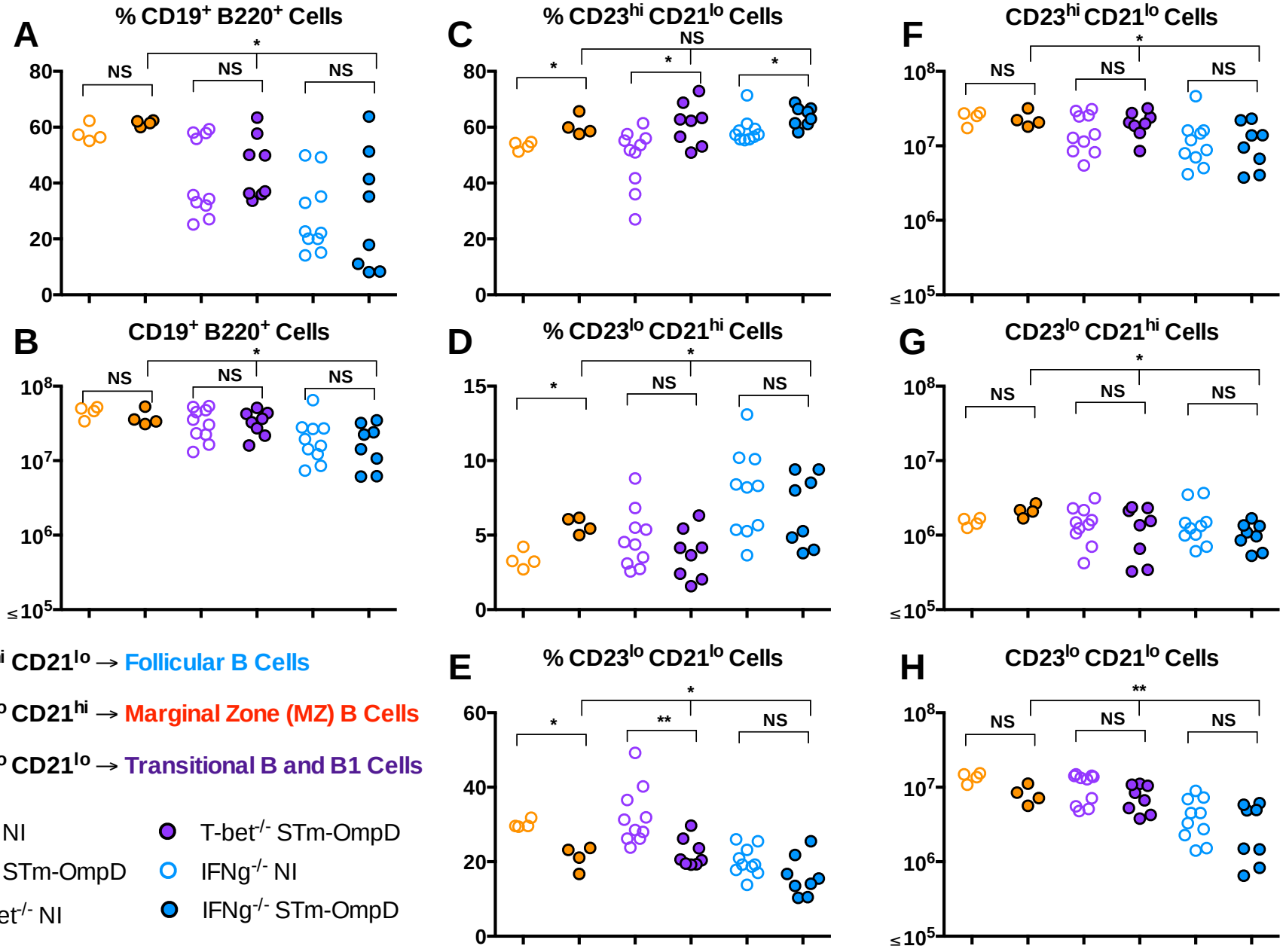
4.2.8 B cell numbers and proportions in the spleen can be influenced by T-bet and IFNg after immunisation with STm-OmpD and infection

Since our data in WT mice suggested immunisation with STm-OmpD and infection could modulate cell proportions of some B cell populations we examined the B cell response in these T-bet$^{-/-}$ and IFNg$^{-/-}$ mice. Splenic B cells were gated on CD19 and B220 and subsequently divided into CD23^{hi} CD21^{lo} (follicular B cells), CD23^{lo} CD21^{hi} (marginal zone B cells), and CD23^{lo} CD21^{lo} (transitional B cells)(Fig. 4.3A). While no significant differences in the proportion and total number of B cells were detected between NI and immunised mice (Fig. 4.7A and B), a reduction was detected in the percentage and number of B cells in immunised IFNg$^{-/-}$ mice compared to WT and T-bet$^{-/-}$ mice that were given STm-OmpD before infection (Fig. 4.7A). Moreover, differences were observed in the proportion of all 3 subsets of B cells in all the STm-OmpD immunised groups (Fig. 4.7C-E) whereas the total number of cells remained similar between NI and immune mice (Fig. 4.7F-H).

4.2.9 Germinal centre (GC) B cell responses induced to STm-OmpD are not T-bet or IFNg dependent

We showed in section 4.2.4 that GC B cell responses are developed after immunisation with STm-OmpD in WT mice. We next examined if this increase in GC B cells was also observed in infected T-bet$^{-/-}$ and IFNg$^{-/-}$ mice that were previously primed and boosted with STm-OmpD. The total number and frequency of GL7^{+} FAS^{+} GC B cells were determined from spleens isolated from WT, T-bet$^{-/-}$, and IFNg$^{-/-}$mice

immunised and infected as illustrated in Figure 4.3A. This revealed that all STm-OmpD immunised mice were able to induce GC B cells, irrespective of the loss of T-bet or IFNg (Fig. 4.7I and J), which indicates that induction of Ab and GC responses to STm-OmpD are not influenced by T-bet or IFNg.


A
% CD19+ B220+ Cells
B
CD19+ B220+ Cells
C
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D
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E
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F
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G
CD23lo CD21hi Cells
H
CD23lo CD21lo Cells
NS
*
**
CD23hi CD21lo → Follicular B Cells
CD23lo CD21hi → Marginal Zone (MZ) B Cells
CD23lo CD21lo → Transitional B and B1 Cells
WT NI
WT STm-OmpD
T-bet-/- NI
T-bet-/- STm-OmpD
IFNg-/- NI
IFNg-/- STm-OmpD

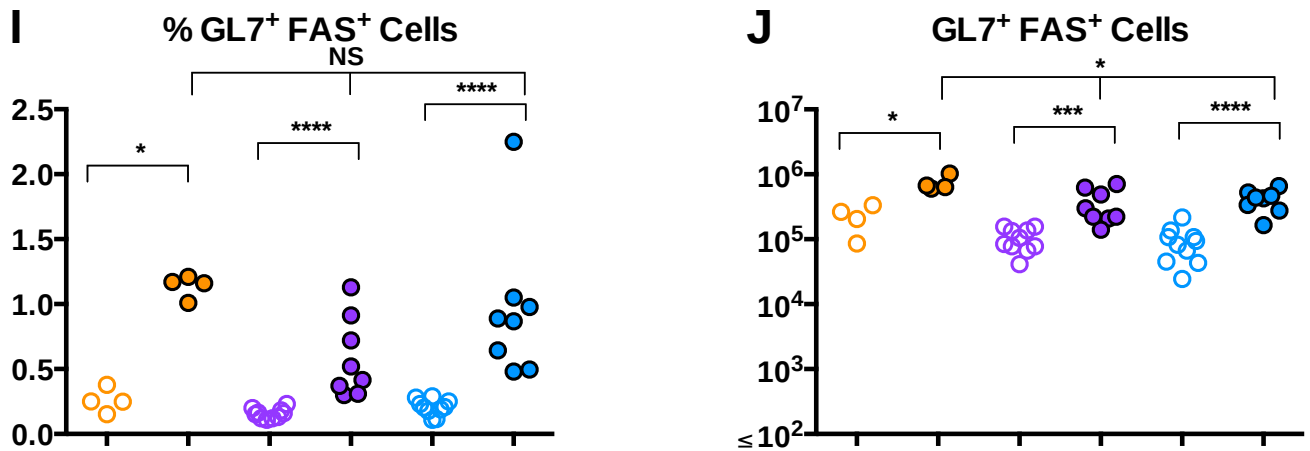

Figure 4.7 T-bet and IFNg influence B cell development but not GC B cells in mice immunised with STm-OmpD
FACS gating strategies used to identify the different B cell subsets and GC B cells are shown in Figure 4.3A. (A and B) Proportion and total number of $CD19^{+}$ $B220^{+}$ splenic cells was determined in WT (orange circles), T-$bet^{-/-}$ (purple circles), and $IFNg^{-/-}$ (blue circles) mice. Frequencies of (C) $CD23^{hi}$ $CD21^{lo}$, (D) $CD23^{lo}$ $CD21^{hi}$, and (E) $CD23^{lo}$ $CD21^{lo}$ are shown. Total number of (F) $CD23^{hi}$ $CD21^{lo}$, (G) $CD23^{lo}$ $CD21^{hi}$, and (H) $CD23^{lo}$ $CD21^{lo}$ were determined. (I and J) Proportion and total number of $GL7^{+}$ FAS^{+} cells are shown. To determine statistical differences, the Mann-Whitney U test was used to compare 2 groups. The ONE-Way ANOVA test was used to compare all STm-OmpD immunised groups * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$, NS = non-significant.

4.2.10 Immunisation with STm-OmpD can provide protection within 24 hours of STm infection

Since protection is established by 4 days we assessed whether the benefits could be detected earlier after infection. Moreover, since IFNg was clearly important for protection we wished to identify the cellular sources of this cytokine. To do this we used IFNg reporter mice that produce eYFP when IFNg is transcribed (299).

We then decided to assess how early protection with STm-OmpD could be appreciated. IFNg reporter mice were immunised i.p. either with PBS or 20 µg of STm-OmpD and boosted at day 14. Fourteen days later they were challenged with STm SL3261 for 1 day (Fig. 4.8A). In STm-OmpD immunised mice there was a decrease in STm bacterial numbers in spleen, liver, and in the peritoneal cavity (PEC)(Fig. 4.8C). At this time point after infection no changes in the size of the tissues were observed (Fig. 4.8B). Similar results were also observed with WT mice (data not shown), indicating that this is not due to the generic make up of these mice. Thus, STm-OmpD is able to protect against infection at different anatomical sites 24 hours after infection.

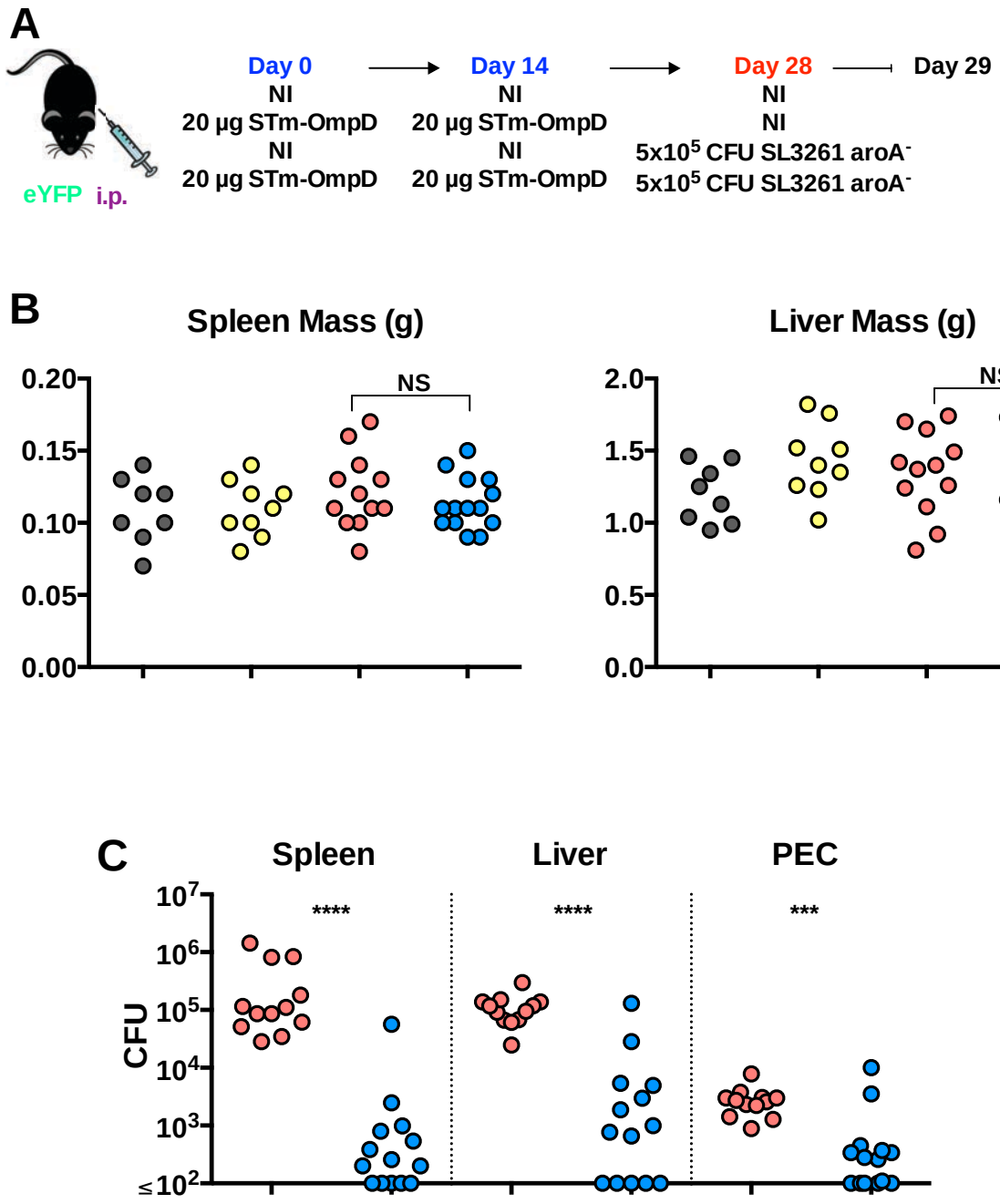

Figure 4.8 Immunisation with STm-OmpD confers protection during early stages of infection

IFNg (eYFP) reporter mice were either infected with STm SL3261 i.p. for 1 day (salmon circles) or immunised and boosted with STm-OmpD and infected for 24 hours (blue circles). Control groups of NI mice (grey circles) and STm-OmpD immunised mice (purple circles) were used. (A) Protocol of immunisation used in these experiments. (B) Spleens and livers were weighed and mass calculated (C) Bacterial numbers of spleen, liver and PEC were counted. Data is from 3 different experiments. Each mouse is represented by one dot. ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$, NS = non-significant.

4.2.11 Cell populations in the spleen and PEC produce similar levels of IFNg in NI and STm-OmpD immunised mice 24 hours after infection

Our data in section 4.2.5 suggested that IFNg was important for offering optimal protection after immunisation with STm-OmpD. To evaluate the sources of IFNg after STm-OmpD immunisation and challenge IFNg (eYFP) reporter mice were immunised as described in Figure 4.8A. The following sections explore this in more detail.

4.2.11.1 $CD4^+$ and $CD8^+$ T cell populations show different responses after immunisation with STm-OmpD and infection

$CD4^+$ and $CD8^+$ T cell populations in the spleen and PEC were analysed after immunisation and infection (Fig. 4.9A). In the spleen both $CD4^+$ and $CD8^+$ T lymphocyte proportions and numbers did not change (Fig. 4.9B-E). In the PEC an increase in the total of proportion of both subsets in mice immunised with STm-OmpD was observed (Fig. 4.9F and G), but still reflected their pre-challenge state. Expression of IFNg from $CD4^+$ and $CD8^+$ T cells in spleen and PEC one day after infection was then examined (Fig. 4.10A). As expected at this time point, no change in the proportion and total number of IFNg+ cells from the $CD4^+$ $CD62L^{lo}$ (Fig. 4.10B and C) and $CD4^+$ $CD62L^{hi}$ (Fig. 4.10D and E) populations in the spleen was observed after infection in those mice immunised with STm-OmpD. A similar result was observed for splenic $CD8^+$ $CD62L^{lo}$ $IFNg^+$ cells (Fig. 4.10F and G). However, a slight increase was observed in the proportion and total number of the $CD8^+$ $CD62L^{hi}$ $IFNg^+$ cells in the spleen (Fig. 4.10H and I). Thus, $CD8^+$ T cells can produce IFNg early after primary infections, but this is lost in mice protected by immunisation. In the PEC, no

change in the proportion of any of the CD4 and CD8 populations was observed (Fig. 4.11A-D), although immunisation or challenge increased the induction of $IFNg^{+}$ cells in the $CD4^{+}$ and $CD8^{+}$ $CD62L^{lo}$ population (Fig. 4.11A and C).

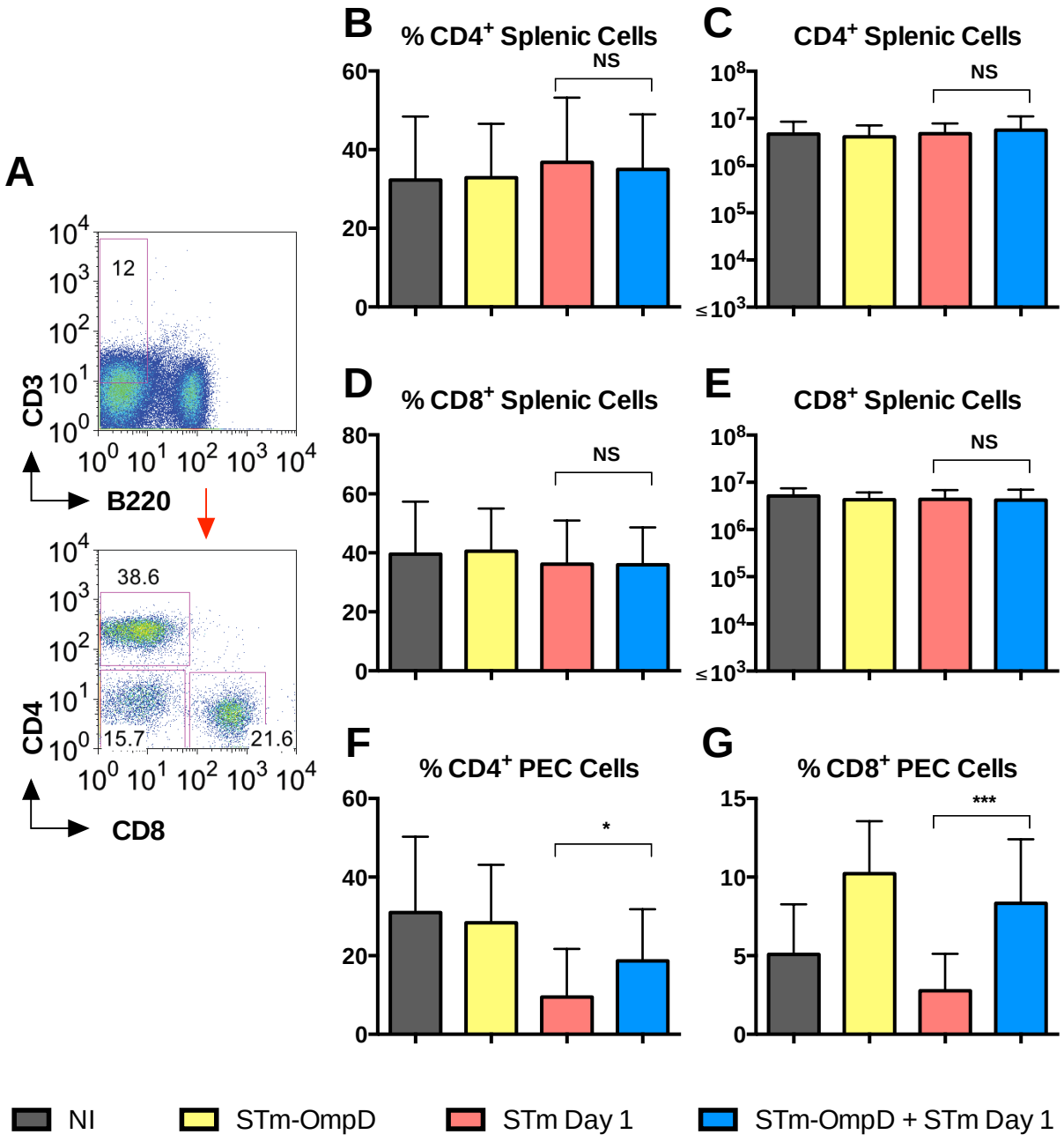

Figure 4.9 CD4$^+$ and CD8$^+$ T cells in the spleen and PEC of NI and STm-OmpD immunised mice 1 day post-infection

(A) Representative FACS gating used for T cells in IFNg (eYFP) reporter mice immunised as mentioned in Fig. 4.8A. Proportion and total number of (B and C) CD4$^+$ and (D and E) CD8$^+$ splenic T lymphocytes were determined. (F and G) Proportion in the PEC of CD4$^+$ and CD8$^+$ T cells is shown, respectively. Data is from 3 individual experiments where minimum 4 mice per group were used. * = $P \leq 0.05$, *** = $P \leq 0.001$, NS = non-significant.

