UNIVERSITY OF BIRMINGHAM

AN INVESTIGATION INTO VACCINE-MEDIATED IMMUNE RESPONSES AGAINST INVASIVE *SALMONELLA* TYPHIMURIUM INFECTION

by

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ABSTRACT

Salmonella enterica serovar Typhimurium is one of the main causative agents of invasive non-typhoidal *Salmonella* (iNTS) disease. Resistance to antimicrobial agents is a rising concern, complicating treatment, and prognosis. The need for effective preventative strategies against iNTS is crucial for controlling spread of infection and preventing severe disease. Outer membrane vesicles (OMVs) are naturally shed outer membrane blebs that mimic the bacterial surface and are being explored as a cost-effective vaccine against many infections. Understanding the immunological mechanisms that constitute successful vaccine responses is key for vaccine design. In this thesis, I explored specific innate immune components that are crucial for generating productive immune responses to vaccination with OMVs, resulting in improved ability to control iNTS infections in mice. I showed that complement component 3 (C3) was essential for protection in OMV-vaccinated and challenged mice. Further, I discovered a novel antigen persistence phenotype in OMV-vaccinated and challenged mice. Further investigation of this phenotype revealed that it is specific to OMVs with intact $LPS_{O\text{-}Ag}$ chains, and not induced by immunisation with subunit vaccines such as sFliC, or *Salmonella* Typhi-derived Vi polysaccharide or conjugated vaccines. This phenotype was not influenced by an absence of lymphoid cells, and depletion of macrophages altered the distribution of persistent antigen, however this phenotype was not associated with protection. The results presented in this thesis identify key immune components required for successful protection against iNTS infections in mice, but also potentially pathogenic phenotypes resulting from OMV-vaccination, which bare importance on vaccine design.

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LIST OF ABBREVIATIONS

µg	Micrograms
µL	Microlitre
Ab	Antibody
Ag	Antigen
AP	Alkaline phosphatase
APC	Antigen presenting cell
ASCs	Antibody secreting cells
BCR	B cell receptor
BSA	Bovine serum albumin
C'	Complement
C1q	Complement component 1q
C3	Complement component 3
C4	Complement component 4
C5	Complement component 5
CD	Cluster of differentiation
CFU	Colony forming unit
CR1	Complement receptor 1
CR2	Complement receptor 2
EDTA	Ethylenediaminetetraacetic acid
EF	Extrafollicular
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FDCs	Follicular dendritic cells
g	gram
GCs	Germinal centre
GMMA	Generalised Modules for Membrane Antigens
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
iNTS	Invasive non Typhoidal Salmonellosis
KO	Knockout
L	litre
LB	Luria Bertani
LMICs	Low-middle income countries
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MBL	Mannose-binding lectin
mg	milligrams
MHC	Major histocompatibility complex

mL	millilitre
MLN	Mesenteric lymph nodes
MZ	Marginal zone
NTS	Non Typhoidal Salmonellosis
O-Ag	O antigen
OD	Optical density
OmpD	Outer membrane protein D
OMVs	Outer membrane vesicles
PBs	Plasmablasts
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCs	Plasma cells
pNPP	*P*-nitrophenyl phosphate
PRRs	Pattern recognition receptors
RP	Red pulp
RPMI	Roswell Park Memorial Institute 1640 medium
s.c.	subcutaneous
SPF	Specific pathogen free
STm	*Salmonella enterica* serovar Typhimurium
TBS	Tris buffered saline
TD	T-dependent
Tfh	T follicular helper
Th	T helper
TI	T-independent
TLR	Toll-like receptor
ViPS	Vi polysaccharide
Vi-TT	Vi tetanus toxin conjugate
WP	White pulp
WT	Wildtype

PUBLICATIONS CONTRIBUTED TO DURING THESIS

Marcial-Juarez *et al*., ***Salmonella*** **infection induces the reorganisation of follicular cell networks concomitant with the failure to generate germinal centres**, 2023. *iScience*, vol. 26 (4): 106310. doi: 10.1016/J.ISCI.2023.106310.

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CHAPTER 1: INTRODUCTION

Non-typhoidal *Salmonella* (NTS) infections are a leading cause of gastroenteritis worldwide. Invasive NTS (iNTS) infections are common in immunocompromised individuals, leading to severe and/or fatal disease. With a rising incidence of antimicrobial resistance in NTS bacteria, treatment options are becoming increasingly limited leading to poor prognosis and disease outcomes. Preventative strategies against infections, such as vaccines, are required to control infection spread and disease. Vaccine development is time- and cost-intensive and requires an understanding of how the immune system responds to bacterial antigens and the effectiveness of the responses elicited. This chapter outlines our current understanding of NTS and iNTS infections, current and prospective vaccines, and the induced immune responses.

1.1 *Salmonella* bacteria

1.1.1 Classification

Salmonella are rod-shaped Gram-negative facultative anaerobic bacteria belonging to the *Enterobacteriaceae* family. The *Salmonella* genus is divided into two species, *Salmonella bongori* and *Salmonella enterica* (World Health Organisation., 2018). *S. bongori* infections are restricted to cold-blooded animals, whereas *S. enterica* species may infect a variety of warm-blooded animals, including humans and birds (Brenner et al., 2000). The *S. enterica* species can be further divided into six subspecies: *enterica, salamae, arizonae, diarizonae, houtenae*, and *indica*, however almost all infections that cause disease in humans and domesticated animals belong to *S. enterica* subsp. *enterica* (Fàbrega and Vila, 2013).

S. enterica is a highly diverse bacterial species, constituting over 2600 distinct serovars that are differentiated by their O (lipopolysaccharide [LPS]) or H (flagellar) antigens (Fàbrega and Vila, 2013; Gal-Mor et al., 2014). Most *S. enterica* serovars that cause disease in humans and other mammals can be classified into typhoidal and non-typhoidal serovars (Gal-Mor et al., 2014). Typhoidal serovars Typhi and Paratyphi are human-restricted pathogens (Feasey et al., 2012; J. Barton et al., 2021; Spanò, 2016), and are the causative agents of typhoid fever. *S. enterica* serovar Typhimurium (STm) and Enteritidis (Sen) are the dominant serovars causing non-typhoidal *Salmonella* (NTS) infections (Saleh et al., 2019; Fàbrega and Vila, 2013; Gal-Mor et al., 2014; Majowicz et al., 2010a).

1.1.2 Cell wall structure

As mentioned previously, serovars of the *S. enterica* species is subdivided by their antigenic presentation of O and/or H antigens. As a Gram-negative bacterium, the *Salmonella* cell wall consists of an inner membrane, a peptidoglycan layer, and an outer membrane (**Figure 1.1**). The two membranes are separated by a viscous aqueous cellular compartment that is densely packed with proteins known as the periplasm (Silhavy et al., 2010). The inner membrane is a phospholipid bilayer where membrane-associated functions such as energy production, lipid biosynthesis, and protein secretion occur in bacteria (Silhavy et al., 2010). The outer membrane, however, is an asymmetric lipid bilayer rich in proteins and glycolipids, predominantly LPS (Silhavy et al., 2010; Kamio and Nikaido, 1976). LPS serves as a major structural component of the outer membrane, a permeability barrier against small hydrophobic molecules (such as antimicrobial compounds) and modulates the host immune response during infection (Silhavy et al., 2010; Kamio and Nikaido, 1976; Bertani and Ruiz, 2018; Kong et al., 2011).