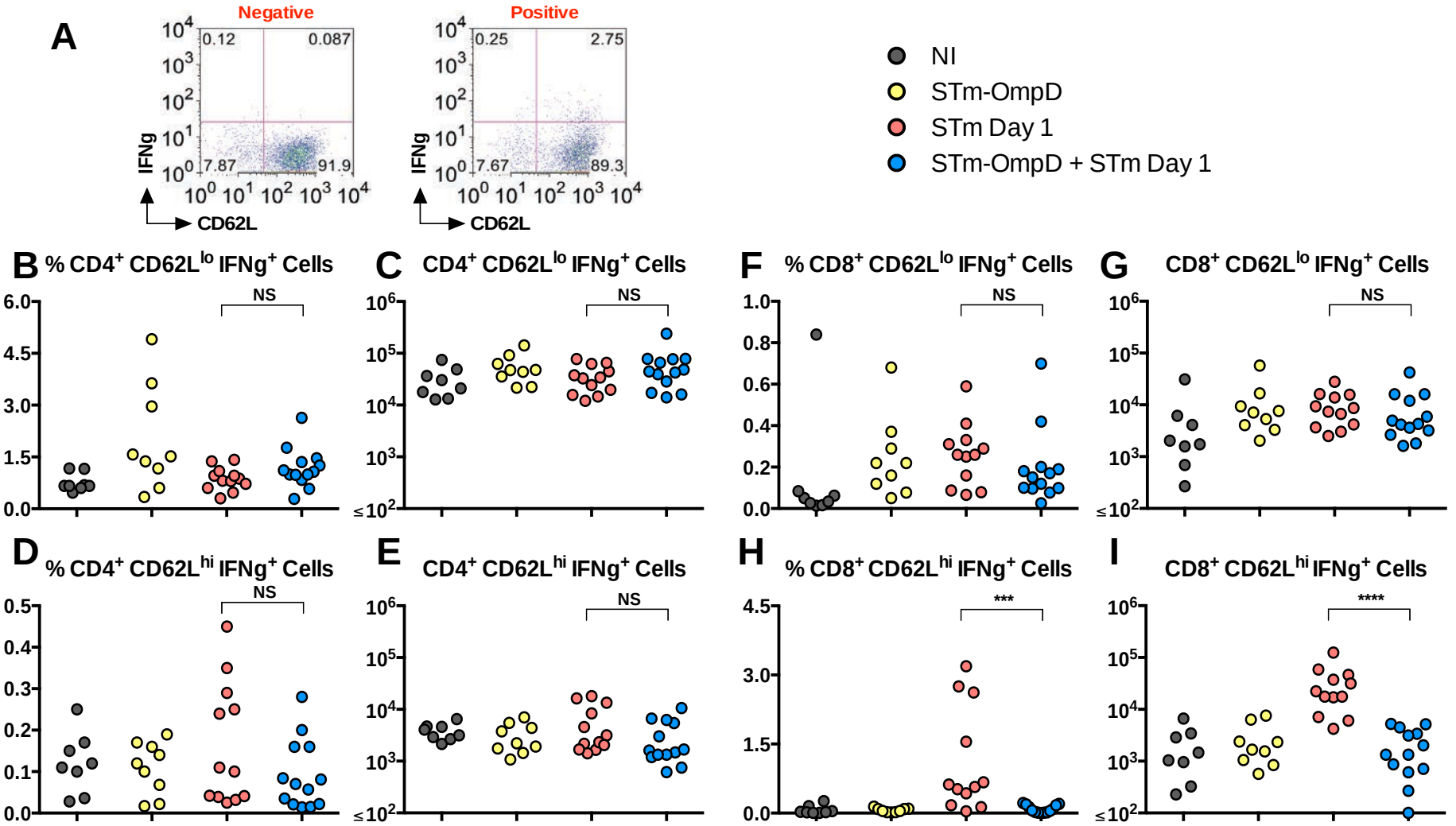

Figure 4.10 Splenic CD4$^+$/CD8$^+$ CD62L^{lo} IFNg$^+$ and CD4$^+$/CD8$^+$ CD62L^{hi} IFNg$^+$ populations in NI and STm-OmpD immunised mice after 1 day of infection

IFNg (eYFP) reporter mice were infected as described in Figure 4.8A. (A) Representative FACS plot of cells stained for CD4$^+$CD8$^+$ CD62L$^{lo/hi}$ IFNg$^+$ T cells. Proportion and total cells of (B and C) CD4$^+$ CD62L^{lo} IFNg$^+$, (D and E) CD4$^+$ CD62L^{hi} IFNg$^+$, (F and G) CD8$^+$ CD62L^{lo} IFNg$^+$, and (H and I) CD8$^+$ CD62L^{hi} IFNg$^+$, were determined. Data is from 3 individual experiments. *** = $P \leq 0.001$, **** = $P \leq 0.0001$, NS = non-significant.

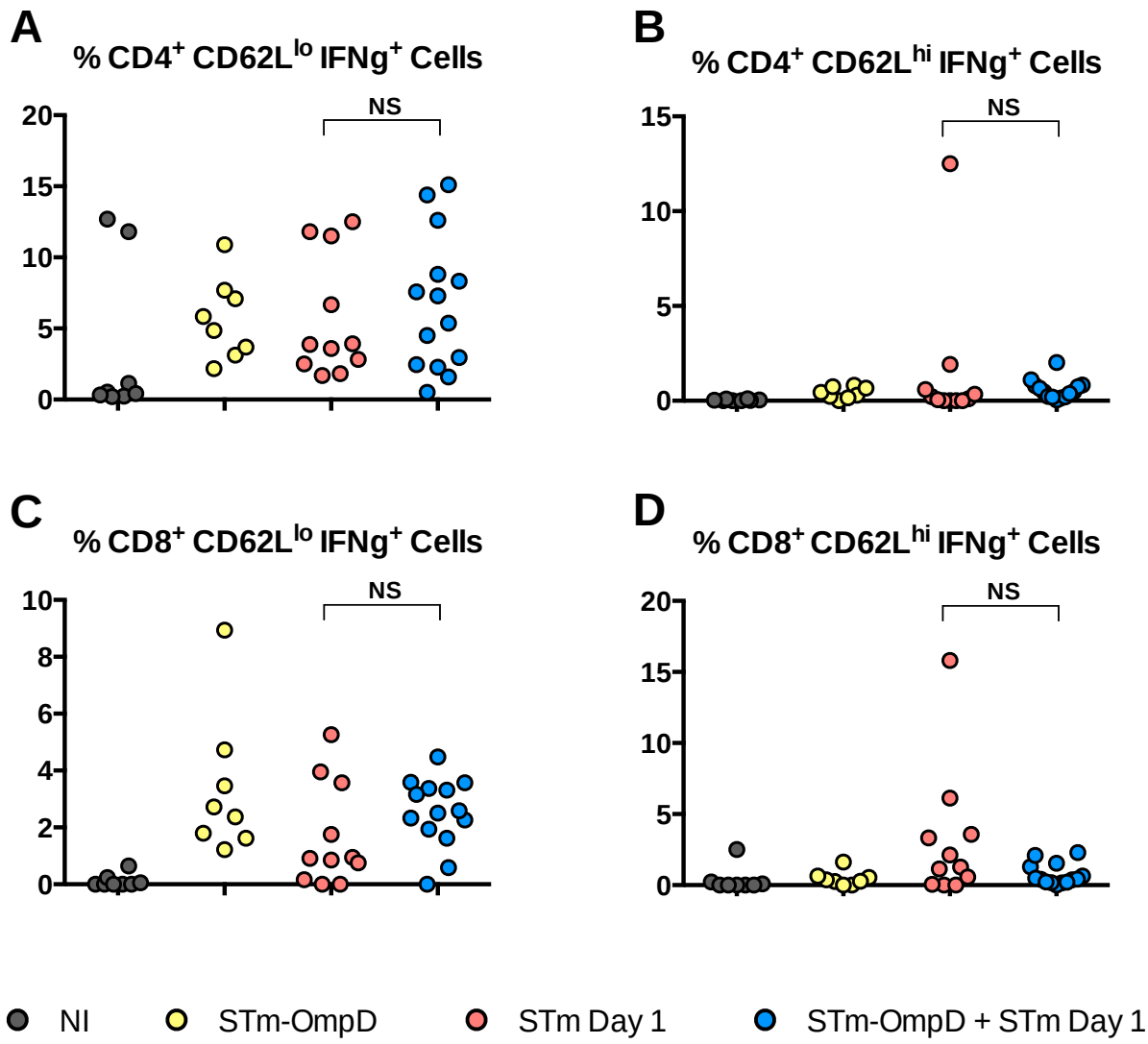

Figure 4.11 $CD4^+/CD8^+$ $CD62L^{lo}$ $IFNg^+$ and $CD4^+/CD8^+$ $CD62L^{hi}$ $IFNg^+$ cells in the PEC in NI and STm-OmpD immunised mice at 1 day post-infection

IFNg (eYFP) reporter mice were infected as showed in Figure 4.8A. FACS analysis of PEC cells stained for $CD4^+/CD8^+$ $CD62L^{lo/hi}$ $IFNg^+$ T cells was performed as shown in Fig. 4.10A. Proportion of (A) $CD4^+$ $CD62L^{lo}$ $IFNg^+$, (B) $CD4^+$ $CD62L^{hi}$ $IFNg^+$, (C) $CD8^+$ $CD62L^{lo}$ $IFNg^+$ and (D) $CD8^+$ $CD62L^{hi}$ $IFNg^+$ are shown. Data is from 3 individual experiments. NS = non-significant.

4.2.11.2 B cells do not produce IFNg after STm-OmpD immunisation

The potential for B cells to produce IFNg was assessed (Fig. 4.12A). B cell numbers and proportions were similar in the spleen and PEC under all conditions (Fig. 4.12B-D). To examine if B cells produce IFNg, $B220^+$ IgM^+ $IFNg^+$ cells were enumerated. Very few B cells produced IFNg in either the spleen or PEC (Fig. 4.12E-G). Therefore, while B cells contribute to protective responses through Ab, they do not do it through the production of IFNg.

GC B cells and plasma cells were identified as $GL7^+$ FAS^+ and $B220^+$ $CD138^+$ cells, respectively (Fig. 4.13A). GC B cells were readily detected after immunisation with STm-OmpD (Fig. 4.13B and C). Nevertheless, few GC B cells or plasma cells were $IFNg^+$ (Fig. 4.13G-I).

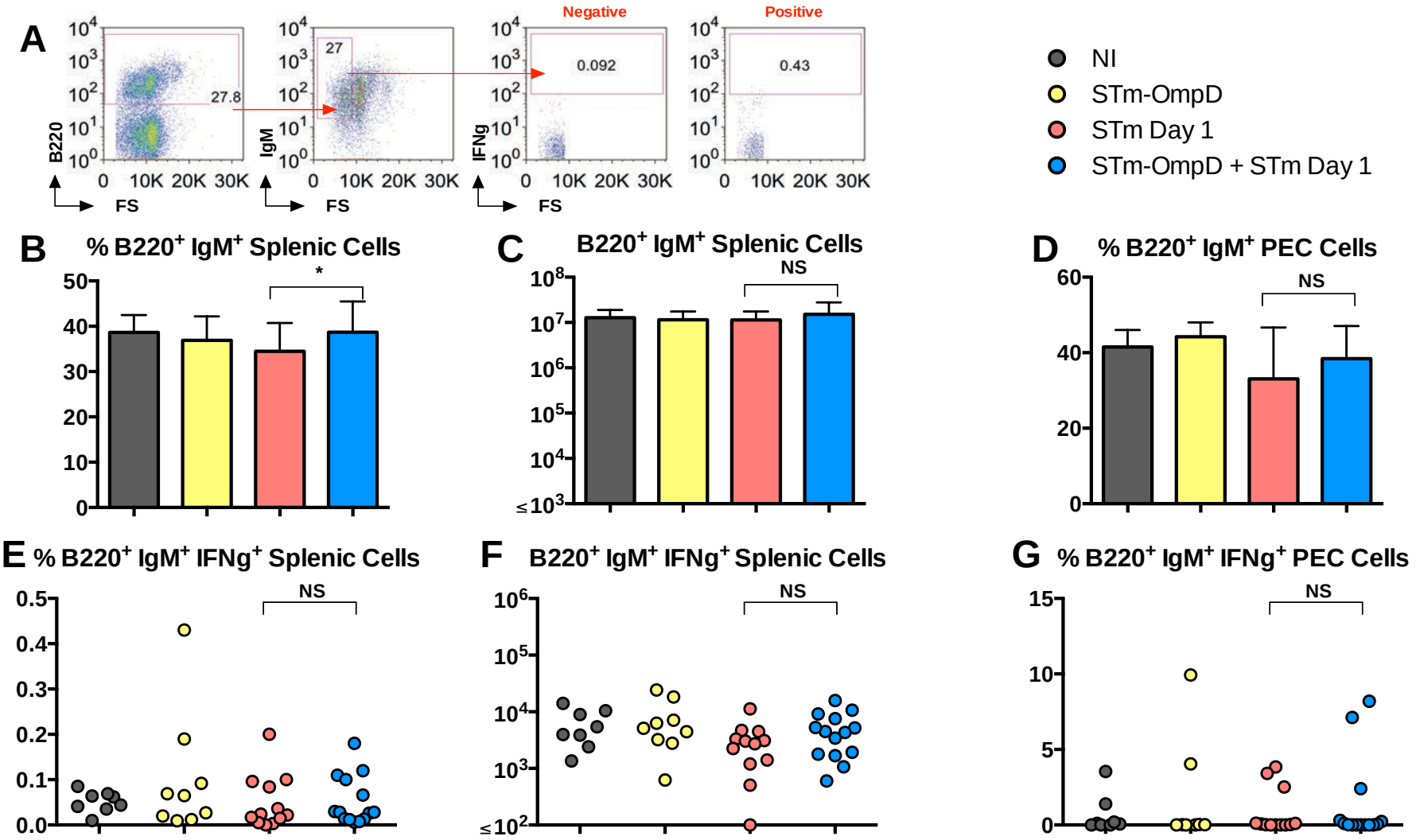

Figure 4.12 Total B cells and IFNg$^+$ B cell populations in the spleen and PEC after 1 day of infection in NI and STm-OmpD immunised mice

(A) Illustration of representative FACS plots used to gate B cells in IFNg (eYFP) reporter mice immunised as mentioned in Figure 4.8A. (B and C) Proportion and total number of splenic B220$^+$ IgM$^+$ B cells. (D) Frequency of B220$^+$ IgM$^+$ B cells in the PEC. (E and F) Proportion and total numbers of B220$^+$ IgM$^+$ IFNg$^+$ splenic cells. (G) Proportion of B220$^+$ IgM$^+$ IFNg$^+$ PEC cells. Data is from 3 individual experiments and 4 mice or more were used per group. * = $P \leq 0.05$, NS = non-significant.

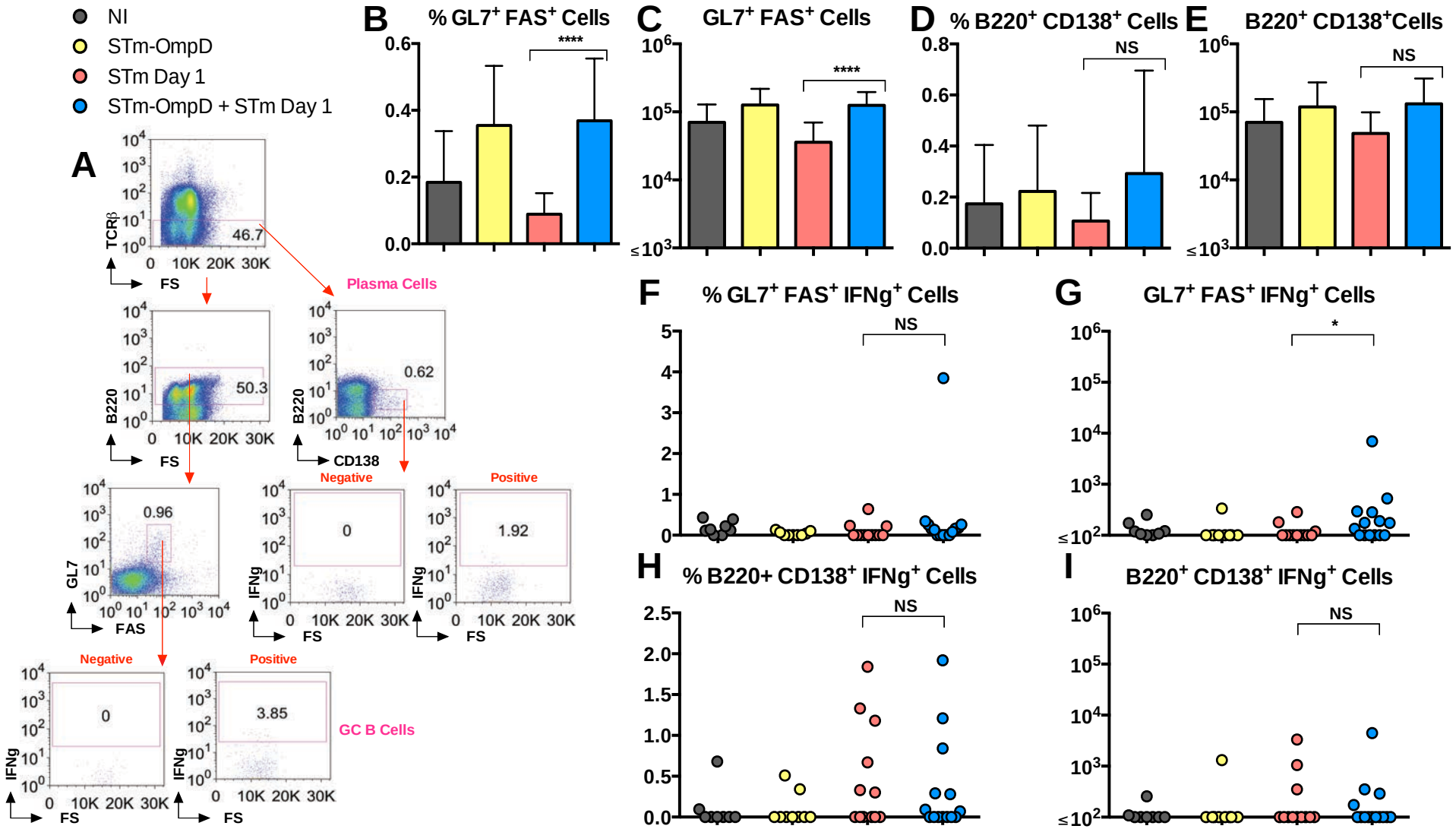

Figure 4.13 GC B cell responses are dependent on STm-OmpD immunisation whereas plasma cells remain the same after 24 hours of infection in immunised mice

Splenic cells stained for GL7$^+$ FAS$^+$ (GC B Cells) and B220$^+$ CD138$^+$ (plasma cells) in IFNg reporter mice (Fig. 4.8A). (A) Representative FACS plots for GC B Cells and plasma cells. Proportion and total number of (B and C) GL7$^+$ FAS$^+$, (D and E) B220$^+$ CD138$^+$, (F and G) GL7$^+$ FAS$^+$ IFNg$^+$, and (H and I) B220$^+$ CD138$^+$ IFNg$^+$ cells are shown. Data is from 3 experiments. * = $P \leq 0.05$, **** = $P \leq 0.0001$, NS = non-significant.

4.2.11.3 IFNg is not produced by dendritic cells (DCs) in STm-OmpD immunised mice

Dendritic cells (DCs) can produce pro-inflammatory cytokines, such as IL-12 and IFNg (344-346). To test if IFNg is induced by splenic DCs after immunisation or 24 hours after infection expression by B220- CD64- Ly6C- $CD11c^{+}$ MHC II^{+} DC cells was assessed (Fig. 4.14A). There was a slight increase in the proportion (Fig. 4.14B) but not in the numbers (Fig. 4.14C) of DCs in those mice that were given STm-OmpD This showed that IFNg was not induced in DCs. (Fig. 4.14D and E).

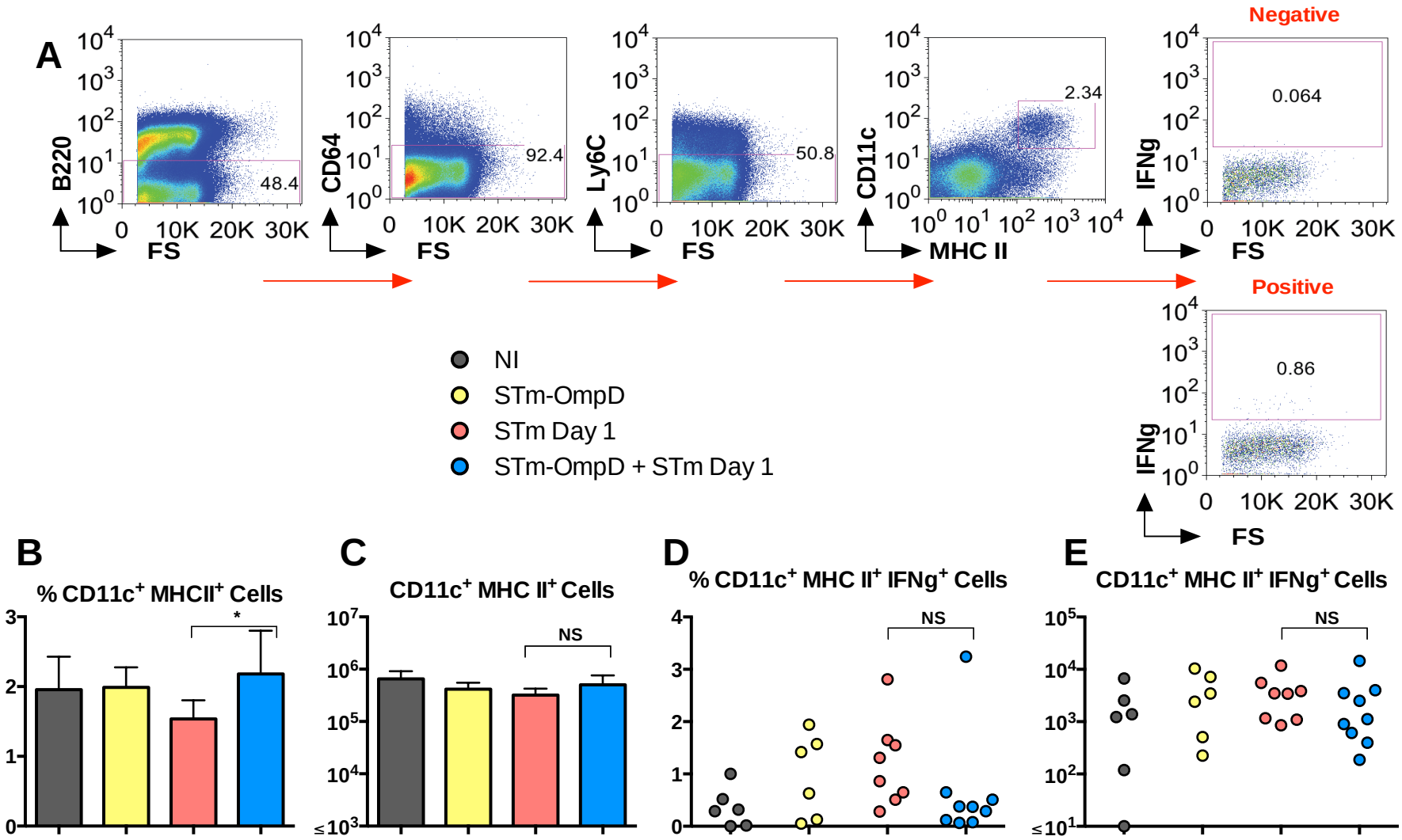

Figure 4.14 DCs expressing CD11c$^+$ MHC II$^+$ IFNg$^+$ in NI and STm-OmpD immunised mice 1 day post-infection
FACS staining for splenic DCs in IFNg (eYFP) reporter mice immunised as in Figure 4.8A. (A) Gating strategy for B220$^-$ CD64$^-$ Ly6C$^-$ CD11c$^+$ MHC II$^+$ splenic cells is illustrated. (B and C) Proportion and total number of CD11c$^+$ MHC II$^+$ DCs is shown. (D and E) Frequency and total cells of CD11c$^+$ MHC II$^+$ IFNg$^+$ DCs. Data is from 2 different experiments. * = $P \leq 0.05$, NS = non-significant.

4.2.11.4 IFNg is induced in F480^{-} GR1^{+} cells after infection

We then examined IFNg expression in macrophages and neutrophils in mice infected with STm for 24 hours, with and without immunisation, as shown in Fig. 4.8A. FACS analysis of F480^{+} GR1^{-} and F480^{-} GR1^{+} cells was performed as described in the spleen and PEC (Fig. 4.15A). In the F480^{+} GR1^{-} population, the proportion and total number of cells were the same for both NI and immune mice in the spleen and PEC (Fig. 4.15B, C, and F). However, while the F480^{-} GR1^{+} population remained the same in the spleen (Fig. 4.15D-E), the proportion of these cells in the PEC was much higher in mice infected with STm, although this proportion was lower in mice that had also been immunised with STm-OmpD (Fig. 4.15G).

The expression of IFNg^{+} cells was then assessed (Fig. 4.16A). The F480^{+} GR1^{-} population was subsequently gated for Ly6C^{+} IFNg^{+} cells (Fig. 4.16B). Ly6C was used based on the gating strategy showed in (347). No statistical significant difference was observed in the proportion of F480^{+} Ly6C^{+} IFNg^{+} cells in the spleen and PEC (Fig. 4.16B and F). Nevertheless, the numbers of F480^{+} Ly6C^{+} IFNg^{+} cells in the infected but non-immunised mice was greater than in the other groups (Fig. 4.16C). A similar set of findings was observed for GR1^{+} cells, with an increase in the proportion and total number of GR1^{+} IFNg^{+} cells in the spleen after infection but that was not observed in immunised mice (Fig. 4.16D A and E). No differences were observed in the proportion of GR1^{+} IFNg^{+} cells in PEC (Fig. 4.16G).

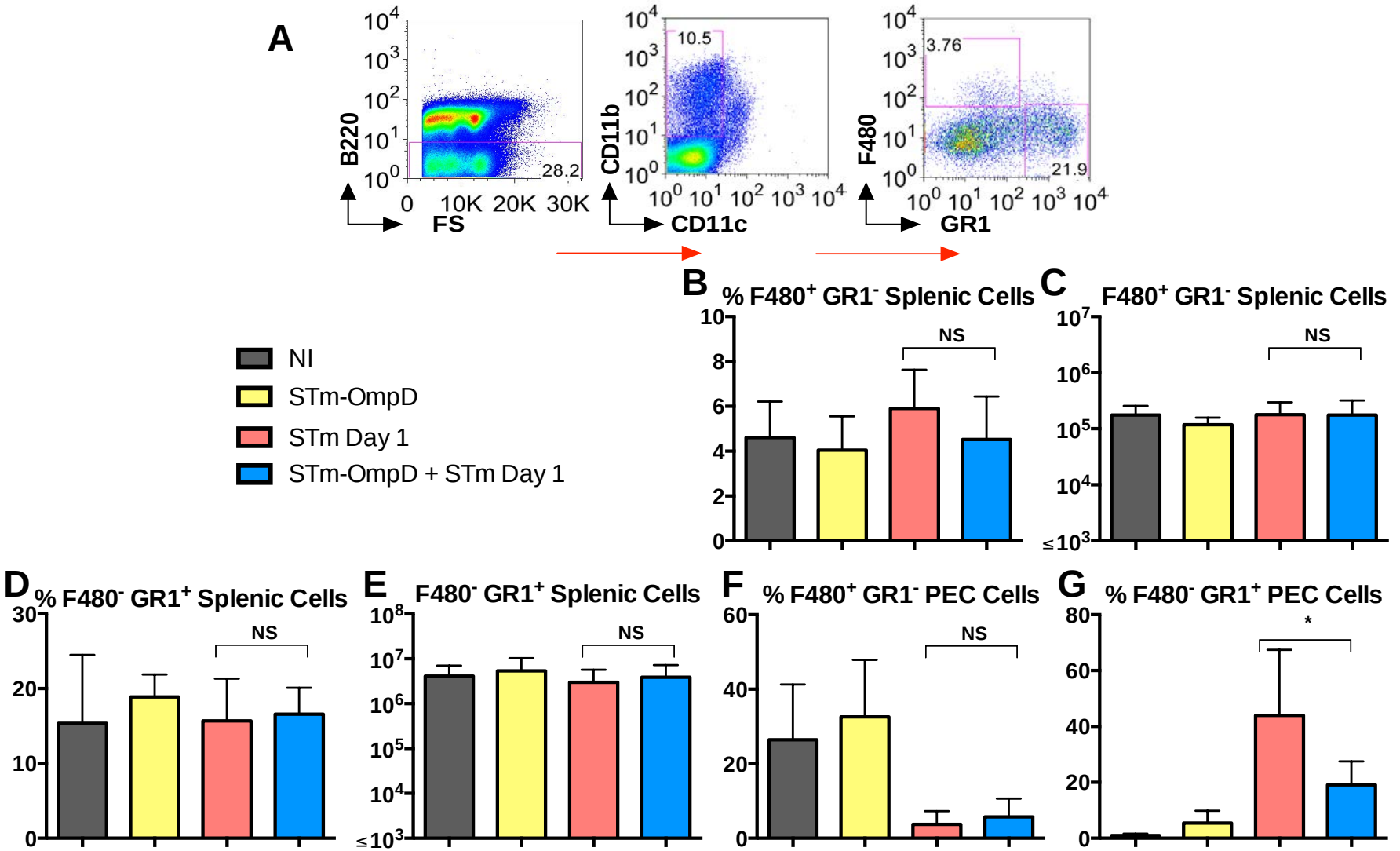

Figure 4.15 Myeloid cells expressing F480$^+$ GR1$^-$ and F480$^-$ GR1$^+$ in spleen and PEC in NI and STm-OmpD immunised mice at 1 day post-infection

Cells from spleen and PEC from IFNg (eYFP) reporter mice used in Figure 4.8A were stained for F480$^+$ GR1$^-$ and F480$^-$ GR1$^+$ cells. (A) Representative gating used to identify subsets of myeloid cells in spleen and PEC. Proportion and total numbers of (B and C) F480$^+$ GR1$^-$, (D and E) F480$^-$ GR1$^+$ (D and E) Percentage of F480$^+$ GR1$^-$ and F480$^-$ GR1$^+$ cells in the PEC, respectively. Data is from 2 individual experiments. * = $P \leq 0.05$, NS = non-significant.

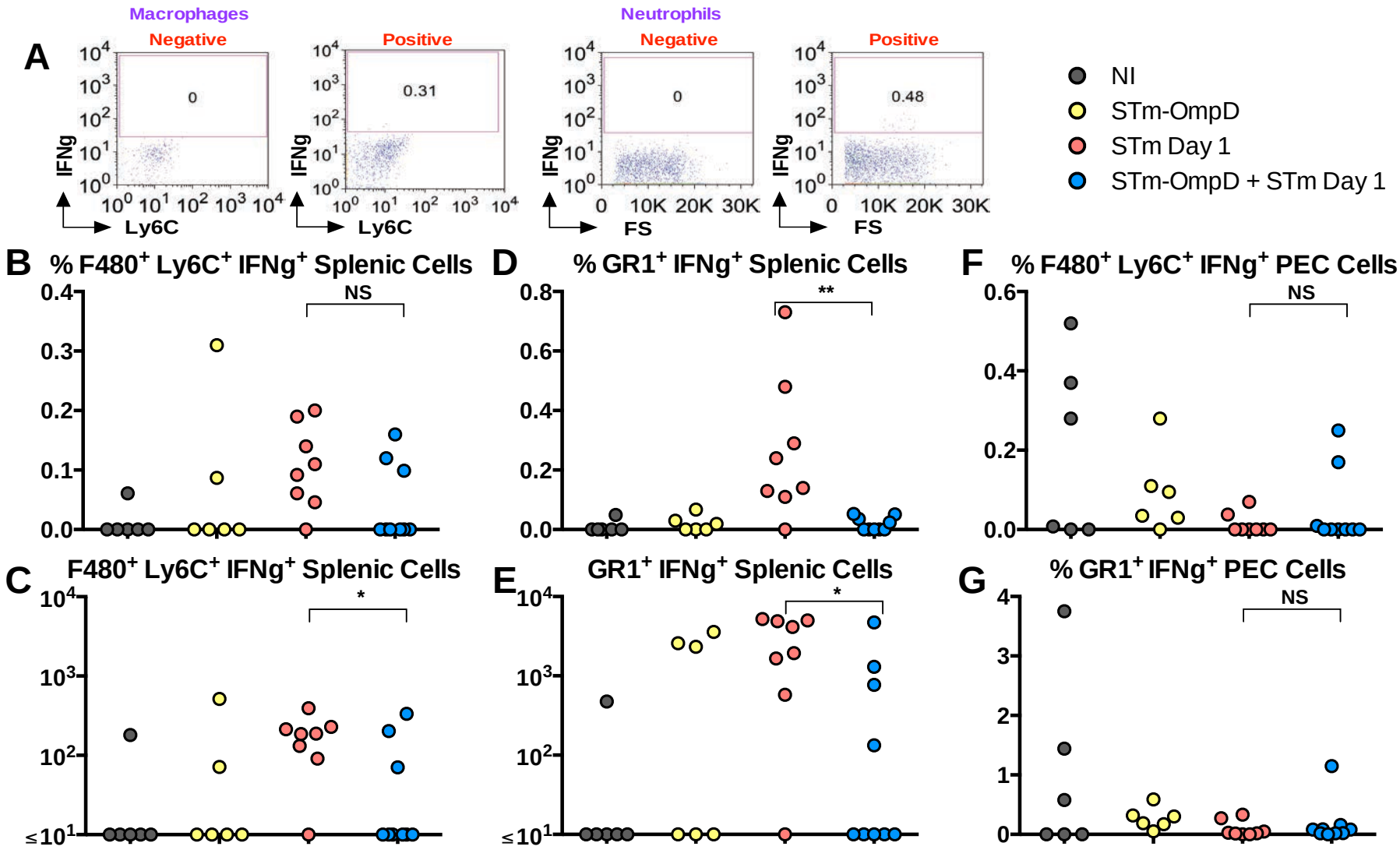

Figure 4.16 IFNg expression by myeloid cells in spleen and PEC in NI and STm-OmpD immunised mice 1 day after infection

Splenic and PEC cells from IFNg (eYFP) reporter mice used in Figure 4.8A were stained for F480$^+$ Ly6C$^+$ IFNg$^+$ and F480$^-$ GR1$^+$ IFNg$^+$ cells. (A) FACS gating used to identify these subsets. Proportion and total numbers of (B and C) F480$^+$ Ly6C$^+$ IFNg$^+$, (D and E) F480$^-$ GR1$^+$ IFNg$^+$ splenic cells are shown. (F and G) Percentages of same subsets in the PEC were determined, respectively. Data is from 2 individual experiments. * = $P \leq 0.05$, ** = $P \leq 0.01$, NS = non-significant.

4.2.12 Protection from immunisation with STm-OmpD is detectable by 4 hours after STm infection

We then examined whether the protective responses to STm-OmpD were effective even earlier after infection, IFNg (eYFP) reporter mice were immunised as described in Figure 4.17A and infected for 4 hours. This time point was chosen to allow us to examine whether different cells had upregulated IFNg. There were no differences between the size and weight of the infected versus the non-infected spleens and livers (Fig. 4.17B). However, there was an approximately 10-fold reduction in bacterial numbers in the spleen, liver, and PEC of the mice that were previously immunised with STm-OmpD (Fig. 4.17C). Therefore, immunisation with STm-OmpD can help control extracellular dissemination rapidly after infection.

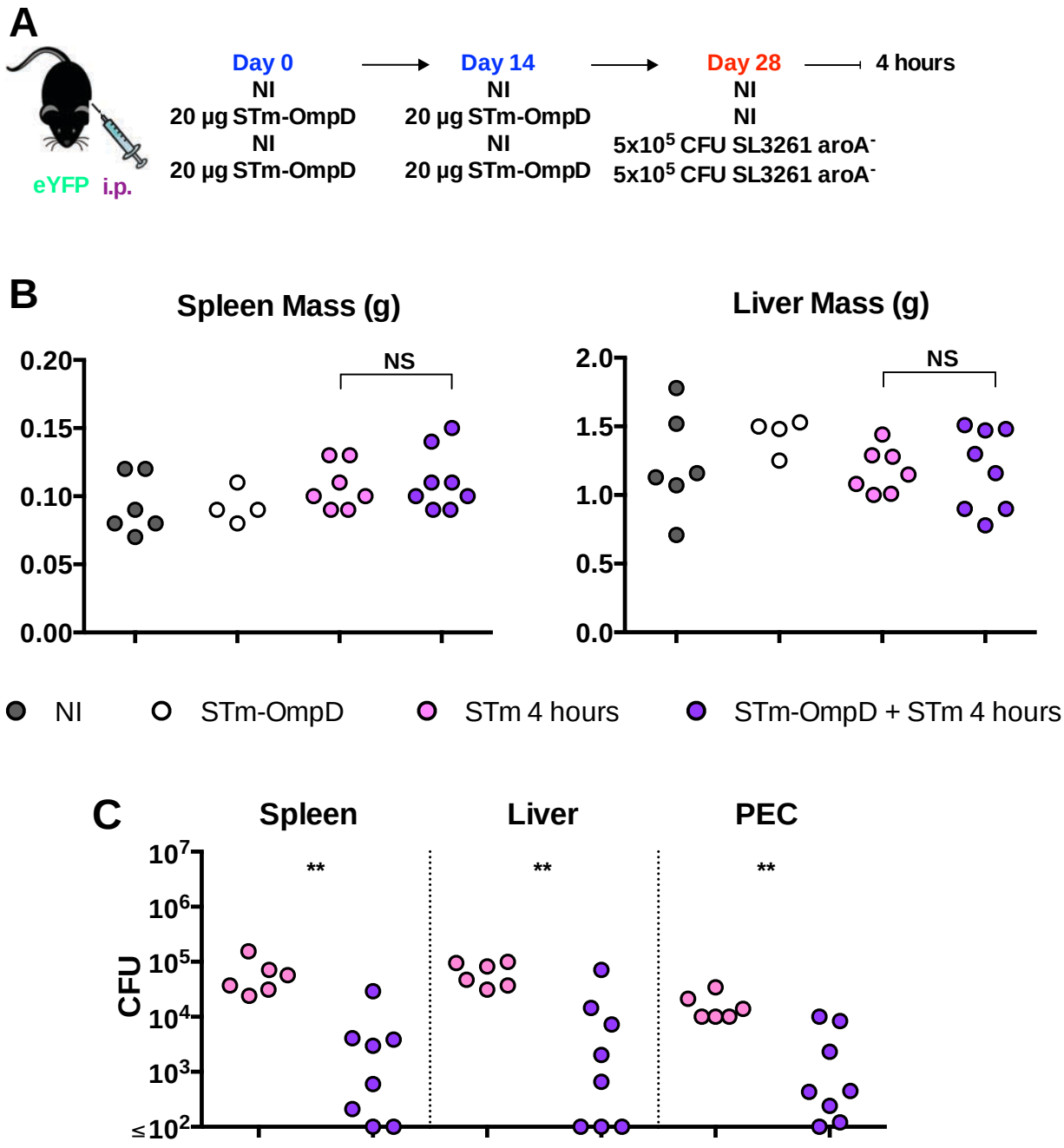

Figure 4.17 Protection after STm-OmpD immunisation is effective in the first hours of STm infection

IFNg (eYFP) reporter mice infected with STm SL3261 i.p. for 4 hours (pink circles) or primed and boosted with STm-OmpD and infected for 24 hours (purple circles). And control groups of NI mice (grey circles) and STm-OmpD immunised mice (clear circles) were used. (A) Protocol of immunisation followed in these experiments. (B) Spleens and livers were weighed and mass determined (C) Bacterial numbers of spleen, liver and PEC were enumerated. Data is from 2 different experiments. Each dot represents one mouse. ** = $P \leq 0.01$, NS = non-significant.