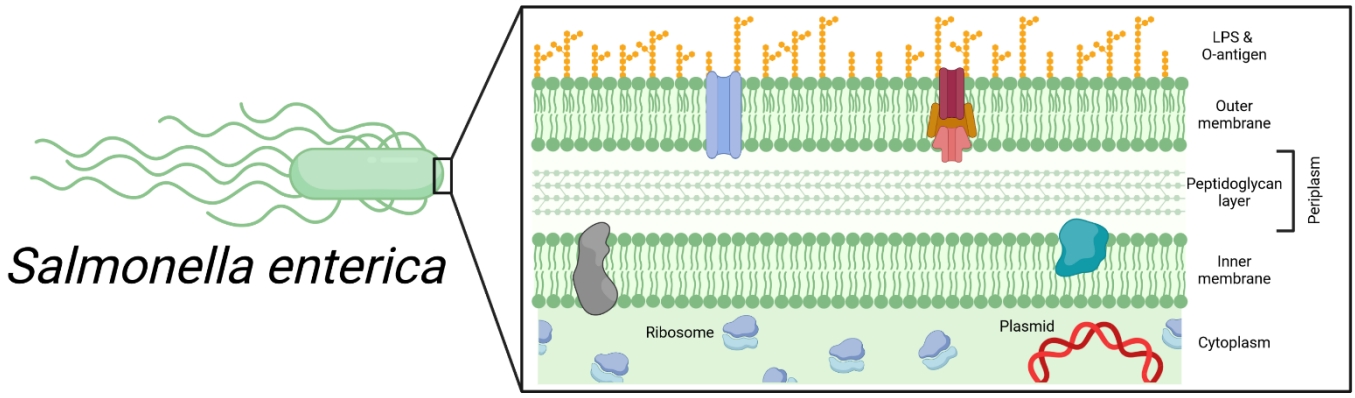

Figure 1.1: Gram negative bacterial cell wall of *Salmonella* bacteria. Figure was created using (Liu et al., 2016) and Biorender.com.

1.1.2.1 Lipopolysaccharide (LPS)

LPS is a key component of the Gram-negative outer membrane. It is comprised of lipid A, core oligosaccharide, and repeating units of the O-antigen (O-Ag) (Kong et al., 2011; Bertani and Ruiz, 2018). Lipid A is a glucosamine disaccharide and secures the LPS molecule to the outer membrane (Bertics et al., 2006). The core oligosaccharide is made of an inner and outer core, which are conserved within *Salmonella* species (Hankins et al., 2013).

The O-Ag is a key virulence factor in many Gram-negative infections, including *Salmonella*. O-Ag is hydrophilic and made of repeating oligosaccharide units (Lerouge and Vanderleyden, 2002). In Gram-negative species, repeating O-Ag units are synthesised via the wzx/wzy pathway. O-Ag is synthesised on the cytoplasmic side of the bacterial membrane. It is then flipped to the periplasmic side by action of Wzx and polymerised with additional polysaccharide units by Wzy (Hong et al., 2015). Mutant *Salmonella* strains lacking these pathways produce LPS that lack O-Ag chains (Collins et al., 1991).

It is important here to bring to attention the difference between LPS and capsular polysaccharide (CPS), and further the difference between LPS O-antigen ($LPS_{O\text{-}Ag}$) and O-antigen capsule. STm lack the anti-inflammatory Vi capsule of *S.* Typhi and Paratyphi, yet they make an alternative CPS consisting of group IV polysaccharide, or O-Ag capsule, that is common among other enteric bacteria (Marshall and Gunn, 2015). Confusion may occur between the names of the two structures, where the group IV capsule is referred to as O-Ag capsule due to its high degree of similarity to the repeating tetrasaccharide units of $LPS_{O\text{-}Ag}$ (Marshall and Gunn, 2015).

The key difference between these two structures is the way in which it is anchored to the membrane – where $LPS_{O\text{-}Ag}$ is associated with an LPS core region, O-Ag capsule is not (Sokaribo et al., 2021). Further differences include the number of repeating units in each structure ($LPS_{O\text{-}Ag}$ have <100 repeating units where O-Ag capsule can have >2000), the lower overall net charge of O-Ag capsule, and finally glycosylation of certain monosaccharide units is specific to O-Ag capsule and does not occur with $O\text{-}Ag_{LPS}$ (Sokaribo et al., 2021). LPS and CPS also differ functionally. LPS plays a role in barrier properties of the cell envelope, activates TLR4 and induces endotoxic shock, and also is involved in resistance to host serum factors such as antibody and complement (Willis and Whitfield, 2013). CPS also mediates serum resistance, but also acts to shield LPS and type 3 secretion systems (T3SS) from immune recognition, reduces inflammation, and delay apoptosis of infected macrophages (Marshall and Gunn, 2015).

For the purposes of this thesis O-Ag refers to that specifically of $LPS_{O\text{-}Ag}$, unless otherwise specified.

1.2 *Salmonella* infection in humans

1.2.1 Typhoidal *Salmonella*

Typhoid fever, or enteric fever, is caused by *S. enterica* serovars Typhi and Paratyphi and is transmitted by the faecal-oral route through consumption of contaminated food or water (Dougan and Baker, 2014; Bhandari et al., 2022). It is a systemic illness that is characterised by fever, abdominal pain, nausea, coughing, vomiting, and diarrhoea (Bhandari et al., 2022). Typhoid fever is endemic to Africa, the Americas, and South and Southeast Asia, causing ~20 million cases per year and approximately 200,000 deaths (Ochiai et al., 2012; World Health Organisation, 2018b; Bhandari et al., 2022).

Virulence of *Salmonella* Typhi and Paratyphi is determined by typhoid toxin, Vi antigen (the capsular polysaccharide), O and H antigen (Bhandari et al., 2022; Dougan and Baker, 2014). The Vi antigen acts as a defence against macrophage phagocytosis (Bhandari et al., 2022). After entering the gut, bacteria invade the intestinal mucosa by microfold (M) cells and establish an initially undetectable infection. The bacteria disseminate systemically in the absence of mucosal inflammation or diarrheal response (Dougan and Baker, 2014; Senthilkumar et al., 2014; Snyder et al., 1963). Asymptomatic carriers may harbour the bacilli for days or even years without inflammatory typhoid symptoms (Senthilkumar et al., 2014; Snyder et al., 1963; Nguyen et al., 2004; Marineli et al., 2013). This lack of inflammatory response distinguishes *S*. Typhi disease from those caused by its non-typhoidal relatives. *S.* Typhi gain access to the underlying lymphoid tissues within the gut. The infection then rapidly disseminates to the spleen, liver, bone marrow and gallbladder by replicating intracellularly within phagocytes (Gal-Mor et al., 2014; Bhandari et al., 2022).

S. Typhi infections can be treated with antibiotics. Antibiotics commonly used to treat typhoid fever include fluoroquinolones, cephalosporins, macrolides and carbapenems.

However, antimicrobial resistance to a range of antibiotic classes is becoming an increasing problem and makes treatment of *S*. Typhi/Paratyphi infections more difficult. Resistance to chloramphenicol, ampicillins, fluoroquinolones and third generation cephalosporins have all been reported (Erdal Akalin, 1999; Mukhopadhyay et al., 2019; Andrews et al., 2018). This, in conjunction with asymptomatic carriage exhibited in *S*. Typhi infections, make preventative measures against infection an attractive approach for quenching the spread of disease.

1.2.2 Non-typhoidal *Salmonella* (NTS)

Non-typhoidal *S. enterica* causes Salmonellosis disease. The two most common serotypes to cause NTS are *Salmonella* Typhimurium (STm) and *Salmonella* Enteritidis (SEn) (Marchello et al., 2022). Transmission occurs by the faecal-oral route by ingestion of contaminated food or water, as with typhoidal *Salmonella*. Salmonellosis, in immunocompetent hosts, is a self-limiting disease characterised by acute onset fever, abdominal pain, nausea, vomiting and diarrhoea (World Health Organisation, 2018a). Unlike typhoidal strains, NTS seek a survival advantage over existing host gut microflora by exploiting the gut mucosal inflammatory response. This is achieved by a myriad of different mechanisms, such as resistance to host antimicrobial peptides and competition for metabolic niches (Feasey et al., 2012). Disease symptoms occur within 72 hours of infection, and last for up to a week. It was estimated that in 2017, NTS-induced enterocolitis accounted for 95.1 million cases of diarrhoeal disease, approximately 50,000 deaths and 3.10 million disability-adjusted life years (DALYs) (Stanaway et al., 2019; Majowicz et al., 2010b). Treatment is not usually necessary, however in immunocompromised hosts or infants/elderly patients, infection can become invasive and life-threatening. In cases where treatment is needed, NTS can be treated with antibiotics (described in detail below).

1.3 Invasive non-typhoidal *Salmonella* (iNTS)

Populations such as infants and young children, elderly, immunocompromised, malnourished or those with concomitant HIV or malaria infections are at higher risk to developing invasive non-typhoidal *Salmonella* (iNTS) disease (Marchello et al., 2022; Mastroeni and Rossi, 2016). As with non-invasive disease, the two most common serovars associated with iNTS were STm and SEn (Ao et al., 2015; Marchello et al., 2022). iNTS is estimated to cause 3.4 million cases annually, accompanied with 15-20% case-fatality ratio (Marchello et al., 2022; Ao et al., 2015; Balasubramanian et al., 2019).

1.3.1 Pathogenesis

The route of pathogenesis is summarised in **figure 1.2**. Orally ingested bacteria enter the gut lumen and invade the intestinal epithelial microfold (M) cells. Through these, the bacteria cross the mucosa and enter the submucosa, where they are endocytosed by both macrophages and dendritic cells. Bacteria then replicate intracellularly within *Salmonella* containing vacuoles (SCVs). At this point, failure to control the infection within the phagocytic cells (e.g., poor neutrophil recruitment, impaired cytokine responses etc.) results in invasive disease. Infected cells and free bacteria migrate to the mesenteric lymph nodes, where they then have access to the lymphatic system and bloodstream. Infection then spreads systemically as both intracellular and extracellular bacteria replicate and expand within the reticuloendothelial system. Periodic recirculation through these systems results in new foci of infection across the host, and the *Salmonella* bacteria colonise multiple organs such as the spleen, liver, and kidneys (Gilchrist et al., 2015; Gordon, 2011; Feasey et al., 2012). Over the course of infection, *Salmonella* bacteria infect immune cells such as macrophages and PMNs,

providing a replicative niche and protection from extracellular host defences such as antibody and complement (Dunlap et al., 1992; Salcedo et al., 2001; Helaine et al., 2014).