4.2.13 IFNg induction is detectable 4 hours post-infection and influenced by immunisation

4.2.13.1 Numbers of IFNg$^+$ CD4$^+$ T cells increase in the spleen 4 hours post-infection

T cell populations were examined in the spleen and PEC were analysed by FACS (gating strategy as shown Figure 4.9A and 4.10A). In the PEC, the proportion of both CD4$^+$ and CD8$^+$ T lymphocytes did not change (Fig. 4.18E and F). However, while in the spleen the CD8$^+$ lymphocytes remain the same (Fig. 4.18C and D), an increase in the total number but not in the proportion of CD4$^+$ T cells in mice immunised with STm-OmpD was observed (Fig. 4.18A and B). Expression of IFNg from CD4$^+$ and CD8$^+$ T cells in spleen was then examined (Fig. 4.19) and PEC (Fig. 4.20) after 4 hours of infection. At this time there was an increase in the proportion but not total number of splenic CD4$^+$ CD62L^{lo} IFNg$^+$ cells in STm-OmpD immunised and challenged mice (Fig. 4.19A and B). However, the CD4$^+$ CD62L^{hi} IFNg$^+$ and CD8$^+$ CD62L^{lo} IFNg$^+$ populations remained the same (Fig. 4.19C-F). In contrast, there was an increase in CD8$^+$ CD62L^{hi} IFNg$^+$ T cells in the mice infected for 4 hours with STm (Fig. 4.19G and H). In the PEC, only minor changes were observed (Fig. 4.20). Therefore CD4$^+$ T cells produce IFNg at 4 hours post-infection in immunised mice but not at 24 hours.

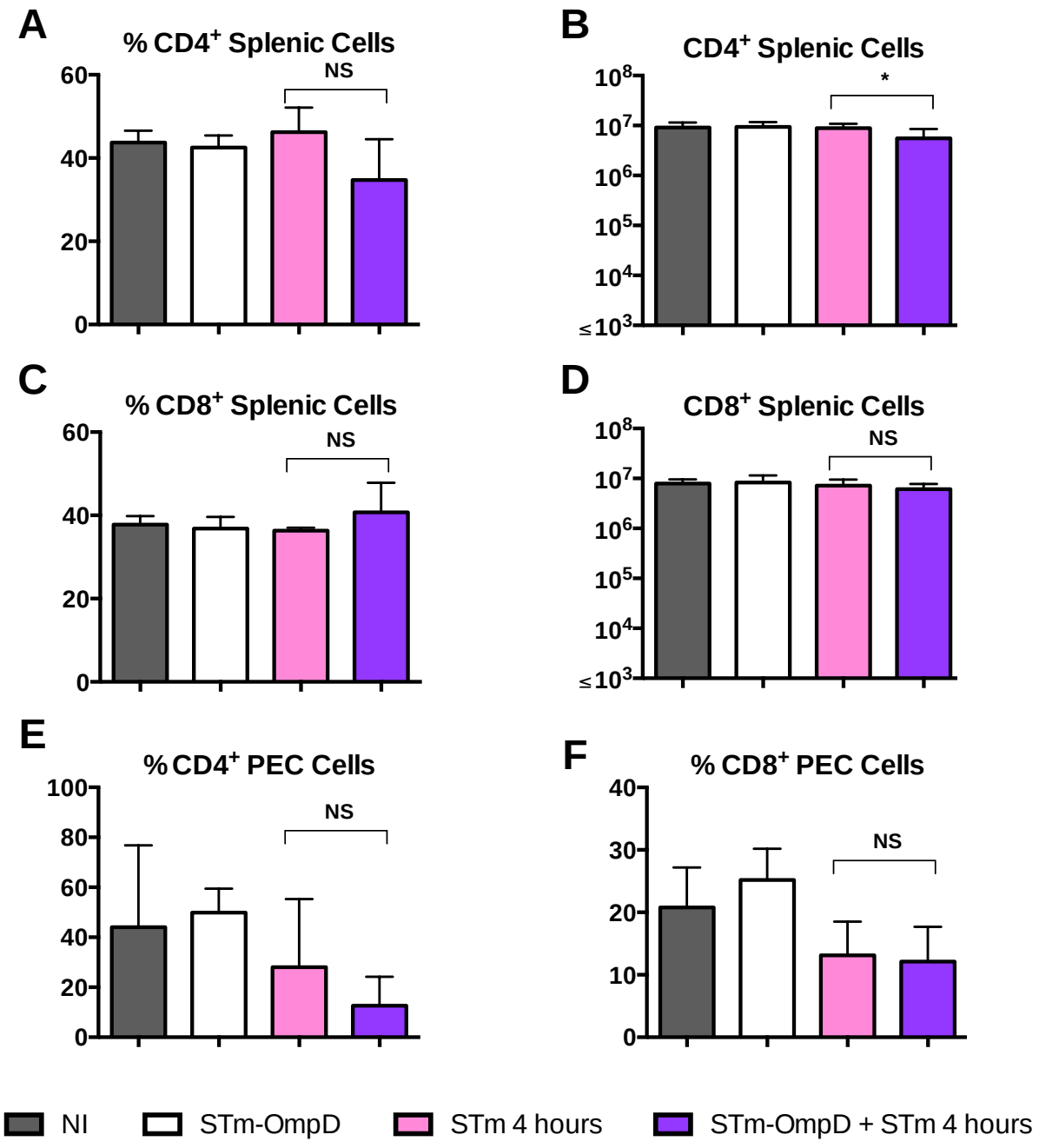

Figure 4.18 CD4$^+$ and CD8$^+$ T cells in NI and STm-OmpD imunised mice infected 4 hours post-infection

For gating strategy used for FACS refer to Figure 4.9A. Proportion and total number of (B and C) CD4$^+$ and (D and E) CD8$^+$ splenic T cells from IFNg (eYFP) reporter mice immunised as in Fig. 4.17A is shown. (E and F) Proportion in the PEC of CD4$^+$ and CD8$^+$ T cells, respectively. Data is from 2 individual experiments. * = $P \leq 0.05$, NS = non-significant.

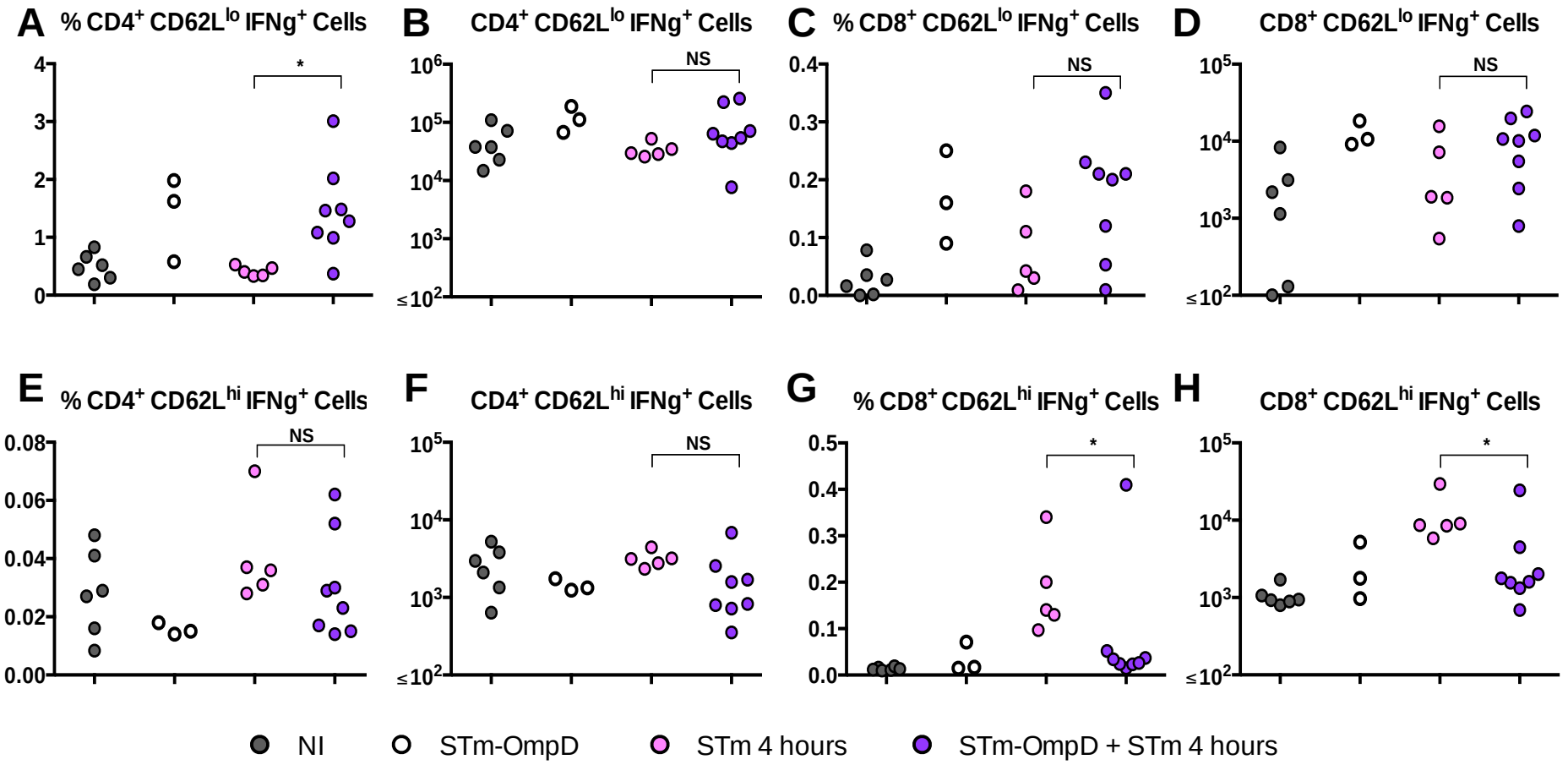

Figure 4.19 Expression of IFNg by CD4$^+$/CD8$^+$ T cells after 4 hours of infection in STm-OmpD immunised and NI mice
IFNg (eYFP) reporter mice were immunised as outlined in Figure 4.17A. For the FACS gating strategy used refer to Fig. 4.10A. Proportion and total numbers of (B and C) CD4$^+$ CD62L^{lo} IFNg$^+$, (D and E) CD8$^+$ CD62L^{lo} IFNg$^+$, (E and F) CD4$^+$ CD62L^{hi} IFNg$^+$ and (G and H) CD8$^+$ CD62L^{hi} IFNg$^+$ are observed. Data is from 2 individual experiments. * = $P \leq 0.05$, NS = non-significant.

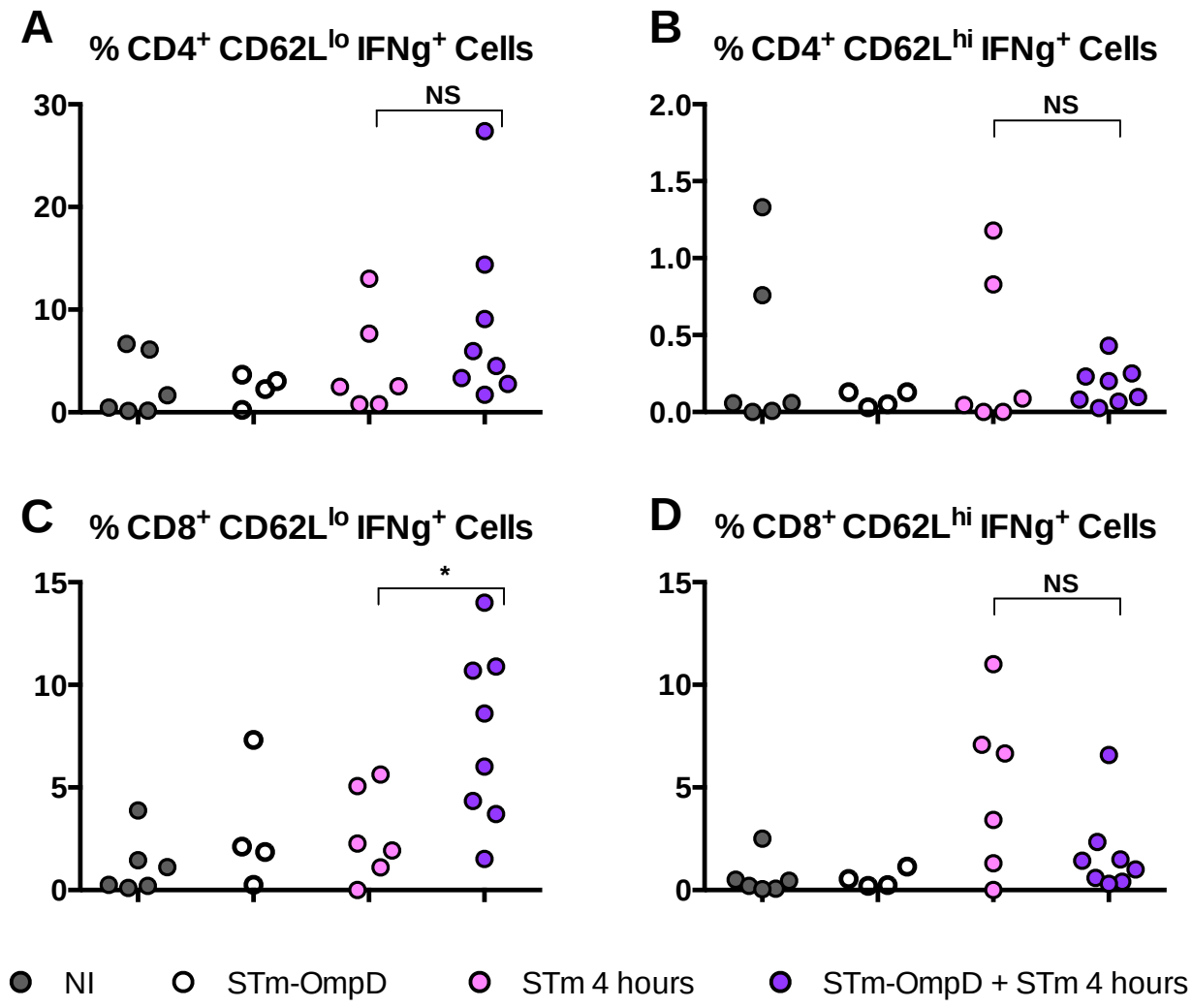

Figure 4.20 CD4$^+$/CD8$^+$ CD62L^{lo} IFNg$^+$ and CD4$^+$/CD8$^+$ CD62L^{hi} IFNg$^+$ cells in the PEC in mice with or without immunisation at 4 hours post-infection
The FACS gating strategy used is outlined in Fig. 4.10A. Proportion of (A) CD4$^+$ CD62L^{lo} IFNg$^+$, (B) CD4$^+$ CD62L^{hi} IFNg$^+$, (C) CD8$^+$ CD62L^{lo} IFNg$^+$ and (D) CD8$^+$ CD62L^{hi} IFNg$^+$ PEC cells from IFNg (eYFP) reporter mice immunised as in Figure 4.17A were determined. Data is from 2 individual experiments. * = $P \leq 0.05$, NS = non-significant.

4.2.13.2 IFNg production for other cells at 4 hours post-infection

We then examined IFNg production from other cell types, using the approaches described in Section 4.2.11. To limit repetition the data is included as Appendix D and the significant findings showed in Figure 4.21. Reflecting our findings at 24 hours no changes in B cell populations or their production of IFNg were found (Fig. D1). Similar results were found for GC B cells (Fig. D2), DCs (Fig. D3) nor for $F480^+$ cells (Fig. D4).

4.2.13.3 $GR1^+$ cells accumulate rapidly in the PEC after immunisation and challenge

The frequencies of $F480^-$ $GR1^+$ cells in the spleen and PEC increased in mice that were given STm-OmpD (Fig. 4.21A and C), although total number of these cells in the spleen did not change significantly compared to NI mice challenged with STm (Fig. 4.21B). When $F480^-$ $GR1^+$ $IFNg^+$ cells were evaluated, a reduction in the proportion of $GR1^+$ $IFNg^+$ cells in the spleen was found (Fig. 4.21D), but this was not the case in the total numbers and proportion of cells in the spleen and PEC respectively where no significant changes were observed (Fig. 4.21E and F). Therefore, immunisation can alter the cell types that produce IFNg after infection.

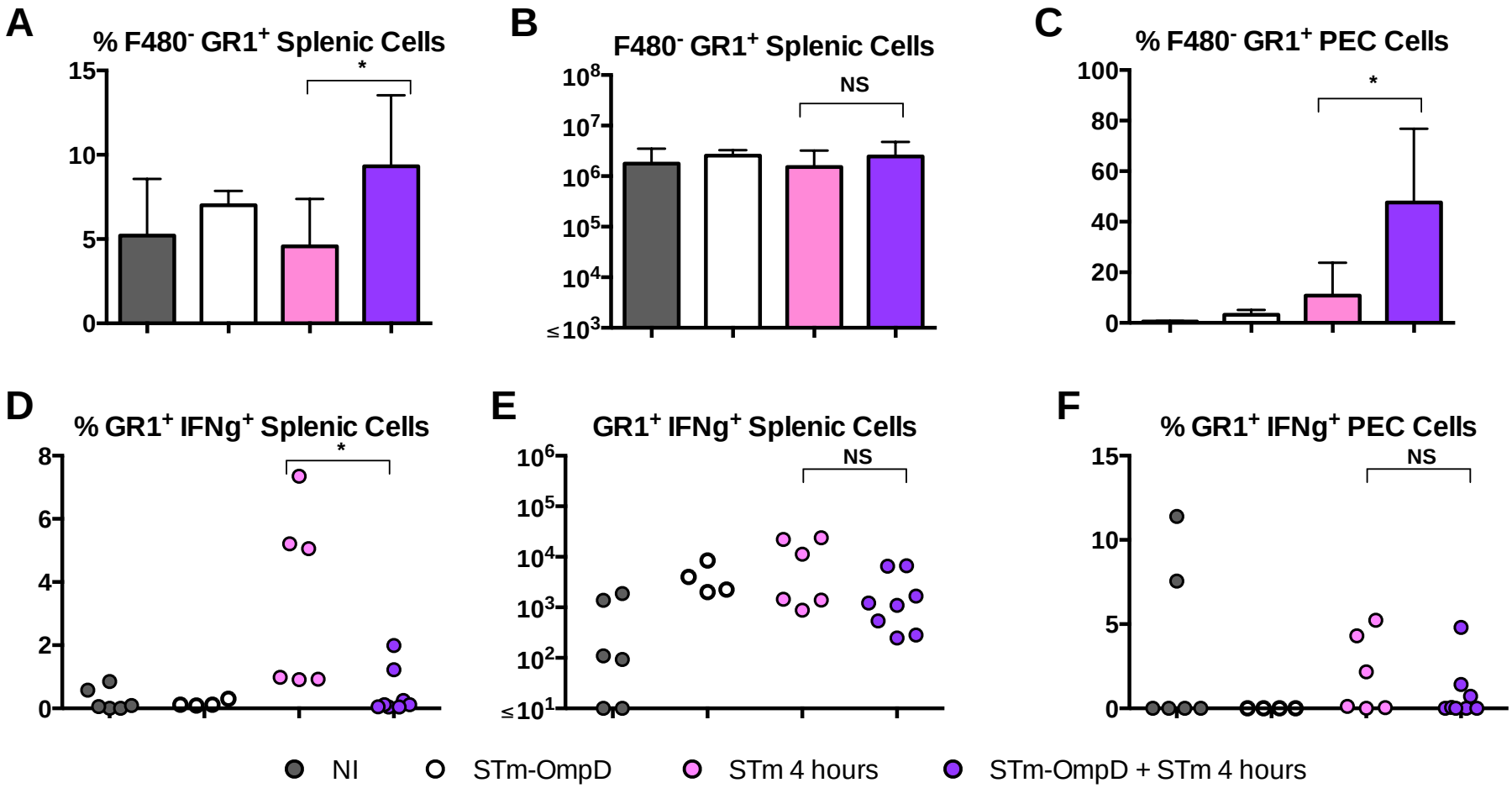

Figure 4.21 F480$^-$ GR1$^+$ neutrophils expressing IFNg in NI and STm-OmpD immunised mice at 4 hours post-challenge
Splenic and PEC cells from IFNg (eYFP) reporter mice were gated for F480$^-$ GR1$^+$ cells. For the FACS strategy used to gate these cells refer to Figure 4.16A. (A and B) Proportion and total number of F480$^-$ GR1$^+$ splenic cells are illustrated. (C) Frequency of F480$^-$ GR1$^+$ cells in the PEC was determined. (D and E) Proportion and total numbers of F480$^-$ GR1$^+$ cells expressing IFNg were determined. (F) Frequency of F480$^-$ GR1$^+$ IFNg$^+$ cells in the PEC is shown. Data is from 2 individual experiments. . * = $P \leq 0.05$, NS = non-significant.