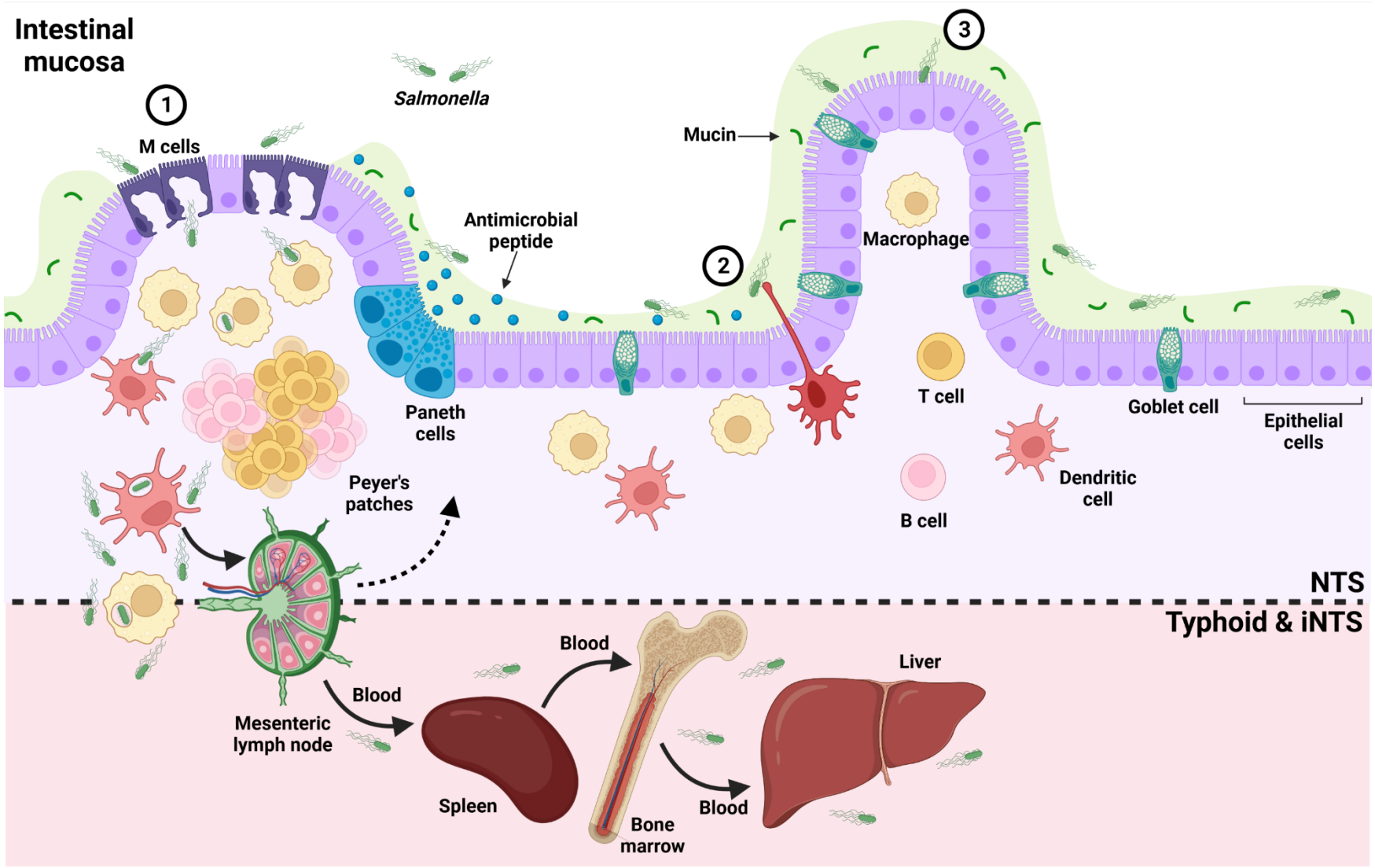

Figure 1.2: Pathogenesis of Salmonella infections. When ingested, *Salmonella* bacteria can infect the intestinal mucosa by (1) transcytosis through M cells, (2) Uptake by CX3CR1^{+} macrophages or dendritic cells, or (3) SPI-I-induced uptake. Figure was created using (Broz et al., 2012) and Biorender.com.

1.3.2 HIV

Human immunodeficiency virus (HIV) status is often associated with an increased risk to iNTS disease. HIV is characterised by a progressive failure of functional immunity, marked by a depletion of $CD4^+$ T cells (Patton, 2003). This makes the individual susceptible to opportunistic infections, and development of acquired immune deficiency syndrome (AIDS). Profound depletion of $CD4^+$ T cells in the gastrointestinal (GI) tract, and specifically those that produce interleukin (IL)-17 (Th17) has been shown to be a key determinant in uncontrolled dissemination of NTS bacteria, leading to invasive disease (Raffatellu et al., 2008). HIV^+ adults also displayed dysregulated cytokine production during intracellular infection which led to persistence and recurrence of iNTS. HIV^+ adults displayed a six-times greater bacterial load in the bone marrow during relapse in infection (Feasey et al., 2012). Bacterial loads negatively correlated with $CD4^+$ T cell counts, and the concentration of cytokines measured, further demonstrating a role for these immune mechanisms in the transition from non-invasive to invasive infection. Schreiber *et al*. demonstrate a marked attenuation of proinflammatory cytokines when they performed transcriptional analysis on whole blood samples of iNTS-infected HIV^+ patients, compared to HIV^- controls and HIV^+ patients with other invasive bacterial infections (Schreiber et al., 2011). Others have demonstrated an impairment in macrophages from HIV^+ patients to produce proinflammatory cytokines, and that the levels of these cytokines were greatly diminished in late-HIV infections when patients were most susceptible to disease (Gordon et al., 2007). Finally, a role for antibodies in serum and intracellular killing of NTS bacteria have been demonstrated. HIV^+ patients demonstrate a defective humoral response that is associated with impaired serum and phagocytic killing of NTS strains. However, these defects were accompanied by a presence of anti-LPS

IgG. It was later found that this IgG was inhibitory, which bound LPS and blocked access to serum proteins to enable serum bactericidal activity (MacLennan et al., 2010).

1.3.3 Age

Age is another significant risk factor for enhanced susceptibility to iNTS infection. Infants, young children, and the elderly are at-risk populations for iNTS infection and disease (Galanakis et al., 2007; Wen et al., 2017; Parisi et al., 2019; Mandomando et al., 2009). Over 30,000 child deaths have been associated with iNTS, and in 2017, 20,000 of these cases were of these cases were reported in sub-Saharan Africa (Das et al., 2022). Various studies have reported that risk factors associated with iNTS included young age, malnutrition, and concomitant malaria infections (Crump et al., 2015; Mandomando et al., 2009). Malaria has been shown to induce immune suppression in patients, where blood stages of the parasite induce an impairment in dendritic cells (DCs) leading to suboptimal T lymphocyte activation (Millington et al., 2006). Maternal Ab against NTS bacteria have been previously demonstrated as protective (de Alwis et al., 2019), and so infants that do not breastfeed or whose maternal Ab have waned are thus susceptible to iNTS disease.

The elderly population is also at increasing risk of opportunistic infections. Age-related thymic involution (the process in which the thymus shrinks in size accompanied by a loss of thymic epithelial cells and reduced thymic hormones), results in dysregulated T lymphocyte function, an increase in the number of immature T lymphocytes, and decreased thymopoeisis (Taub and Longo, 2005; Saltzman and Peterson, 1987). As noted above, T cell responses are critical in controlling NTS infections and preventing the progression to invasive infection and disease. Therefore, increased NTS-related

case-fatality rates seen in elderly people (Stanaway et al., 2019) may be a result of age-related immunological deterioration.

1.4 Antibiotic treatment and AMR

Salmonella infections (both typhoidal and non-typhoidal) are treated with a range of antimicrobials. These include aminopenicillins, tetracyclines, aminoglycosides, chloramphenicol, cephalosporins, and sulphonamides.

Ampicillin is a β-lactam antibiotic in the aminopenicillin class that inhibits cell wall synthesis of bacteria by binding to penicillin-binding proteins (PBPs) and interrupting their function (Peechakara et al., 2022). Resistance to ampicillin is mediated by several mechanisms; reduced access to PBPs, reduced binding affinity for PBPs due to mutations, and enzymatic degradation by β-lactamases (Kaushik et al., 2014). In cases of typhoid fever, indiscriminate use of ampicillins led to development of multidrug resistant (MDR) *Salmonella* Typhi strains, where ampicillin resistant was encoded by a plasmid in the H1 incompatibility group (Kaushik et al., 2014).

Chloramphenicol is a bacteriostatic antibiotic whose mechanism of action inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit (Abdollahi and Mostafalou, 2014). Enzymatic inactivation of chloramphenicol by acetyltransferases can confer resistance, in addition to target site mutations, decrease permeability of the bacterial cell wall, and expression of chloramphenicol efflux pumps (Fernández et al., 2012).

Tetracyclines (including glycylcyclines) are a bacteriostatic class of antibiotics that also inhibit protein synthesis, but instead bind the 30S ribosomal subunit to specifically inhibit tRNA binding (Speer et al., 1992; Schnappinger and Hillen, 1996). They are a broad-spectrum antibiotic and a large class containing a range of naturally occurring,

semi-synthetic and synthetic members (Shutter and Akhondi, 2022). Several genes in *Salmonella* have been defined as conferring resistance to tetracycline. The *tet* genes have been linked to efflux, ribosomal protection proteins (RPPs) or enzymatic modification of tetracycline to render it inactive (Hedayatianfard et al., 2014). As many as 30 distinct tetracycline-specific efflux pumps have been described in various bacterial species (Grossman, 2016).

Fluoroquinolones are broad-spectrum antibiotics. Systemic fluoroquinolones that are available for clinical use include ciprofloxacin, norfloxacin and moxifloxacin (Baggio and Ananda-Rajah, 2021). Fluoroquinolones act to inhibit bacterial topoisomerases II & IV, inhibiting DNA replication and causing bacterial cell death (Fluoroquinolones, 2020). Resistance to fluoroquinolones occur when mutations in the genes encoding these enzymes occur. For example, resistance to ciprofloxacin was associated with point mutations in various DNA-synthesis related genes, with the most-probable mutation associated with resistance being a S83F single nucleotide polymorphism (SNP) in the *gyrA* which codes for a DNA gyrase (Kakatkar et al., 2021).

Cephalosporins, like ampicillin, are β-lactam antimicrobials that were initially derived from *Cephalosporium sp.* They are large, bactericidal antibiotics that also bind PBPs to inhibit cell wall synthesis (Bui and Preuss, 2022) and show activity against both Gram-negative and Gram-positive bacteria (Stewart and Bodey, 1976). As with ampicillin, resistance to cephalosporins arise from β-lactamase expression. Additional resistance mechanisms include increased impermeability of the outer membrane in Gram-negative bacteria, and target modification of PBPs by trans-, carboxy-, and endopeptidases (Livermore, 1987).

Streptomycin, an aminoglycoside, is also used in treatment of *Salmonella* infections. Other aminoglycosides include kanamycin, neomycin, framycelin, paraomomycin and

tobramycin (Ball et al., 1975). Streptomycin is bactericidal, and serves to inhibit protein synthesis, by irreversibly binding the 16S RNA in the 30S subunit (Sharma et al., 2007). However, streptomycin is only active against aerobic bacteria due to its dependency on an electron transport system to cross the hydrophobic bacterial membrane (Waters and Tadi, 2022). Enzymatic inactivation is a key mechanism of resistance to streptomycin (Spagnolo et al., 2016; Pezzella et al., 2004) conferred by streptomycin-resistance genes *strA* and *strB* (Spagnolo et al., 2016; Pezzella et al., 2004). These genes encode for phosphotransferases, but modification to streptomycin can also be achieved through acetyl- and nucleotidyltransferases (Shaw et al., 1993). Resistance to streptomycin can also arise from decreases membrane permeability and ribosomal modification (Shaw et al., 1993).

Sulphonamides belong to a broad-spectrum synthetic antimicrobial class (Ovung and Bhattacharyya, 2021). Their names derive from their $-SO_2NH_2$ or $-SO_2NH$ functional groups. Sulphonamides show effective activity against Gram-negative bacteria, and some Gram-positives (White and Cooper, 2005). Sulphonamides are structural analogues and competitive inhibitors of *p*-aminobenzoic acid, which is a key component in the synthesis of folic acid (Ovung and Bhattacharyya, 2021). The folate pathway is essential for DNA synthesis, and so sulphonamides act to inhibit DNA synthesis and arrest cell growth in bacteria. Mutations of the target enzyme has been showed to bestow resistance to many distinct bacterial species, even if at the cost of enzyme efficiency (Sköld, 2000).

Resistance to one or multiple of these antibiotics have been reported frequently in previous years. Resistance to tetracyline, chloramphenicol and ampicillin has been reported as early as 1962 (Vatopoulos et al., 1994; Yurack, 1964; Voogd et al., 1968). Resistance patterns to both streptomycin and sulphonamides were reported as early

as 1968 (Anderson, 1968). Between 2004-2014, *Salmonella* isolates in Korea were investigated and showed that of the 22 serotypes, 40.5% were resistant to ampicillin and 31.6% to tetracycline (Yoon et al., 2017). This study also showed that STm species were the most likely to present with multidrug resistance patterns. Wei *et al.* showed that 42% of STm isolates had reduced susceptibility to ceftriaxone, a 3rd-generation cephalosporin (Wei et al., 2019).

Reported case fatality ratio (CFR) of iNTS infections remain high even where treatment is available. iNTS in Africans reported an overall CFR of 20.6% (Uche et al., 2017). Others have reported that in children and those with concurrent HIV infections, mortality rates vary between 10-30% (Kariuki et al., 2015). Most importantly, microbiologically confirmed iNTS infections treated with appropriate antibiotics have CFRs of 10-47% in African adults and children (Gordon et al., 2002; Mandomando et al., 2009). This highlights a need for alternative treatments or preventative measures such as vaccines against iNTS infection and disease.

1.5 Vaccines against *Salmonella*

Currently, only three vaccines are available for use in humans against *Salmonella* infections, but these are limited to *Salmonella* Typhi. These include the live-attenuated Ty21a, Vi capsular polysaccharide (ViCPS), and Vi-conjugate vaccines (Perera et al., 2021). In the past a heat-phenol inactivated whole cell vaccine against typhoid fever was also available, however its use is no longer recommended due to high reactogenicity (Levine et al., 1989; Engels et al., 1998). The live-attenuated Ty21a vaccine has proven useful with reported efficacy of ~60% for the first two years post-immunisation (Black et al., 1990), however this vaccine has limited thermostability and requires cold-chain distribution (Perera et al., 2021). ViCPS vaccines consist of

purified Vi capsular polysaccharide. ViCPS vaccines demonstrate a protective efficacy of approximately 70%, depending on dosage (Yang et al., 2001), and that seropositive status can be increased upon booster immunisation 3 years after the first dose had been received (Zhou et al., 2007). However, ViCPS vaccines have limited immunogenicity in infants due to their TI-independent nature, and so are only recommended for children >2 years old (Perera et al., 2021; MacLennan et al., 2014). Vi-conjugate vaccines are synthesised by conjugation of the ViCPS to a carrier protein, such as tetanus or diphtheria toxin (Thiem et al., 2011). Conjugate vaccines elicit robust protective responses, showing robust anti-Vi Ig responses with ~90% efficacy in children aged 2-5 years and limited adverse events (Lin et al., 2001; Thiem et al., 2011; Crump and Oo, 2021).

Whilst multiple vaccines exist against Typhoid fever, no vaccine is licensed for human use against NTS, though many are under study. As mentioned by Perera *et al.*, though humoral responses are sufficient to clear typhoid infections, a combination of both cellular and humoral responses are required for proper clearance of iNTS infections (Perera et al., 2021). Potential vaccine candidates for NTS species include live attenuated, glycoconjugates, subunit protein, and multiple antigens presenting systems (MAPS) such as outer membrane vesicles (OMVs) (Baliban et al., 2020; Perera et al., 2021).

1.5.1 Live-attenuated vaccines

Live attenuated vaccines provide ideal immunostimulatory profiles for infection, replicating almost exactly the responses necessary to clear real infections. Oral immunisation with the Ty21a *Salmonella* Typhi vaccine showed cross-protective mucosal antibody production to NTS strains (Kantele et al., 2012). Preclinical analysis

of live attenuated NTS vaccines demonstrated antibody induction in most healthy volunteers – however bacterial shedding persisted until almost 2 weeks post-immunisation (Angelakopoulos and Hohmann, 2000; Hindle et al., 2002). Advantages of live-attenuated vaccines lie in their relatively low production cost, however finding a balance between appropriate immunogenicity and attenuation can prove difficult.