4.2.14 F480^{+} cells are dispensable for protection after immunisation with STm-OmpD

Myeloid cells can aid the control of STm replication during primary responses. Nevertheless, the relative contribution of different myeloid cells in Ab-mediated protection after STm infection is unclear. Moreover, our data suggests that myeloid cells could produce IFNg. Therefore, we assessed protection in mice depleted of macrophages after injection of clodronate liposomes. WT mice were immunised with STm-OmpD as shown in Figure 4.22A. F480^{+} cells were depleted with clodronate liposomes one day prior challenge. Control groups of NI mice, with and without depletion, with clodronate liposomes were included. Cells were gated as illustrated in Figure 4.22B and depletion of F480^{+} cells confirmed in the spleen and PEC, respectively (Fig. 4.22C-E). Depletion of F480^{+} cells did not alter the Ab response detected (Fig. 4.23A and F). After challenge bacterial numbers in the spleen, liver, and PEC of mice immunised with STm-OmpD with and without the clodronate-liposome treatment were similar although there was a tendency for bacterial numbers to be higher in the spleen (Fig. 4.24). Therefore, clodronate-sensitive cells are not essential for the control of STm in STm-OmpD immunised mice.

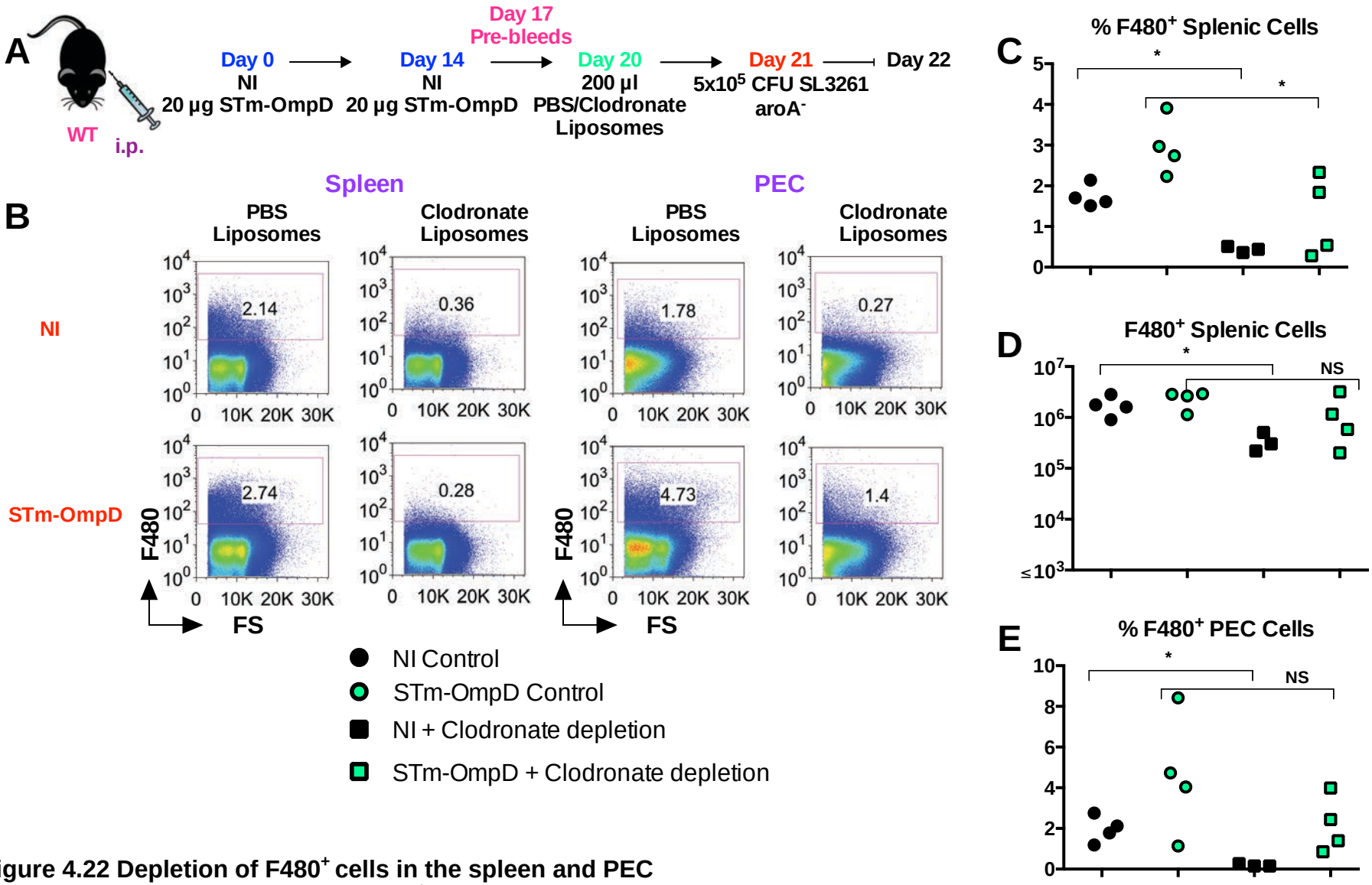

Figure 4.22 Depletion of F480$^+$ cells in the spleen and PEC

(A) Protocol used for depletion of F480$^+$ cells. Groups of mice were immunised with STm-OmpD or PBS and depleted with clodronate liposomes. Control groups in which PBS liposomes were injected were also added. (B) Representative FACS gating used for F480$^+$ cells in the spleen and PEC. (C and D) Proportion and total number of F480$^+$ cells in the spleen were determined. (E) Proportion of F480$^+$ cells in the PEC is shown. * = $P \leq 0.05$, NS = non-significant.

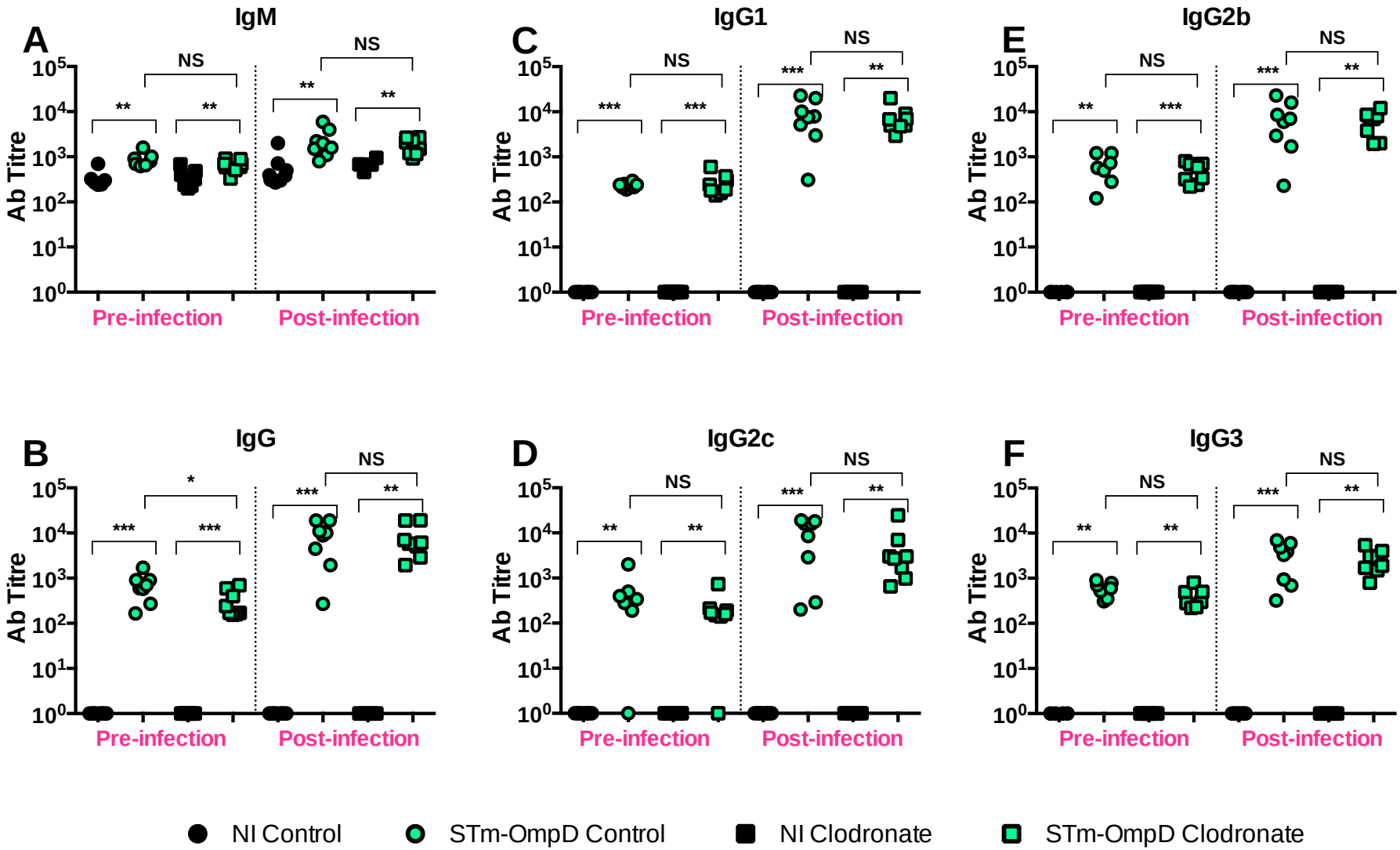

Figure 4.23 Depletion with clodronate liposomes does not affect Ab responses to STm-porins in immunised mice
ELISA from sera of mice immunised as in Figure 4.22A. (A-F) Ab titres for IgM, IgG, and IgG subclasses were detected to STm-porins from sera obtained before and after depletion with clodronate liposomes. Data is from 2 individual experiments. Each dot represents one mouse. * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, NS = non-significant.

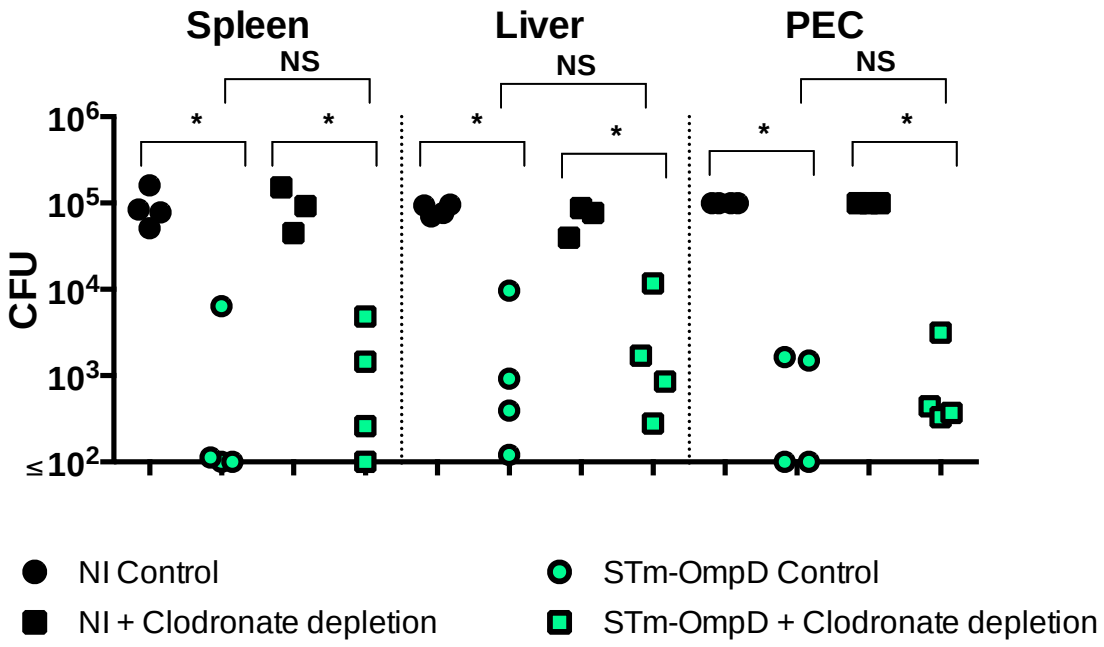

Figure 4.24 F480$^+$ cells are dispensible for protection after immunisation with STm-OmpD
Bacterial numbers were enumerated in the spleen, liver, and PEC. Data is representative from 2 different experiments. Each dot represents one mouse. * = $P \leq 0.05$, NS = non-significant.

4.2.13 Protection after STm-OmpD immunisation is reduced when GR1$^+$ cells are absent

Since clodronate-liposome treatment did not significantly alter bacterial clearance we examined the role of neutrophils. To do this we depleted neutrophils using anti-GR1 Ab in NI mice and mice that were previously immunised with STm-OmpD as shown in Figure 4.25A. Cells were gated as shown in Figure 4.25B to confirm depletion in the spleen and PEC (Fig. 4.25C-E). Depletion of cells with anti-GR1 Ab did not affect STm-OmpD IgG titres or of individual isotypes (Fig. 4.26A and D). After challenge, mice immunised with STm-OmpD, that were treated with a control Ab showed a reduction in bacterial numbers of 100-fold in the spleen, liver, and PEC compared to NI mice (Fig. 4.27). In contrast, immunised mice treated with anti-GR1 Ab had higher bacterial numbers in these sites. Therefore, GR1$^+$ cells are important in maintaining protection after STm-OmpD immunisation during earliest stages of infection.

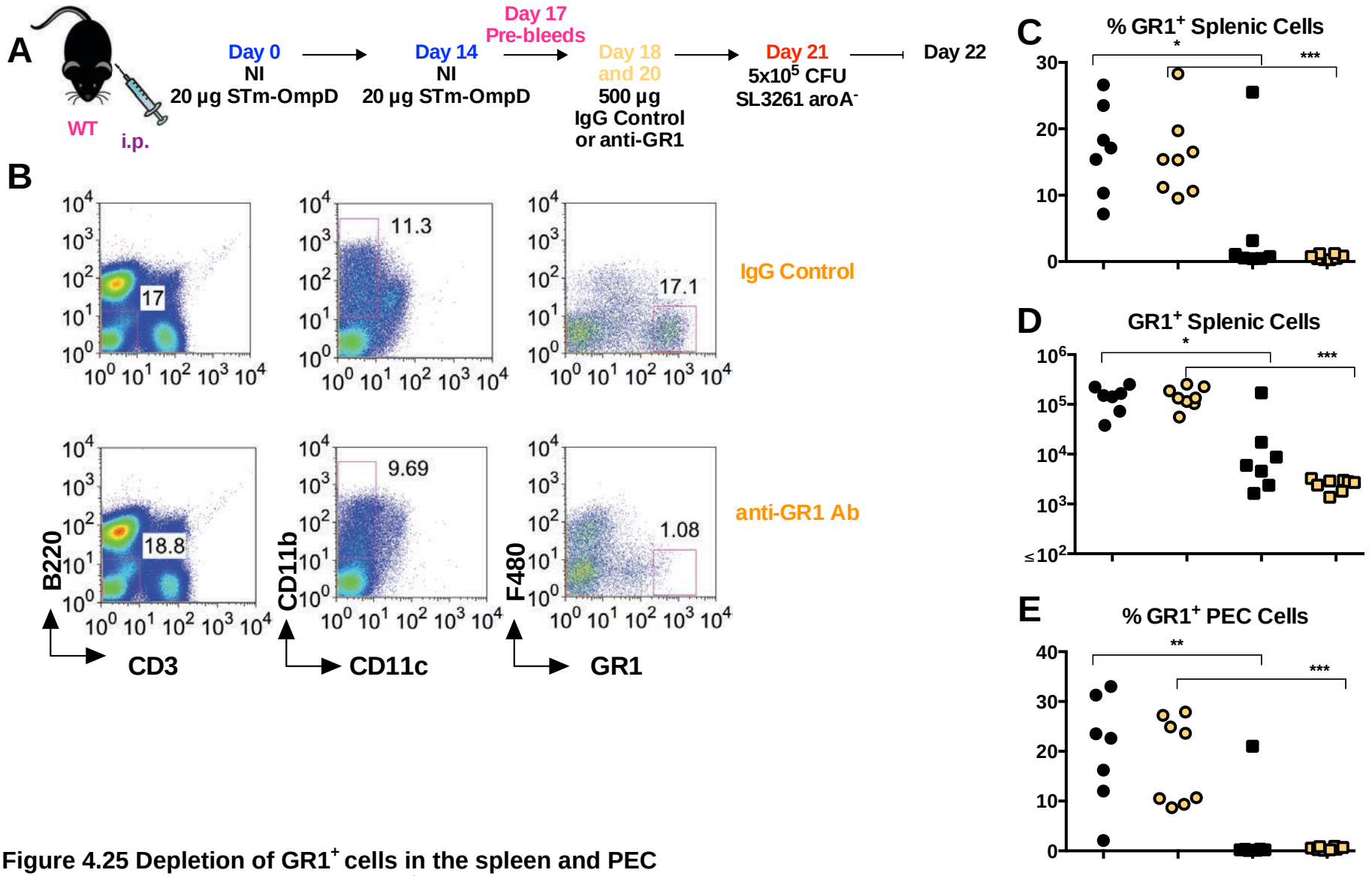

Figure 4.25 Depletion of GR1$^+$ cells in the spleen and PEC

(A) Protocol used for depletion of GR1$^+$ cells. Groups of mice were immunised with STm-OmpD or PBS and depleted with anti-GR1 Ab. Control groups in which IgG control Ab was injected were also used. (B) Representative FACS gating used for GR1$^+$ cells in the spleen and PEC. (C and D) Proportion and total number of GR1$^+$ cells in the spleen were analysed. (E) Proportion of GR1$^+$ cells in the PEC is shown. * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

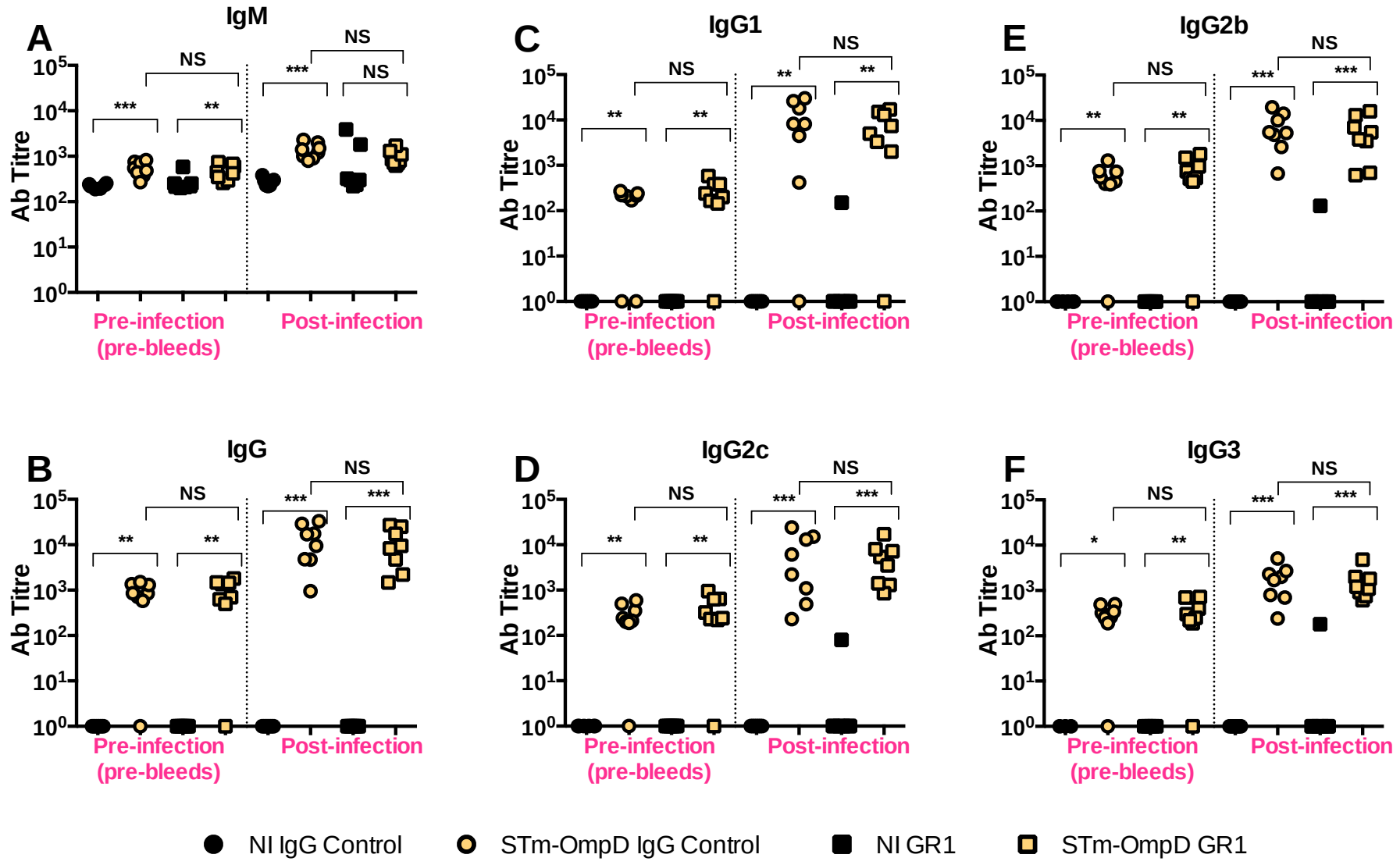

Figure 4.26 Ab to STm-OmpD is able to induce robust Ab responses to porins after depletion of GR1^{+} cells
ELISA using sera from mice immunised as in Figure 4.25A. (A-F) Ab titres for IgM, IgG, and IgG subclasses were detected to STm-porins from sera obtained before and after depletion with anti-GR1 Ab. Data is from 2 different experiments. Each dot represents one mouse. * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, NS = non-significant.

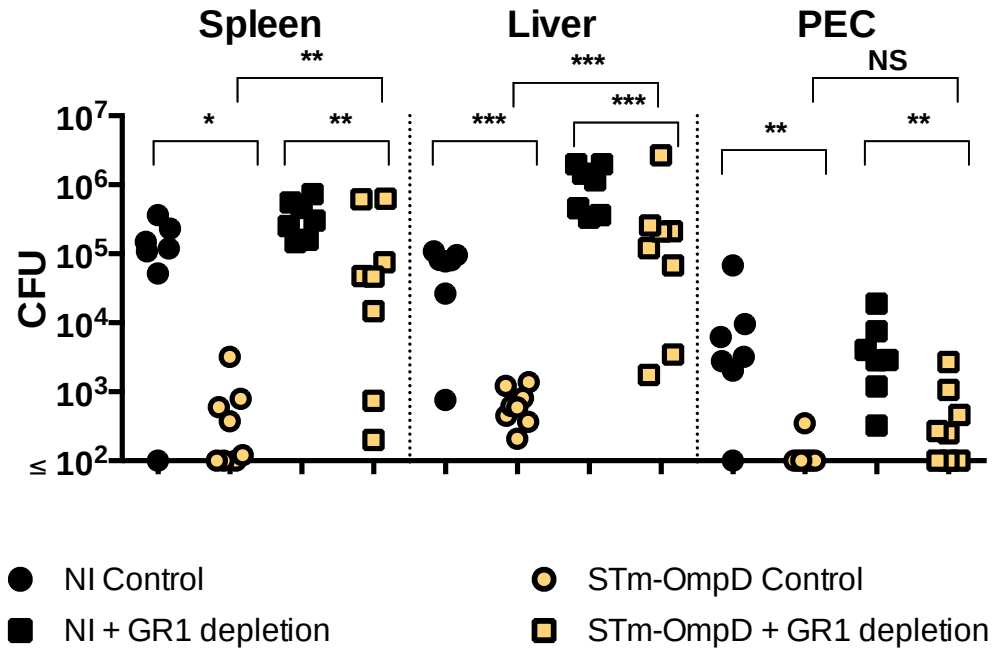

Figure 4.27 Protection is impaired in mice immunised with STm-OmpD after depletion of GR1$^+$ cells

Bacterial numbers were counted in the spleen, liver, and PEC. Data is from 2 individual experiments. Each dot represents one mouse. * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, NS = non-significant.

4.3 Discussion

The aim of the work in this chapter was to identify how the interaction between different cells, along with their production of IFNg provided protection after STm-OmpD immunisation. Once infection is established, Th1 responses and IFNg production are required for clearance of the bacteria (220). However, we found that although protection was not as effective as in WT mice, T-bet and IFNg were absolutely not necessary for some protection in the spleen and liver after immunisation with STm-OmpD.

An important finding was the induction of multiple isotypes after immunisation with STm-OmpD, which contrasts to other antigens, such as flagellin, which induces a Th2 response of a predominant IgG1 isotype (76). This was not dependent on the absence of T-bet or IFNg. Immunisation with porins can induce TI responses (72). We did not assess the TI features of the response to STm-OmpD or which isotypes were induced in a TI manner; however, previous work in our lab has demonstrated that TI responses can be protective (72).

While switching to IgG2a/c is important during natural infection (287), control of infection after immunisation with STm-OmpD is not dependent in this isotype. Although, studies have shown that T-bet$^{-/-}$ mice can increase IgG1 production in order to compensate the lack of IgG2a/c (297), this was not seen in our studies. Furthermore, our results correlate with data from experiments performed in conjunction with Prof. Kai-Michael Toellner in which IgG1 was identified as the main isotype involved in protection after STm-OmpD immunisation. Together, this highlights the importance of Ab switching in protection and the contribution of

different IgG isotypes to specific antigens. Immunisation with STm-OmpD also induced an early GC B cell response, which differs to the response after live bacteria (254). This GC response was not T-bet and IFNg-dependent, although these factors were necessary for IgG2a/c switching.

Since IFNg played a significant role in protection, we examined in what cell types it was observed. Moreover, Ab to STm-OmpD was able to protect as early as 4 hours after infection, which identified the rapid effect of Ab to STm-OmpD, and suggested IFNg may be important at this time. Therefore, understanding which cells were important in contributing to protection at earlier stages needed assessment. IFNg plays a crucial role in conferring protection during early stages of primary infection (223) and different sources of IFNg have been identified. Moreover, early protective responses to *Salmonella* are normally TI whereas final clearance of the bacteria is TD but may be IFNg independent (348). Furthermore, IFNg induced by $CD8^+$ T cells and NK cells is sufficient to control *Salmonella* infections at initial stages of bacterial growth (191, 349). While NK cells were not assesed here, we detected an increase in splenic $CD8^+$ $CD62L^{hi}$ $IFNg^+$ cells in mice at 24 hours post-infection but this was lost in mice that were given STm-OmpD. Our data correlates with findings in which induction of IFNg by noncognate memory $CD8^+$ T cells is mainly mediated by flagellin, IL-12, IL-18, and NLRC4 inflamasomme activation after STm infection. These responses are regulated by $CD8\alpha^+$ DCs, which may be important in regulating the innate immune response (349), However, the decrease observed in this population in immunised mice could be due to other factors.

In contrast, $CD4^+$ $CD62L^{lo}$ $IFNg^+$ T cells were identified as IFNg producers 4 hours post-infection in both NI and immunised mice. Moreover, studies have shown that

activated $CD4^+$ T cells, in response to innate stimuli, rapidly induce IFNg after *Salmonella* infection and this is also dependent on IL-12 and IL-18 (350, 351). This could explain the production of IFNg by $CD4^+$ T cells at this time of infection. Therefore, a better understanding of whether these innate responses induced by both T cell subsets directly contribute to clearance of the bacteria would be helpful for understanding vaccine function.

It has been shown that myeloid cells can also produce IFNg at early stages of *Salmonella* infections (352). Nevertheless, using IFNg (eYFP) reporter mice we could only detect production of IFNg from the $GR1^+$ population in NI yet infected mice, although accumulation of neutrophils was detected in the PEC of both NI and immunised infected mice.

Myeloid cells, such as macrophages and neutrophils, are important at early stages of primary infections and depletion experiments have indicated the importance of these cells in the control of bacterial dissemination (184, 186, 187). Furthermore, when mice were depleted using clodronate liposomes, median bacterial numbers in the spleens of STm-OmpD immunised mice tended to be higher than non-depleted mice, but this did not reach significance. However, our clodronate data cannot be considered conclusive, as depletion seemed to be affected in the clodronate-treated mice immunised with STm-OmpD. Thus, other approaches such as immunochemistry are needed to confirm macrophage depletion in the spleen. In contrast, loss of protection in mice depleted of neutrophils suggests a key role for these cells in limiting extracellular bacterial replication. This highlights the importance of neutrophils in controlling infection, as HIV positive patients with neutropenia, are more susceptible to bacteraemia (189).

In conclusion, immunisation with STm-OmpD induces an IgG response that involves switching to all isotypes. After infection, Ab to STm-OmpD acts quickly and therefore controls infection. Moreover, this protection is not dependent on T-bet but requires IFNg from other sources at earlier stages of infection. However, it is unclear at this stage from what source IFNg is needed to control infection and other sources such as NK and NKT cells also need further assessment. Further, since STm-OmpD is considered a TD antigen, establishing how long a TI response is maintained after immunisation until T cell help is required still needs to be assessed.

CHAPTER 5: CONSTRUCTION OF A TRIPLE OMPC, OMPF O-ANTIGEN DEFICIENT *SALMONELLA* TYPHIMURIUM STRAIN

5.1 Introduction

In Chapter 3 we identified the importance of LPS O-antigen (O-Ag) in limiting and/or restricting access of protective Ab to conserved epitopes in Gram-negative bacteria. Although O-Ag is immunogenic (140-142, 333), it also blocks the interaction of protein-specific antibodies to exposed epitopes on the cell wall through steric hindrance (291-294). However, the stability and permeability of the bacterial outer membrane (OM) is significantly affected when LPS is truncated exposing OMPs and outer leaflet anchored lipoproteins (334, 335), increasing the susceptibility of the bacterium to complement killing and making it less capable of colonising host tissues (38). Despite this, our main aim was to generate a virulent strain without O-Ag and the other two major porins (OmpC and OmpF) to reduce contaminants and simplify the purification of OmpD for future experiments.

Purified OMPs from *Salmonella* strains with truncated LPS are able to induce cross-protection after infection and several studies have suggested porins could be used to mediate protection against homologous and heterologous strains (312, 321, 336, 337). To further our studies we therefore wished to generate a triple OmpC, OmpF, O-Ag deficient *Salmonella* Typhimurium (STm) strain more amenable for antibody-target interaction studies under physiologically relevant conditions. In this chapter, the generation of the mutant is described.

5.2 Results

5.2.1 Construction of a *S.* Typhimurium double *ompC* and *ompF* mutant

In order to investigate the specific role of STm-OmpD in protection, we first needed to construct a strain whereby the other two major porins, OmpC and OmpF, are absent. The attenuated strain STm SL3261 *ompC::aph ompF::cat* was kindly provided by Dr. R. A. Kingsley (University of Cambridge). The mutations were transduced into the virulent STm SL1344 by P22 (Fig. 5.1) and confirmed by PCR (Fig. 5.2). Colonies obtained were screened using specific *ompC* and *ompF* flanking primers in conjunction with primers internal to the kanamycin and chloramphenicol cassettes. Fragments corresponding to undisrupted gene can be observed in the STm SL1344 parental strain control: *ompC* (1339 bp) and *ompF* (1449 bp)(Fig. 5.2A). The disruption of *ompC* and *ompF* genes by kanamycin and chloramphenicol antibiotic resistance cassettes, respectively, result in fragments of 1555 bp (*ompC::aph*) and 1372 bp (*ompF::cat*)(Fig. 5.2A).

To further confirm the validity of the double mutation, the colonies were subjected to additional PCR using gene specific flanking primers with internal kanamycin or chloramphenicol primers, whereby only mutants with *ompC* and *ompF* disrupted by the correct resistance cassette would generate a fragment (Fig. 5.2B). Furthermore, to confirm the process of P22 transduction had not induced inadvertent phenotypes (including loss of O-Ag), which may affect the downstream applications, silver staining confirmed the presence of O-Ag (data not shown).

This strain was used in Chapter 3 with other mutants, where the *ompA* and *ompR* genes are disrupted (Table 2.3), to show the ability of complement and anti-STm-OmpD antibodies to kill in the absence of the other porins (Fig. 3.3)

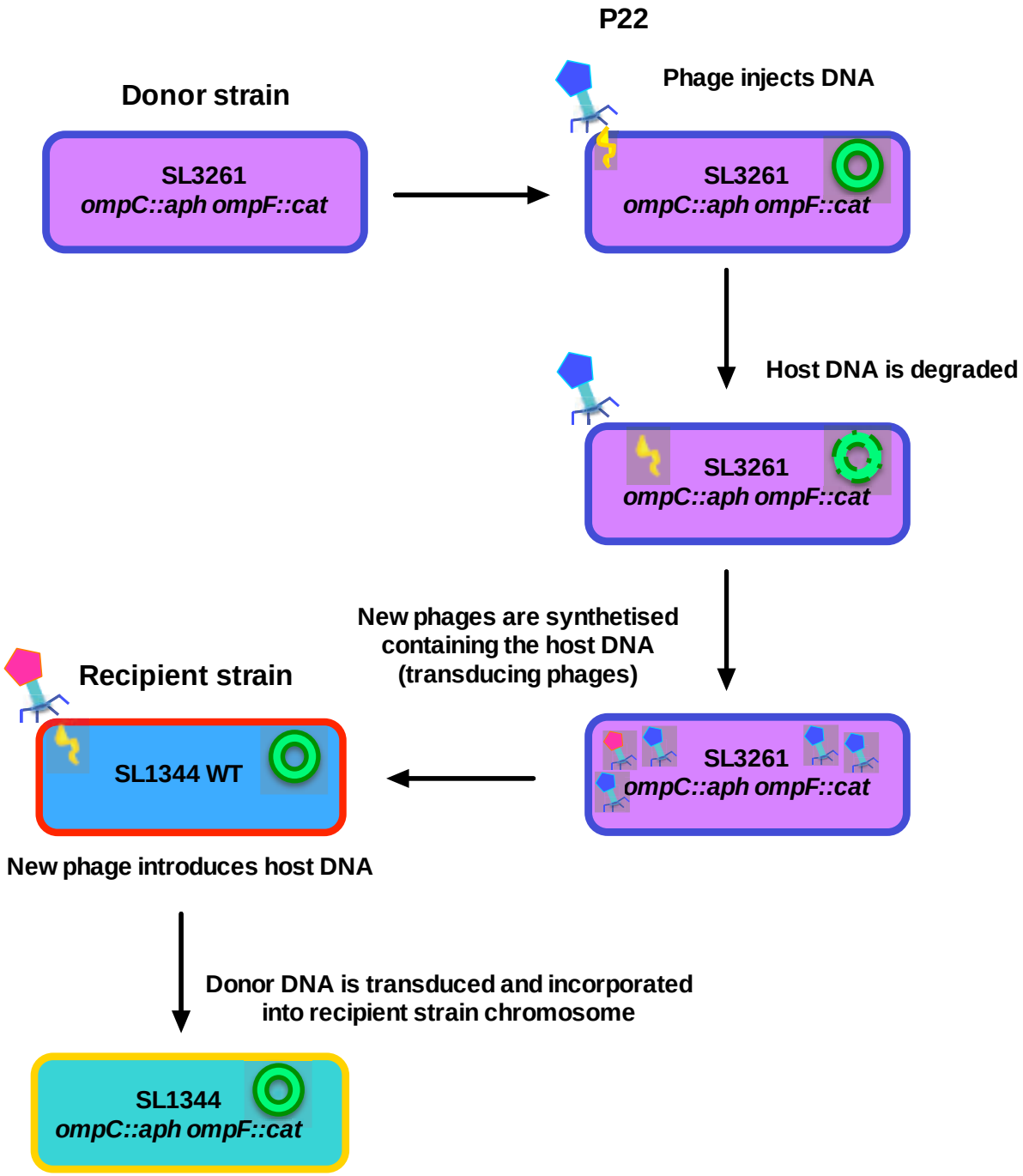

Figure 5.1 Diagram of the process used to generate the SL1344 *ompC::aph ompF::cat* mutant by P22 transduction
P22 transduction was performed to introduce the ompC::aph ompF::*cat* double mutation from the attenuated SL3261 into the SL1344. Because the two genes were already disrupted in the donor cell by insertion of a kanamycin and chloramphenicol resistance cassette respectively, no extra step was needed to incorporate this mutation into the SL1344 strain.

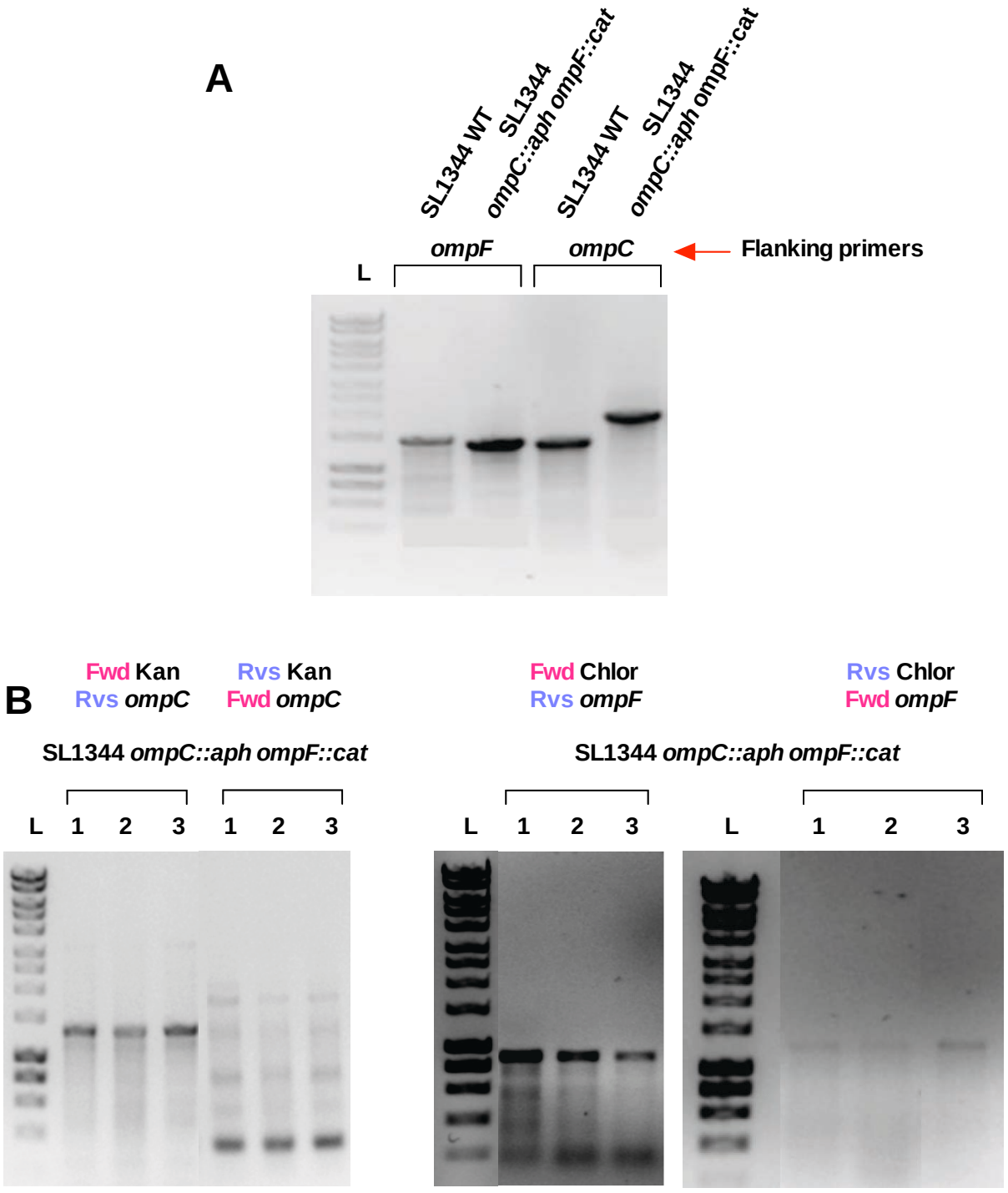

Figure 5.2 Confirmation of the P22 transduced STm SL1344 *ompC::aph ompF::cat* double mutant

PCR confirmation of the generated STm strain. (A) Comparison of the fragment sizes between the STm SL1344 WT and double mutant using flanking primers specific to the *ompC* (1339 bp) and *ompF* (1449 bp) genes. (B) Confirmation using internal Fwd kanamycin and chloramphenicol primers combined with the specific gene Rvs primer and Rvs Kanamycin and Chloramphenicol primers in conjunction with the Fwd gene primer. Fragments size: 1555 bp (*ompC::aph*) and 1372 bp (*ompF::cat*).

5.2.2 Disruption of the *wbaP* gene

Following the confirmation of the mutations in the virulent STm, the resistance cassettes disrupting the *ompC* and *ompF* genes were removed as described previously to create the strain SL1344 *ΔompC ΔompF* (300). Recombination resulting in the loss of the resistance cassette and deletion of the genes was confirmed by PCR using *ompC* and *ompF* specific flanking primers (Fig. 5.3A and B).

To disrupt the *wbaP* gene and stop the synthesis of the O-Ag, we elected to utilise gene doctoring methodology (301) summarised in Figure 5.4. Briefly, the kanamycin cassette was PCR amplified from pDOC-K using primers containing 50 nucleotides of homology to *wbaP*. The resultant fragment was cloned into pDOC-C at I-SceI restriction site. The resultant plasmid, termed pDOC-C *wbaP*, and vector pACBSE were co-transformed into our STm SL1344 *ΔompC ΔompF*. The addition of arabinose to the growth media induced the expression of both the I-SceI restriction enzymes and λ red recombinase genes from the pACBSE vector. Expression of I-SceI restriction enzyme linearises the *wbaP*-kanamycin chimeric DNA fragment from the pDOC-C vector, with the λ red recombinase enhancing the recombination between the linear fragment and the chromosome at the *wbaP* gene position. The mutants were confirmed by PCR using *wbaP* specific flanking primers, generating a fragment of the expected size for the *wbaP* gene disrupted by the insertion of a kanamycin resistance cassette (1431 bp)(Fig. 5.3C). A complete analysis of the whole genome of the mutant was established with the help of MicrobesNG at the University of Birmingham. This mutant has been shipped to Mexico to our collaborators and we are currently waiting to get new batches of purified STm-OmpD for future experiments.