Another form of live-attenuated vaccines called recombinant attenuated *Salmonella* vaccines (RASV), have been explored in recent years. These systems utilise attenuated *Salmonella* strains to deliver antigens of other bacterial species. Phenotypically, these RASV strains are wildtype at the point of immunisation but become attenuated after colonisation of the host-tissues by regulation of virulence factors (Li et al., 2009). These vaccines have shown promise by not only inducing highly robust immune responses to the recombinant antigens of other bacteria, but high Ig titres against both *Salmonella* LPS and O-Ag (Wang et al., 2010, 2013).

1.5.2 Glycoconjugate vaccines

Purified polysaccharide vaccines can elicit poor immunogenicity due to their T-independent nature. However, as shown with Vi-conjugate vaccines, conjugation to an immunogenic protein carrier can bolster immune responses to the polysaccharide components. Further, it has been reported that conjugation of core O-polysaccharide to *Salmonella*-derived protein carriers such as flagellin (FliC) can elicit robust $CD4^+$ T cell help and dramatically improve polysaccharide immunogenicity (Baliban et al., 2020; Perera et al., 2021). However, FliC may not represent an ideal carrier protein due to their high variability between strains in addition to their phase-variable expression (Perera et al., 2021). Others have revealed that conjugation of O-Ag 4 and 12 (O:4 and O:12, respectively) to bovine serum albumin (BSA) can induce O:4-

specific antibody when administered with Freund's adjuvant (Svenson and Lindberg, 1981). The same group had previously demonstrated that conjugation of O-Ag to porins improves immunogenicity in mice compared to both purified polysaccharide and O-Ag conjugated to diphtheria toxin (Svenson et al., 1979).

1.5.3 Subunit vaccines

Purified protein subunit vaccines are attractive methods of generating protective immune responses and have a proven track record with several infectious diseases such as meningococcal and pneumococcal infections. Highly conserved protein epitopes can be identified by bioinformatical analysis and reverse vaccinology techniques (Perera et al., 2021), which would then confer a cross-protective immunity against multiple strains of bacterial species. OmpD, a highly conserved porin found on the outer membrane of *Salmonella* Typhimurium, has been identified as a key protein that elicits strong antibody responses by T-independent B1b cell-dependent mechanisms (Gil-Cruz et al., 2009). However, it has been shown that not all antibody isotypes induced to OmpD confer equivalent protection, and that this correlates with the antibody's ability to access OmpD in the presence of densely packed LPS molecules (Domínguez-Medina et al., 2020). Indeed, interaction of these porins with O-Ag on bacteria maximise the antigen diversity within individual species – a diversity which cannot be achieved by immunisation with singular subunit vaccines.

1.5.4 Outer membrane vesicle vaccines

Outer membrane vesicles (OMVs) are produced when the bacterial cell membrane 'blebs' to produce small spherical structures. They are non-replicating and theoretically display a similar antigen density and pathogen-associated molecular patterns (PAMPs) as whole cell bacteria (Baliban et al., 2020). Therefore, they would

produce similar immunostimulatory patterns required for clearance of real infections. The GSK Vaccines for Global Health (GVGH) have produced a low-cost, scalable, GMP-quality method of producing STm OMVs, known as GMMA (Generalised Modules for Membrane Antigens). The method exploits the hyperblebbing *ΔtolR Salmonella* Typhimurium mutant (Meloni et al., 2015). Subcutaneous immunisation of mice with STmGMMA have been shown to produce robust humoral responses to LPS, O-Ag, and porins, and these responses were associated with enhanced protection in the spleens and livers in animals that were systemically challenged with STm (Schager et al., 2018; Fiorino et al., 2021; Zhang et al., 2017). Further, subcutaneous delivery of OMVs produced a balanced Th1/Th2 immune response (Baliban et al., 2020), which has been previously noted as essential for proper STm clearance (Perera et al., 2021).

Others have explored the potential of GMMA-CPS (capsular polysaccharide) vaccines against NTS infections. Capsular O-Ag in NTS had been found to be comprised of multiple surface polysaccharide molecules, including O-Ag capsule, colanic acid and LPS (Sokaribo et al., 2021). Colanic acid consists of repeating units of glucose, galactose, and fucose, and is produced at temperatures <30ºC for survival in adverse environmental conditions (Hanna et al., 2003; Steenackers et al., 2012). This study used random mutagenesis of STm aimed at increasing O-Ag capsule production to be purified and used in vaccine development and produced a hyper-colanic acid producing mutant. Where immunisation with purified colanic acid did not induce protective immune responses in mice, immunisation with OMVs purified from the colanic acid over-producing STm reduced bacterial colonisation in mice, of which $LPS_{O\text{-}Ag}$ was a key antigen targeted (Sokaribo et al., 2021). OMVs therefore provided sufficient protective immunity against STm.

1.6 Murine model of iNTS infection

Mouse models to study invasive *Salmonella* infections are widely used. Studies have demonstrated that prior treatment with antibiotics such as streptomycin induce symptoms of enterocolitis in mice similar to NTS in immunocompetent humans (Bohnhoff et al., 1954; Bohnhoff and Miller, 1962). In other cases, STm infections in mice produce typhoid-like fever symptoms and systemic illness characteristic of iNTS in humans (Santos et al., 2001). The clinical presentation of STm infection is largely dependent on the route of infection, the susceptibility of the mouse strain, and the dose and virulence of the bacteria.

C57BL/6 and Balb/c mice are mouse strains hypersusceptible to *Salmonella* intracellular infection due to their deficiency in intracellular divalent cation transporter, Nramp1 (Higginson et al., 2016; Bellamy, 1999). Nramp1 is thought to impact infection of intracellular bacteria by sequestering iron and stimulating the inflammatory response (Higginson et al., 2016). The susceptibility of these mice makes them ideal models for studying acute invasive infections.

Salmonella infection can be administered by either intraperitoneal (i.p.), intravenous (i.v.), or subcutaneous (s.c.) routes (Simon et al., 2011). I.p. infection allows for superior systemic dosing of NTS bacteria (Dominguez Medina, 2017), which is more relevant in the context of studying invasive disease.

Use of attenuated laboratory strains of STm such as *aroA*$^{-}$ strain SL3261 allows for study of systemic disease in both acute and chronic infection. AroA is required for synthesis of aromatic amino acids that are not available in the host, making mutants of this protein attenuated but still able to colonise mice (Brown et al., 1987; Hoiseth and Stocker, 1981). Large doses (3×10^{6}) of SL3261 administered i.p. to otherwise *Salmonella*-susceptible mouse strain Balb/c is not lethal (Hoiseth and Stocker, 1981).

Further, humoral responses elicited by attenuated strain SL3261 allowed for robust protection against virulent strains in susceptible mice (Hormaeche et al., 1990).