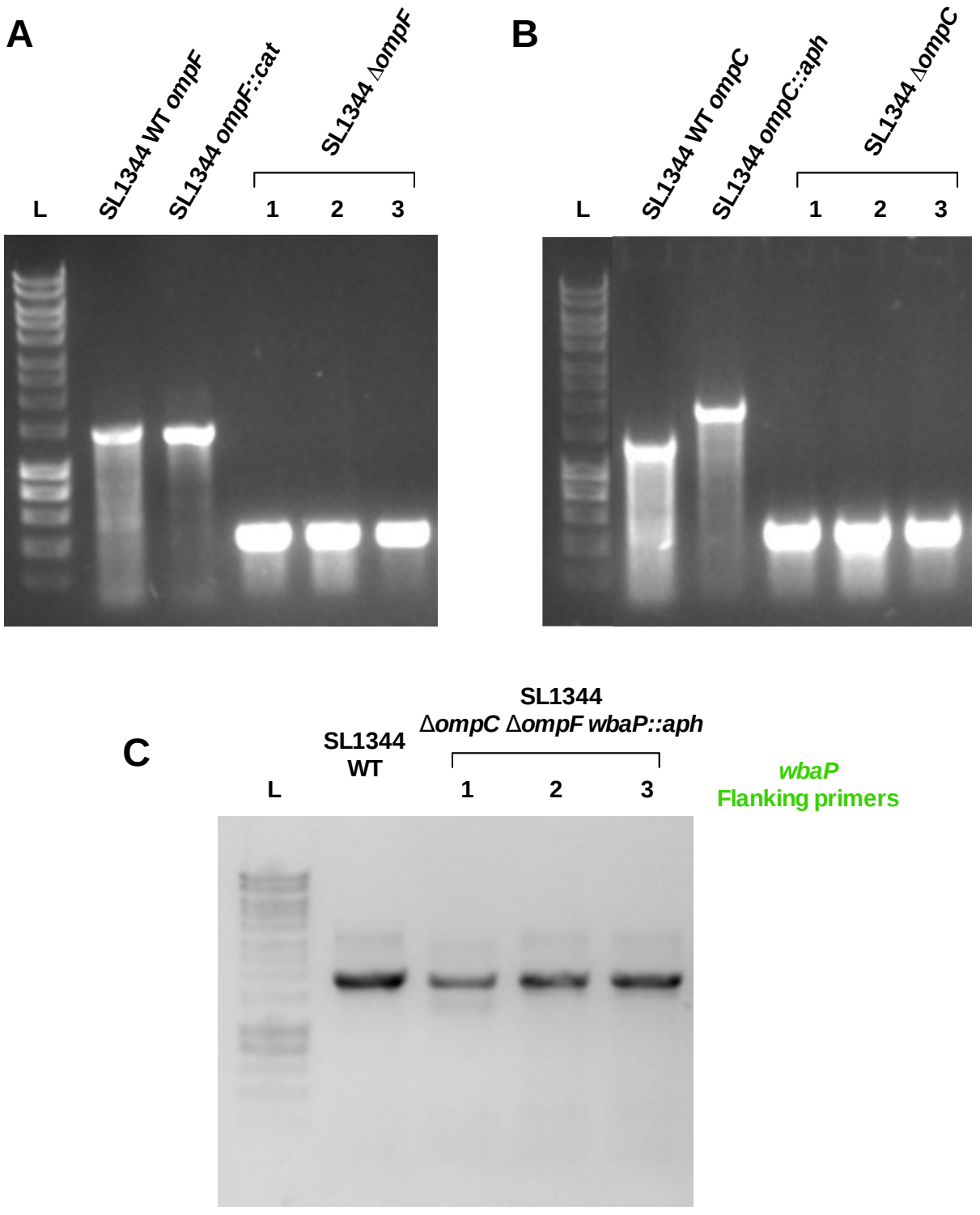

Figure 5.3 Construction of the STm SL1344 *ΔompC ΔompF wbaP::aph* mutant
(A and B) PCR confirmation of the removal of the antibiotic resistance cassettes resulting in deletion of the *ompF* and *ompC* genes using flanking primers for *ompF* and *ompC,* respectively. (C) PCR to verify the disruption of the *wbaP* gene in the double STm SL1344 knockout mutant. Fwd and Rvs *wbaP* flanking primers were used (1431 bp).

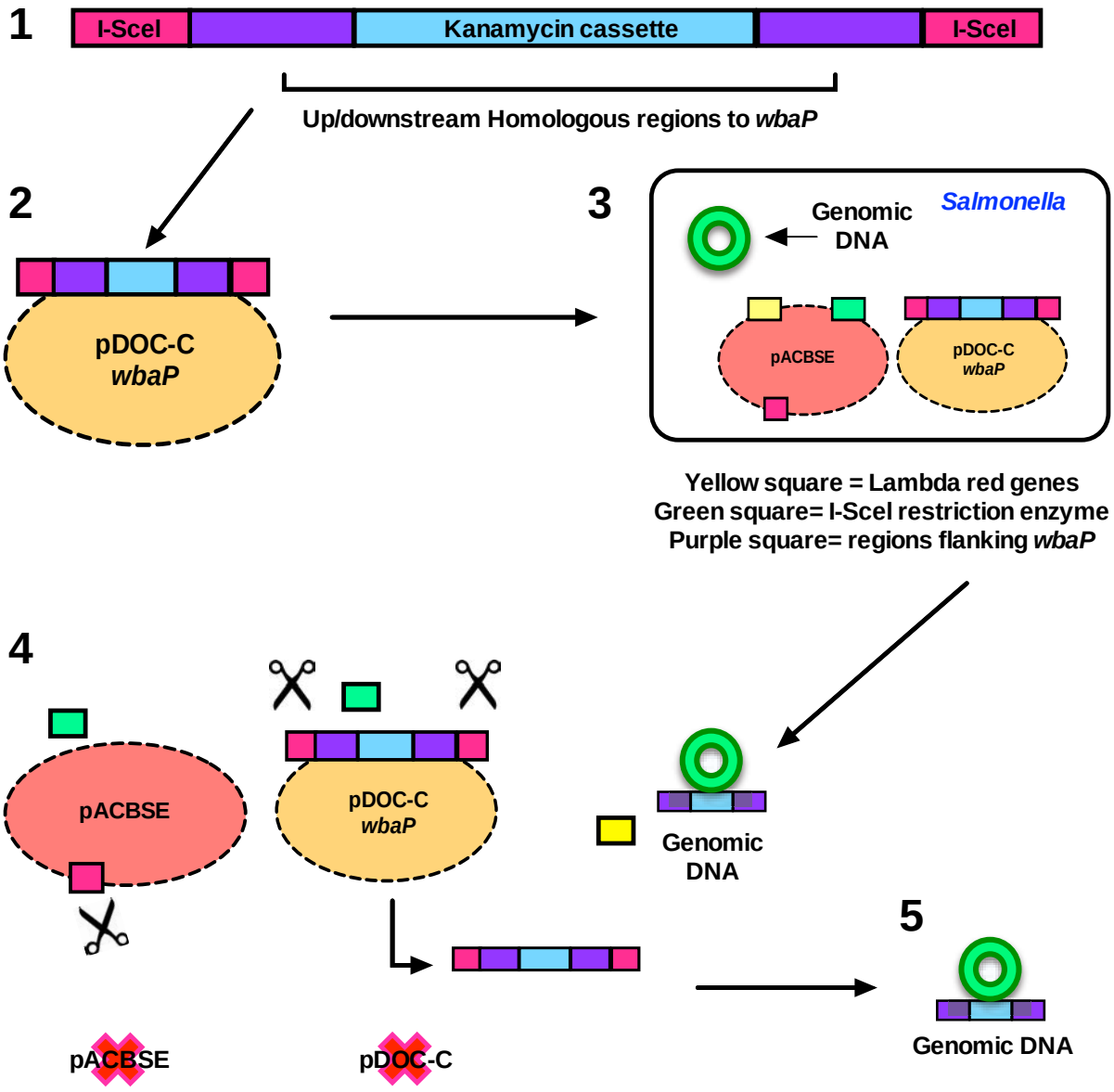

Figure 5.4 Schematic illustrating the process of gene doctoring to generate the SL1344 *ΔompC ΔompF wbaP::aph* mutant

(1) Kanamycin resistance cassette is amplified from pDOC-K using primers with homology to *wbaP*. (2) Cloning of the wbaP-kanamycin fragment into a pDOC-C vector between the I-SceI sites. (3) Electrocompetent SL1344 *ΔompC ΔompF* transformed with pDOC-C *wbaP* and pACBSE. (4) Addition of arabinose to induce expression of λ Red genes and the I-SceI restriction enzyme from pACBSE. The restriction enzyme "cuts" the plasmids and linearises the fragment for recombination with the genomic DNA. (5) The fragment recombines with the chromosome and substitutes the place of the *wbaP* gene in the cell.

5.3 Discussion

It has been shown previously that defects in aromatic amino acid biosynthesis attenuate *Salmonella* resulting in strains effective as live vaccines (159). Nevertheless, *aro* mutants can have defects in their cell membrane barrier function making them more susceptible to immune responses (338). Furthermore, strains with defects in the shikimate pathway have an increase of murA and a failure in endotoxin biosynthesis (339). In order to generate OmpD from a bacterial strain without the other major porins or O-Ag that may inadvertently affect our results, we generated this mutant in a WT background.

For purification of OmpD, the SL3261 mutant is grown in minimal salts medium containing yeast extract, glucose, and $MgSO_4$ and supplemented with the corresponding amino acids. Because it grows slower than SL1344 bacteria and supplementation of media is required, a pathogenic strain may simplify this step while at the same time conserving the integrity of the cell wall.

The presence or absence of LPS O-Ag influences the exposure of the OMPs to the extracellular environment and truncation of this molecule can be achieved by inhibition of the glycotransferanse activity (334, 340). In order to permanently inhibit O-Ag biosynthesis, we disrupted the *wbaP* gene. This gene was selected for mutation as opposed to those associated with O-Ag processing, i.e. *GalE* (340, 341).

The expression of λ red recombinase in strains is thought to increase the propensity of random mutations. To circumvent this issue, phage transduction offers an effective means for transferring mutations to a “clean” background (i.e. the parental strain), which has not been previously exposed to λ red recombinase (331). For the

generation of our *wbaP* mutant this was not possible, as the P22 phage specifically recognises the O-Ag, as the receptor for its interaction with the host *Salmonella* cell. Since our mutant does not synthesise this molecule it was not possible. As an alternative, in addition to confirming the *wbaP* mutation using PCR, we elected to perform whole genome resequencing, analysing the genome sequence for insertions, deletions and single nucleotide polymorphisms (SNPs), with the assistance of MicrobesNG at the University of Birmingham. Sequence analysis indicated the *wbaP*, *ompC* and *ompF* genes were disrupted or deleted, respectively, and no additional SNPs (beyond those present in our parental strain) had accumulated.

Altering the LPS chains of the SL1344 will produce a strain with increased sensitivity to complement-mediated killing and altered *in vivo* colonisation kinetics that differ from that of the WT strain (38, 39). However, the aim of our study was to produce a strain suitable for the purification of native OmpD, in the absence of potentially contaminating factors (such as OmpF/C and O-Ag). This strain will be used for future studies to investigate the potential for cross-protection against *Salmonella* serovars to be achieved by exclusively targeting OmpD.

Although modification of the LPS structure can affect the composition of the cell wall in Gram-negative bacteria, as shown by studies investigating the differences in OMPs profiles between rough and smooth strains (335, 342), it has been shown that truncation of the LPS does not influence the conformation and structure of these OMPs (336). Ultimately, the generation of this strain will simplify the production of highly purified OmpD.

6. FINAL DISCUSSION

One of the aims of this project involved studying how Ab to OmpD protects and what its potential strengths and limitations are as a vaccine against multiple *Salmonella* serovars. STm-OmpD has been previously identified as a protective Ag (72). However, its ability to induce cross-protection against other serovars remained unclear.

Here we showed the importance of Ab in protection after STm-OmpD immunisation. As we found that the OmpD from STm and SEn had >99% of homology differing by only one amino acid (A263S)(Chapter 3), we hypothesised that immunisation with STm-OmpD would protect against both serovars. Although we detected cross-reactive IgG to STm and SEn OMPs, this was not the case when analysed against whole bacteria or in live infections. Thus, it is important to consider that responses induced to an Ag in isolation do not always resemble those observed inside the host. Furthermore, with the use structural models shown in Chapter 3, we were able to identify the importance of LPS-porin complexes in restricting access to these protective epitopes, which raised the possibility of the existence of chimeric epitopes. Thus, while LPS O-Ag is a target of protective Ab (140-143, 333), it also inhibits access of Ab to other cell-surface antigens.

One of the limitations in this study was the possible contamination of STm-OmpD with traces of O-Ag as this porin was purified from a WT strain. Nevertheless, the results shown in Chapter 3 indicated that protective Ab targets OmpD and not O-Ag. Therefore, this raised the need of generating a strain lacking contaminating factors

such as OmpC, OmpF, and O-Ag. The strain generated as explained in Chapter 5 will be used to obtain purified OmpD for future experiments.

As discussed in Chapter 3, antigens are recognised by antibodies after natural infection. However, not all these antibodies are protective due to intrinsic differences in affinity or specificity (327, 328). Moreover, immunisation with STm-OmpD generates a rapid polyclonal response in mice. In Chapter 4 we showed the importance of this response. For instance, protection afforded after immunisation with STm-OmpD was not dependent on Th1 responses as normally seen in a natural infection (287) and T-bet and IFNg were partially dispensable. IgG1 and IgG2b were the isotypes most readily detected in immunised mice. However, the role of these isotypes in protection in mice was not studied here. The IgG1 response has already been assesed, in collaboration with Prof. Kai-Michael Toellner, using $IgG1^{-/-}$ mice. This indicated the importance of IgG1 in protection with STm-OmpD. Thus, the induction of different IgG isotypes may compensate for the lack of others and potentially provide an effective protective response after vaccination.

In Chapter 4 we showed the rapid effect of anti-STmOmpD Ab after 4 hours of infection and identified non-cognate $CD8^{+}$ T cells along with neutrophils as possible inducers of IFNg during early phases of infection. Nevertheless, the results using IFNg (eYFP) reporter mice in this study cannot be considered definitive. The signal produced by some of the surface markers used in these studies in addition with the signal expressed thanks to the eYFP mutation in these mice, were poor sometimes. Therefore, other approaches such as intracellular staining using WT mice may provide a better signal of this response. Moreover, as mentioned before, other cell types, including NK and NKT cells, also need to be analysed.

While our data from depletion experiments showed that neutrophils are important in controlling infection after STm-OmpD immunisation, this was not the case for macrophages. This was observed by flow cytometry and although $F480^{+}$ cells were reduced in clodronate-depleted STm-OmpD immunised mice, these was not equal to the level of depletion observed in NI mice. This could be due to interactions between clodronate liposomes and STm-OmpD that could inhibit the effect of clodronate liposomes but assessment of this is required.

In conclusion, this project is the basis of future work in the context of Ab-epitope-Ag complexes. Not only in the context of OmpD but for other conserved antigens in *Enterobacteriaceae*, for instance OmpA and SadA, which are also highly conserved among serovars. Moreover, it is important for understanding how Ab binds to the surface of the bacteria, how natural barriers such as LPS influence this binding, and the nature of protective epitopes in conserved antigens. All this with the purpose of being able to develop a successful vaccine against a wide range of serovars with the same conserved antigens. In addition, STm-OmpD is considered a TD antigen, but surprisingly generates a TI response. This may be important in the generation of a successful vaccine as it may be able provide protection to different age groups and immunocompromised individuals based on the type of response involved.

6.1 Future directions

This project can be exploited from different perspectives and with the use of different approaches it would give us a better understanding of the nature of Ab binding to different proteins and how this is influenced along with Ag variability. However, some

of the approaches cited here would need collaboration with more speciallised groups in each area.

Dissecting the mechanism of Ab binding to OmpD

Since we have shown that the O-Ag in LPS influences access to the epitope in OmpD (A263S), generating monoclonals that target this epitope in OmpD of STm and SEn and developing structural models to mimic LPS and OmpD interactions would be helpful to answer how this substitution affects lack of binding and influences changes in the arquitecture of this protective epitope as well as how different O-Ags interact with OmpD in the outer membrane.

Understanding the nature of Ab binding to different proteins and Ag variability

To do this, purifying conserved antigens, such as OmpD, OmpA, SadA, that have been previously identified as inducers of protective responses (72, 331) could help identify the level of binding to these antigens using different *Salmonella* strains: WT and O-Ag mutants with the use of techniques such as ELISA and flow cytometry. Moreover, mice would be immunised with these proteins and immune sera tested against these bacteria as well as the assessment of monoclonals to these proteins in isolation and in different O-Ag mutants. The OmpD will be obtained using the strain generated in Chapter 5. Furthermore, generating a mutant of STm expressing the OmpD of SEn and viceversa would help us understand the influence of LPS in blocking protective epitopes or the generation of chimeric epitopes with these porin. Furthermore, infecting mice with these strains after immunisation with STm-OmpD would evaluate the level of protection against each mutant.

Importance of antibody switching

Understanding the role of different Ab isotypes can be achieved with techniques such as fluorescense microscopy and structural imaging. Modullating Ab structures in order to maintain their specificity but modify their heavy chains would give us an insight of how this also affects binding to antigens and protective responses. All this, in order to examine whether access to the cell wall is available equally to all isotypes or if it is dependent on the structure of each isotype.

Additional immunological aspects of the STm-OmpD response

Ab to porins from *S.* Typhi induces a long-lasting Ab response (60). Nevertheless this has not been assessed to STm-OmpD. We have only assessed the response after immunisation with STm-OmpD during early stages of infection. Therefore, analysing this response at later stages is essential along with the protective immune mechanisms involved.

APPENDIX A: BUFFERS AND MEDIA

All buffers and media used throughout this study and that are not described in Chapter 2 are listed below:

Luria-Bertani (LB) broth

20 g of LB broth (Lennox) powder (Sigma-Aldrich)

1 L dH_2O

LB agar

35 g of LB (Lennox) agar (Sigma-Aldrich)

1 L dH_2O

Broth and agar were autoclaved at 121 ºC

10x Phosphate Buffered Saline (PBS)

8.5 g/L NaCl

10.7 g/L Na_2HPO_4

3.9 g/L NaH_2PO_4

Diluted in dH_2O

1x Phosphate Buffered Saline (PBS)

100 ml of 10x PBS per 1 L of dH_2O

ELISA Carbonate Coating Buffer

1.66 g/L Na_2HCO_3

2.84 g/L NaH_2CO_3

0.1% NaN_3

Diluted in dH_2O

FACS Buffer

1% Heat-inactivated foetal bovine serum (HiFBS)

2 mM EDTA

Diluted in PBS

APPENDIX B: EXTRACTION AND PURIFICATION OF PROTEINS

I. Outer membrane proteins (OMPs)

OMPs from STm and SEn were extracted and purified by Ms. Charlotte N. Cook (University of Birmingham). Preparations were made by 2% Triton X-100 extraction (303). When an OD_{600} of 1.0 was reached, cells were harvested at 10,000 x g for 10 minutes at 4 ^{o}C and washed with 10 mM Tris-HCL (pH 7.4) containing 2 mM PMSF (phenylmethylsulfonyl fluoride)(Roche). A French Press was used to disrupt the cells at 20,000 psi and centrifuged at 6,000 x g for 10 minutes at 4 ºC to remove unbroken cells. The supernatant was centrifuged at 30,000 x g for 90 minutes at 4 ºC to separate the envelope and cytoplasmic fractions. Pelleted envelopes were re-suspended in 10 mM Tris-HCL with 2% Triton X-100 and incubated at room temperature for 15 minutes. This was harvested at 30,000 x g for 90 minutes at 4 ºC and washed in 10 mM Tris-HCL. The final preparation was re-suspended in the same buffer. Concentration of the proteins was determined with the BCA assay (Appendix C) and assessed on a 4-12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Total OMPs were stored at -20 ºC.