1.7 The innate immune response

Upon invasion by a Gram-negative bacterium, the first line of defence is the innate immune system. These responses are independent of B and T lymphocytes and occur rapidly. Innate immunity can be categorised further into anatomical barriers (intact skin, low stomach pH, lysozyme in tears and saliva, mucous and mechanical flushing of cilia), humoral barriers (complement, mannose-binding lectins (MBLs), antimicrobial peptides (LPS-binding protein, C-reactive protein, cytokines, and chemokines), and cellular barriers (granulocytes, myeloid cells, NK cells etc.) (Turvey and Broide, 2010; Marshall et al., 2018). The innate immune response is immediate, and responses are the same with each exposure (i.e., no immunological memory). Receptors include Toll- and Nod-like receptors (TLRs and NLRs, respectively). Failure in any of these first-line defences results in a greatly increased susceptibility to infection.

1.7.1 Innate immune cells

Innate immune cells are rapidly recruited to the site of infection through production of cytokines and chemokines (Marshall et al., 2018). Cytokines are small, secreted proteins released by cells that induce immune responses locally and the migration of additional immune cells to the site of infection (Zhang and An, 2007; Ramesh et al., 2013; Marshall et al., 2018).

The cells that are classed as innate immune cells can be divided into subgroups, including phagocytes, granulocytes, natural killer (NK) cells, and innate lymphoid cells (Marshall et al., 2018). Phagocytes are cells that engulf foreign microbes and kill

through multiple bactericidal pathways and are largely comprised of two main cell types: neutrophils and macrophages (Marshall et al., 2018).

Infection of the GI epithelial layer results in upregulation and secretion of proinflammatory cytokines to the site of infection (Hughes and Galán, 2002). This release of cytokines leads to the recruitment of macrophages and PMNCs (O'Brien et al., 1979), which control bacteria through their phagocytic and antimicrobial mechanisms.

1.7.1.1 Macrophages

Macrophage ontogeny is highly variable and complex. Circulating blood monocytes will enter tissues during inflammation and differentiate into macrophages or dendritic cells when stimulated by growth factors and pro-inflammatory cytokines (Shi and Pamer, 2011). Tissue-resident macrophages develop from embryonic precursors during embryogenesis; however, certain tissue-resident macrophages populations may be replenished by blood monocytes in tissue homeostasis (Ginhoux and Guilliams, 2016; Ginhoux and Jung, 2014).

Macrophage populations are heterogeneous, and their phenotype is largely affected by their surrounding environment leading to specific functions depending on the tissue of residence (Shapouri-Moghaddam et al., 2018). For example, Kupffer cells are liver-resident macrophages and are first and foremost involved in controlling infections, yet they are also able to enter a tolerogenic state and prevent excessive immune activation against innocuous antigens absorbed from the gastrointestinal system (Nguyen-Lefebvre and Horuzsko, 2015). Kupffer cells also aid in tissue homeostasis and aid in clearance of damaged or dead cells by their phagocytic function (Nguyen-Lefebvre and Horuzsko, 2015). The spleen possesses multiple subsets of

macrophages, including red pulp, marginal zone, and marginal metallophilic macrophages, all of which perform specialised functions (Borges Da Silva et al., 2015). Red pulp macrophages, as the name suggests, reside in the splenic red pulp and have proven to play an important role in the control of bloodborne infections (Kirby et al., 2009; Schnitzer et al., 1972). Marginal zone (MZ) macrophages are a smaller macrophage subset that assist in clearance of apoptotic material from circulation, therefore regulating autoimmunity (McGaha et al., 2011). Both MZ macrophages and marginal metallophilic macrophages act as scavenger cells, engulfing pathogens and other molecules and developing pro- or anti-inflammatory phenotypes depending on the nature of the interaction (Borges Da Silva et al., 2015). Marginal metallophilic macrophages also are essential for activation of cytotoxic T cell responses through presentation of blood-borne antigens to dendritic cells (Backer et al., 2010). As phagocytes, macrophages are also recognised as effective antigen presenting cells (APCs), which help activate and bolster the adaptive immune response (Martinez-Pomares and Gordon, 2007).

Macrophages are a key responder to *Salmonella* infections due to their extensive repertoire of receptors, pro-inflammatory cytokines, in addition to phagocytic ability. Release of proinflammatory cytokines such as TNFα, IFNγ, IL-1α, IL-1β, IL-12, IL-15, and IL-18 and antimicrobial enzymes such as iNOS by resident and recruited immune cells have also shown to have protective roles in controlling *Salmonella* infections (Eckmann and Kagnoff, 2001; Hughes and Galán, 2002; Rydström and Wick, 2007; Pham and McSorley, 2015). The ability of murine macrophages to produce extracellular traps has also been demonstrated (Mónaco et al., 2021), adding to the macrophage's protective arsenal against *Salmonella* infections. Adversely, macrophages provide an intracellular survival niche for *Salmonella* infections due to

the bacteria's ability to subvert phago-lysosomal fusion (Gogoi et al., 2019), making the role of a macrophage a double-edge sword in *Salmonella* infections.

1.7.1.2 Neutrophils

Neutrophils derive from myeloid progenitors and can be classed as both phagocytes and granulocytes by their dual ability to engulf microbes and release granules during infections or allergic reactions (Eberl and Davey, 2023; National Cancer Institute, 2023). Neutrophils are the most abundant white blood cell in both humans and mice (Eberl and Davey, 2023). They circulate the body and are one of the first recruited immune cell to a site of tissue damage or infection (Fine et al., 2020). During acute inflammation, such as at the initial stages of infection, neutrophils migrate through the blood and extravasate into the tissues through the interstitial space via chemotaxis mechanisms (Parent and Devreotes, 1999; Lehmann et al., 2008). Neutrophils populations are suggested to be important in controlling initial stages of *Salmonella* infection in tissues, however, were not essential for bacterial clearance due to the bacteria's ability to infiltrate protective intracellular niches (Cheminay et al., 2004).

Receptors allow neutrophils to detect chemical gradients of cytokines such as interleukin-8 (IL-8), interferon gamma (IFNγ), and complement fragments C3a and C5a (Kolaczkowska and Kubes, 2013; Futosi et al., 2013). Cytokines secreted by tissue resident immune cells recruit neutrophils to the site of infection, leading to neutrophil swarming and a pro-inflammatory positive feedback loop (Uderhardt et al., 2021). Neutrophils directly phagocytose microbes (Silva and Correia-Neves, 2012), destroying them using antimicrobial mechanisms such as secretion of reactive oxygen/nitrogen species (ROS/RNS) or lytic enzymes into the phagosomal compartment (Zeng et al., 2019). A specific example of this is the use of neutrophil elastase, a serine protease which is effective against *Enterobacteriaceae* species

such as *Salmonella* (Weinrauch et al., 2002). Neutrophil elastases have also been shown to have high affinity for DNA, allowing it to localise to neutrophil extracellular traps (NETs) and improve bactericidal activities (Thomas et al., 2014). NETs are sticky webs of DNA and chromatin, able to trap and immobilise *Salmonella* bacteria and circumvent infection (Brinkmann et al., 2004).

Alternatively, neutrophils can also undergo the process of degranulation, in which they release granules (Eichelberger and Goldman, 2020). Granules possess innate antimicrobial activity, for example myeloperoxidase (MPO) is abundantly expressed in neutrophils and is cytotoxic, enabling killing of pathogens (Aratani, 2018).