II. STm-porins and STm-OmpD

Proteins were isolated and purified from STm strain ATCC 14028 (STm-porins) and STm RAK146 SL3261 *ompC::aph ompF::cat* (STm-OmpD) by repeated extraction with sodium dodecyl sulfate (SDS)(61, 353). Bacteria were grown in minimal salts medium (MAM)(0.5% glucose, 0.1% yeast extract, 0.1% $MgSO_4$) at 200 rpm at 37 ºC

until stationary phase or OD_{600} of 1.0. Bacteria were diluted 1:10 in MAM and incubated at 37 ºC to reach stationary phase. Cells were centrifuged at 6,000 x g for 20 minutes at 4 ºC, washed and re-suspended in 50mM Tris-HCL (pH 7.4). To disrupt the cells a French Press was used at 20,000 psi and cells spun at 6,000 x g for 20 minutes at 4ºC. DNase and RNase were added to the supernatant to then be centrifuged at 30,000 x g for 50 minutes at 4 ºC. The pellet was re-suspended in solubilisation buffer (50 mM Tris-HCL, 2% SDS) and incubated for 30 minutes at 120 rpm at 37 ºC. Following this, the soluble inner membrane (IM) and insoluble outer membrane (OM) fractions were separated at 30,000 x g for 50 minutes at 4 ºC and pellet (containing the OM) was re-suspended in Nikaido's solubilisation buffer (50 mM Tris-HCL, 1% SDS, 3.25 Mm EDTA, 0.5% β2-mercapthoethanol). This step, including the 30 minutes incubation at 37 ºC, was performed 3 times. In the last step, prior to centrifugation at 30,000 x g for 50 minutes, pellet was re-suspended and incubated at 37 ºC at 120 rpm for 2 hours. Isolation of the proteins from the resulting supernatant (OD_{600} of 2.0) was achieved by fast protein liquid chromatography (FPLC) on a Sephacryl S-200 column using Nikaido's purification buffer (50 mM Tris-HCL, 0.5% SDS, 3.25 mM EDTA, 200 mM NaCl). Concentration of proteins was determined with the BCA assay and confirmed with Coomassie blue on a 12% SDS-PAGE. The Limulus amoebocyte lysate (LAL) assay was used to determine any LPS contamination. Identity of the proteins was confirmed by trypsin digest and mass spectrometry (School of Biosciences Functional Proteomics Unit at the University of Birmingham). Proteins were stored at 4 ºC.

APPENDIX C: BICINCHONIC ACID ASSAY (BCA)

For protein quantification the Pierce BCA assay (Thermo Fischer Scientific) was used. This method was adapted to be used in microplates. Briefly, 30 µl of the purified protein or whole bacteria were added per well (by triplicate) and mixed with 200 µl of BCA working reagent (BCA/copper complex). Samples were incubated for 30 minutes at 37 ºC. Bovine serum albumin (BSA) protein standards were prepared according to manufacturer's instructions. Plates were read at λ545 nm on an Emax microplate reader (Molecular Devices) and concentration of the proteins/bacteria was determined with reference to BSA protein standards using a standard curve of absorbance.

APPENDIX D: CELL POPULATIONS IN THE SPLEEN AND PEC IN NI AND STM-OMPD IMMUNISED MICE AT 4 HOURS POST-INFECTION

This appendix contains supplemental FACS data from experiments performed in Chapter 4 using IFNg (eYFP) reporter mice immunised and infected as shown in Figure 4.17A. However, no significant changes were observed in these cell populations.

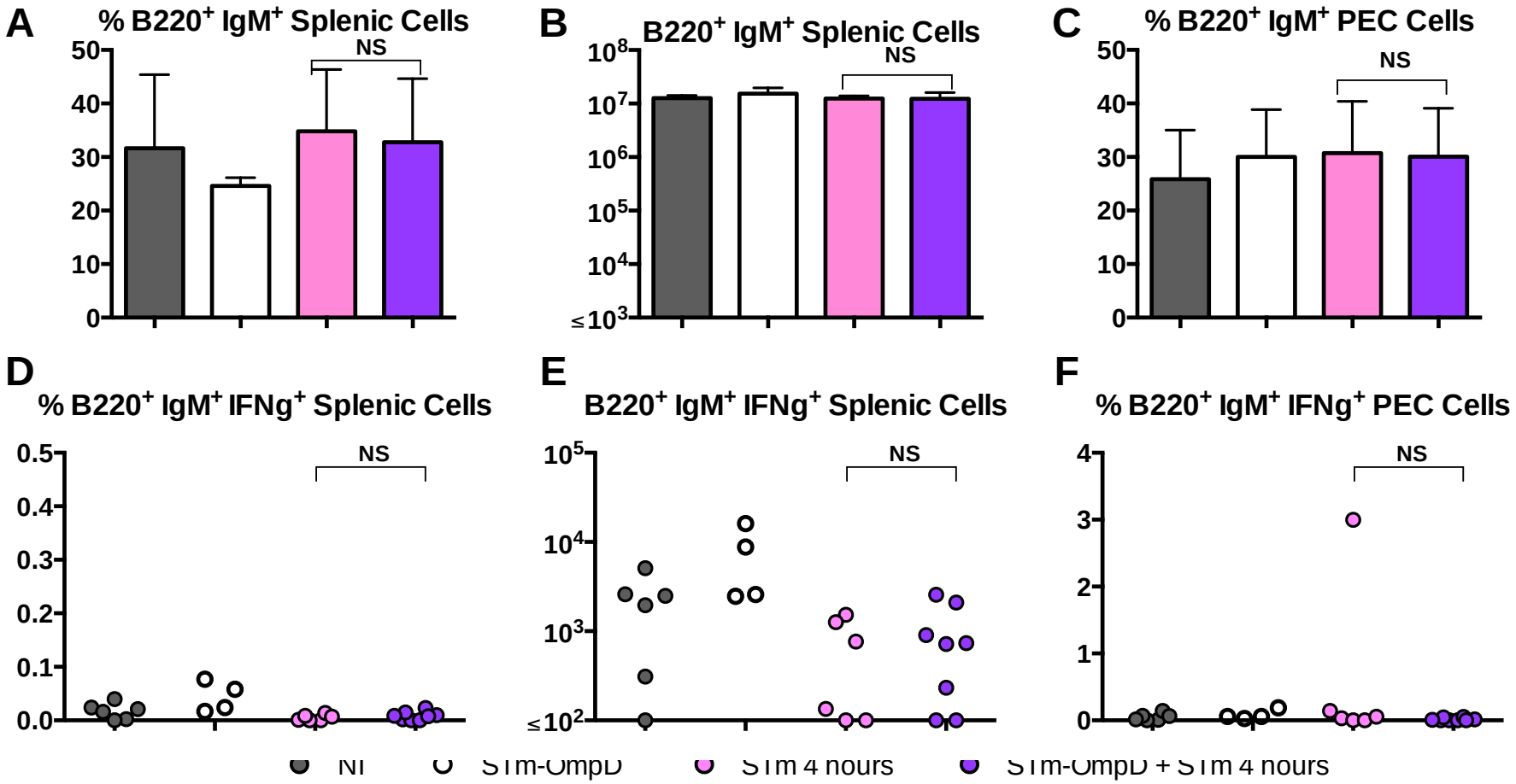

Figure D1 B cells in the spleen and PEC after 4 hours of infection in NI and STm-OmpD immunised mice
For FACS gating strategy refer to Fig. 4.12A. IFNg (eYFP) reporter mice immunised as mentioned in Figure 4.8A were used. (A and B) Proportion and total number of $B220^+$ IgM^+ $IFNg^+$ splenic cells were determined. (C) Proportion of $B220^+$ IgM^+ $IFNg^+$ PEC cells are shown. (D and E) Proportion and total numbers of $B220^+$ IgM^+ $IFNg^+$ in the spleen were analysed. (F) Frequency of $B220^+$ IgM^+ $IFNg^+$ cells in the PEC was determined. Data is from 2 experiments. NS = non-significant.

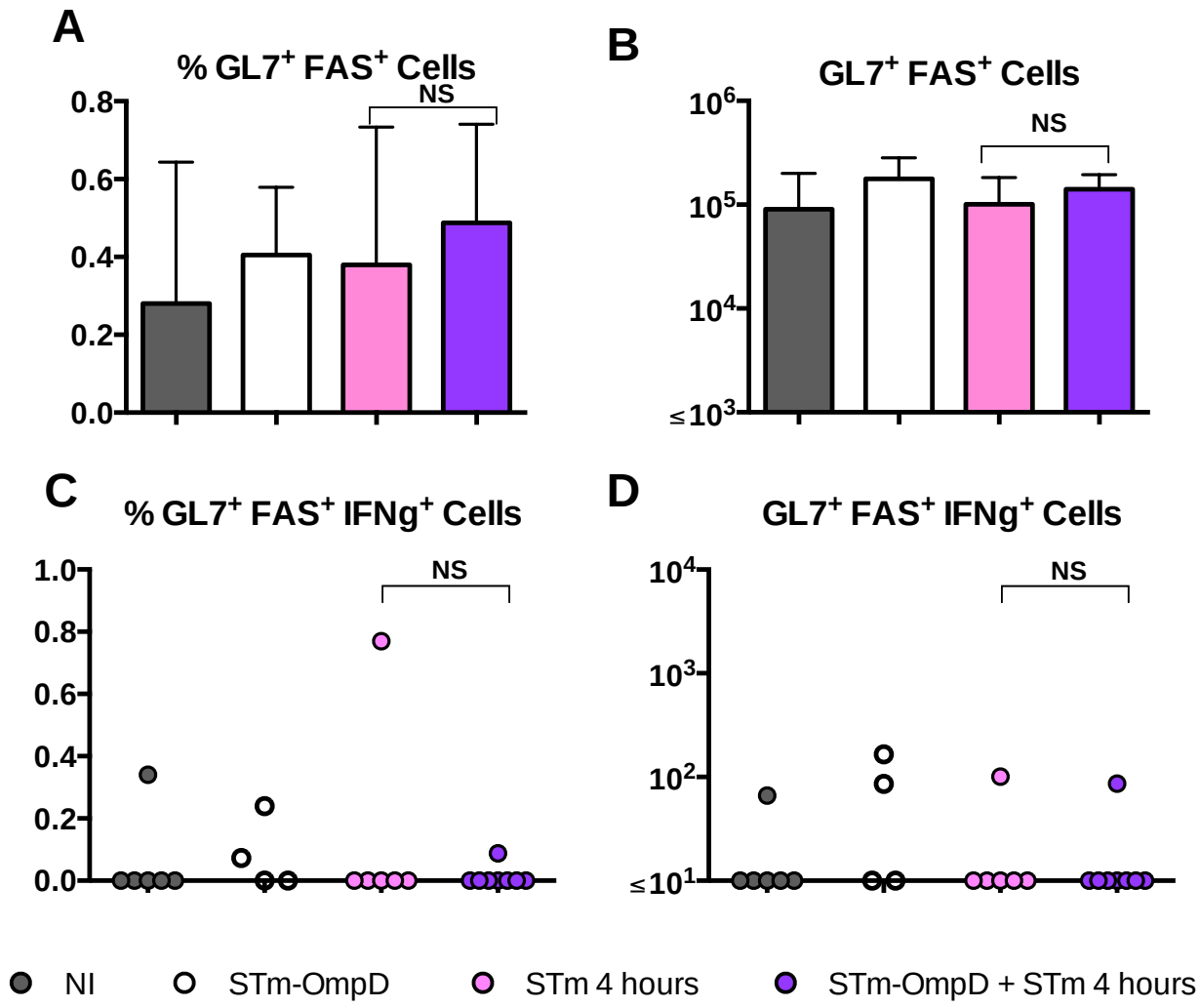

Figure D2 Splenic GC B cells 4 hours post-infection in NI and STm-OmpD immunised mice

For FACS gating strategy refer to Fig. 4.13A. IFNg (eYFP) reporter mice immunised as mentioned in Figure 4.8A were utilised. (A and B) Proportion and total number of $GL7^+$ FAS^+ splenic cells are shown. (C and D) Percentage and total number of $GL7^+$ FAS^+ $IFNg^+$ cells in the spleen were analysed. Data is from 2 experiments. NS = non-significant.

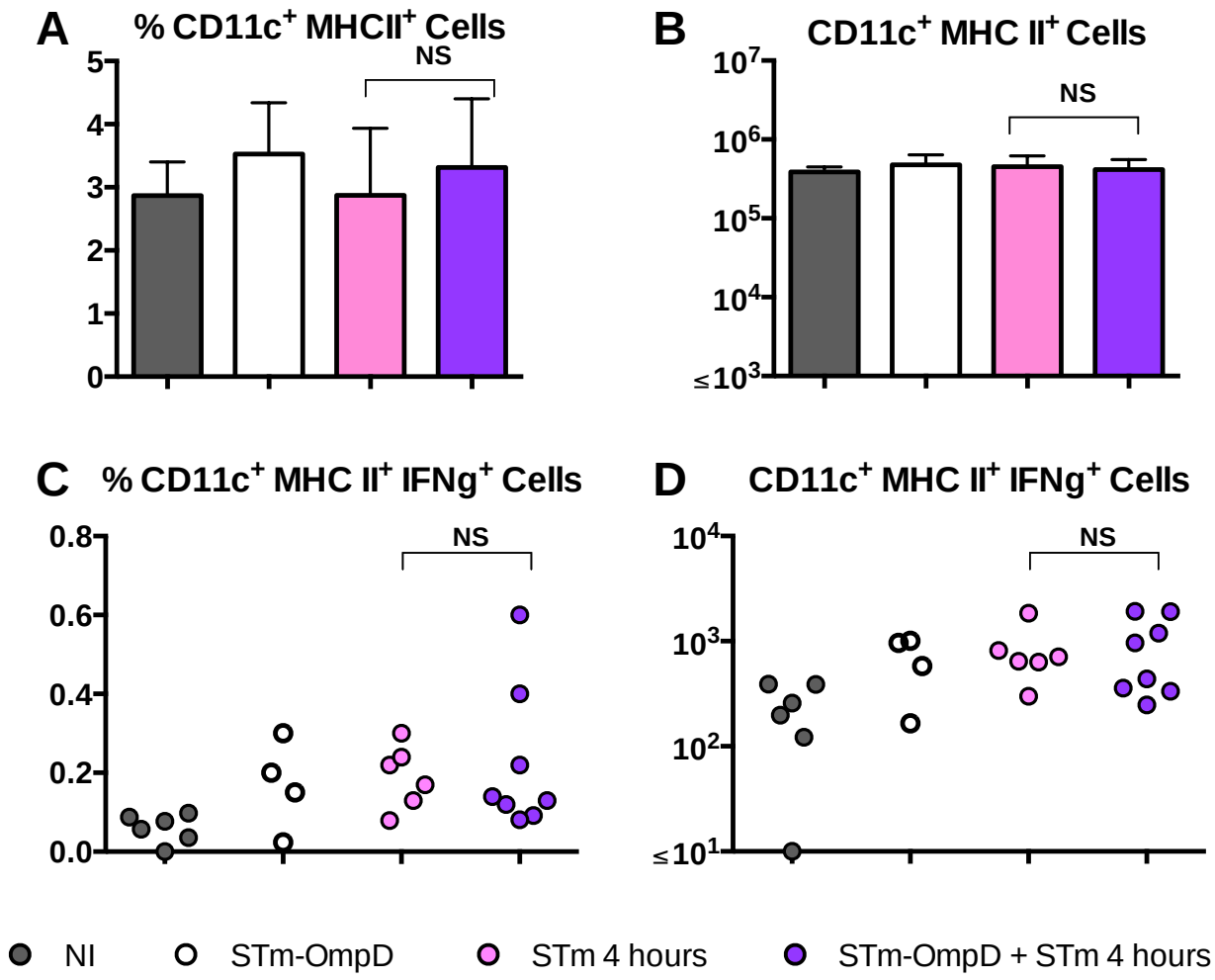

Figure D3 Population of splenic DC cells 4 hours after infection in NI and STm-OmpD immunised mice

For FACS gating strategy see Fig. 4.14A. IFNg (eYFP) reporter mice immunised as mentioned in Figure 4.8A were used. (A and B) Frequency and total number of $CD11c^+$ MHC II^+ splenic cells were determined. (C and D) Percentage and total number of $CD11c^+$ MHC II^+ $IFNg^+$ splenic cells is shown. Data is from 2 experiments. NS = non-significant.

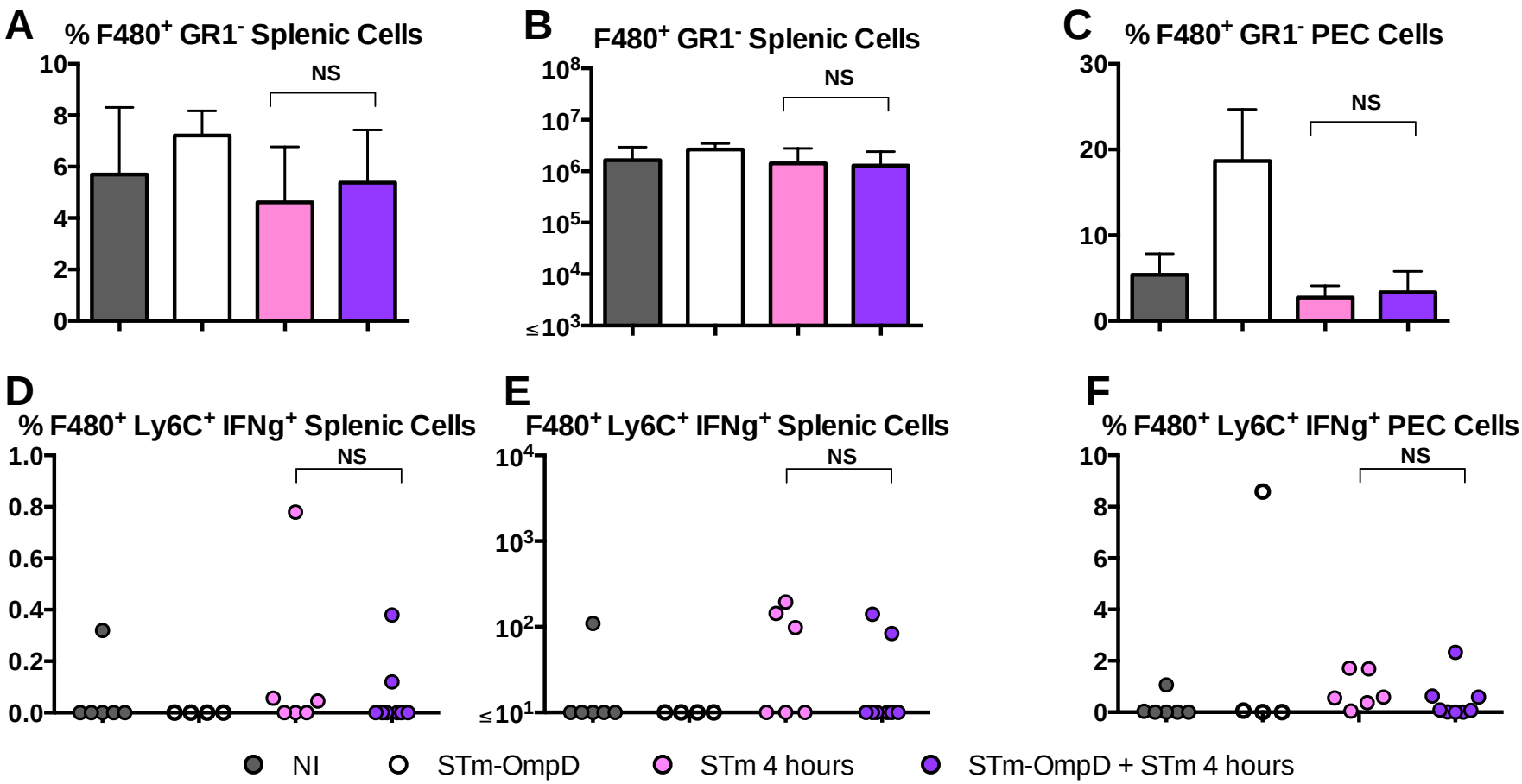

Figure D4 F480^{+} GR1^{-} macrophages expressing IFNg in NI and STm-OmpD immunised mice at 4 hours post-infection
Splenic and PEC cells from IFNg (eYFP) reporter mice were gated for F480^{+} GR1^{-} cells. For the FACS strategy used to gate these cells refer to Figure 4.16A. (A and B) Proportion and total number of F480^{+} GR1^{-} splenic cells are shown. (C) Frequency of F480^{+} GR1^{-} cells in the PEC was determined. (D and E) Proportion and total numbers of F480^{+} Ly6C^{+} IFNg^{+} cells are illustrated. (F) Percentage of F480^{+} Ly6C^{+} IFNg^{+} cells in the PEC is shown. Data is from 2 individual experiments. NS = non-significant.

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