1.7.2 The complement system

The complement system consists of ~50 plasma phase proteins and are largely important for initial cell-free mediated protection and clearance of foreign pathogens (Sarma and Ward, 2011). The complement system can be grouped into three distinct pathways depending on their mode of activation and which specific proteins are involved (Kumar, 2016; Sarma and Ward, 2011; Kerr, 1981).

The complement system is discussed in detail in chapter 3.

1.8 The adaptive immune response

Adaptive immune responses have been described as the basis for effective immunisation against foreign diseases (Marshall et al., 2018). Adaptive immunity functions to recognise specific foreign antigen and distinguish them from self-antigens, and to generate pathogen-specific responses. This arm of the immune system involves B and T cells, and their collaboration to induce immunity specifically tailored

to the infection. This arm of immunity also induces immunological memory, where subsequent adaptive responses are immediate.

1.8.1 The bridge between innate and adaptive responses

The interface between the innate and adaptive immune responses is highly interactive. Communication via specific cells bridges the gap between the two branches, inducing highly targeted adaptive responses whilst innate immunity tackles the acute stages infection. Antigen presenting cells (APCs) are a heterogeneous group of cells that mediate cell-driven immunity by processing and presenting foreign antigen to lymphocytes (Mann and Li, 2014). Professional APCs include dendritic cells (DCs), macrophages, and B cells.

APCs express major histocompatibility complex (MHC) proteins on their surface. MHC class I (MHCI) are found on all nucleated cells, and MHC class II (MHCII) are found on professional APCs (Marshall et al., 2018). APC activation via pattern-recognition receptors (PRRs) upregulates MHC expression and migration to secondary lymphoid organs (Swain et al., 2012). MHCI present endogenous foreign antigen and are a signal of intracellular infection (Hewitt, 2003), and are recognised by $CD8^+$ T cells, initiating differentiation into cytotoxic effectors (Schmidt and Varga, 2018). MHCII present exogenous antigen that has been processed and presented by APCs and presented to $CD4^+$ T cells in lymphoid tissues resulting in activation into a T helper (Th) cell (Marshall et al., 2018; Alberts et al., 2002).

DCs initiate adaptive immune responses to *Salmonella*. Stimulation with *Salmonella*-derived flagellin has been shown to increase expression of CD80, CD86 and CD40 in DCs (McSorley et al., 2002; Bueno et al., 2008) promoting T cell interactions and co-stimulation. *Salmonella* flagellin has also been shown to trigger CCL20 secretion from

epithelial cells, triggering DC chemotaxis and recruitment to the site of infection (Sierro et al., 2001), leading to adaptive response activation.

1.8.2 T cells

T cells derive from bone marrow lymphoid progenitor cells and migrate to the thymus for maturation and selection (Kumar et al., 2018). Mature naïve T cells circulate between blood and peripheral lymphoid tissues until they encounter their cognate antigen (Charles A Janeway et al., 2001). The T cell receptor (TCR) cannot recognise antigen alone, and instead it must be presented by professional APCs on their surface MHC molecules (Tai et al., 2018; Shah et al., 2021). T cells coordinate various aspects of adaptive immunity depending on their subset. Mice deficient in T cells have a higher susceptibility to *Salmonella* infections (Sinha et al., 1997; Hess et al., 1996).

$CD4^+$ Th cells facilitate B cell activation, maturation, plasma cell (PC) differentiation and survival during immune responses, whilst also providing competitive selection of B cells within germinal centres (GCs) to promote evolution of high-affinity B cells receptors and antibodies (Crotty, 2015). The cytokines present in the microenvironment at the time of T cell stimulation influences the subset a Th will develop into. These include, but are not limited to, T-helper 1 (Th1), 2 (Th2), 17 (Th17) and follicular helper T cell (Tfh) (Luckheeram et al., 2012; Magee et al., 2012). Cytokines that influence Th subset developement can be found in **table 1**. APCs and other immune cells are the initial source of cytokines, but eventually positive feedback loops will be established by differentiating cells (Luckheeram et al., 2012).

Table 1.1: Th cell subsets and influential cytokines

Th Subset	Cytokines	References
Th1	IL-12, IFNγ	(Trinchieri et al., 2003)
Th2	IL-4, IL-2	(Cote-Sierra et al., 2004; Kaplan et al., 1996)
Th17	IL-6, IL-21, IL-23, TGF-β	(Tesmer et al., 2008; Luckheeram et al., 2012)

Th1 $CD4^+$ T cell subset has been implicated as a key player in control of *Salmonella* infections in an IFNγ-dependent manner (Pie et al., 1997). Th1 cells largely produce IFNγ, TNFα, and IL-2, and are involved in coordinating cellular immunity and clearance of intracellular pathogens and viral infections (Magee et al., 2012; Romagnani, 1999; Zeng et al., 2017). Administration of exogenous IFNγ to *Salmonella*-infected mice exhibited a bacteriostatic effect (Matsumura et al., 1990), and $IFN\gamma^{-/-}$ mice show a heightened susceptibility to infection (Hess et al., 1996). Additionally, immunisation with flagellin-based subunit vaccines against *Salmonella* have been shown to augment Th1 immunity noted by increased IFNγ-producing $CD4^+$ T cells which correlated with improved bacterial clearance (Bobat et al., 2011).

$CD8^+$ cytotoxic T cells destroy intracellularly infected cells presenting foreign antigen on their MHCI molecules (Schmidt and Varga, 2018). $CD8^+$ T cells on the other hand are not essential for acquired immunity to *Salmonella* but assist in the clearance of primary infections with attenuated bacteria (Lee et al., 2013).

1.8.3 B cells

B cells are derived from lymphoid progenitor cells in the bone marrow. Unlike T cells, B cells can directly recognise foreign antigen (Marshall et al., 2018) and act as APCs to induce T cell help. B cells are responsible for production and coordination of humoral responses to infection. Antibody produced by B cells can neutralise and

opsonise microbes whilst also stimulating complement activation and deposition (Forthal, 2014).

B cells undergo extensive functional rearrangement of the immunoglobulin loci during their development, having to pass through strict selection checkpoints to discard those that risk recognising self-antigen (Lebien and Tedder, 2008). The combined rearrangement of the V, D, and J gene segments in the H chain locus allow for generation of a wide array of B cell receptors (BCR) in the B cell repertoire, each with highly specific ability to recognise foreign antigen (Lebien and Tedder, 2008). When activated by their cognate antigen, the BCR initiates intracellular B cell signalling leading to changes in gene expression such as upregulation of costimulatory cell surface markers CD86 and CD80, adhesion, migration, pro-survival, and cell-cycle genes (Cyster and Allen, 2019). B cells will then undergo massive proliferation and differentiation, ultimately to produce antibody secreting cells (ASCs) or take up residence in the bone marrow as long-lived B memory cells, that are quickly engaged and reactivated upon secondary infections (Marshall et al., 2018). This response accumulates as more naïve B cells encounter antigen and are further stimulated by T cells, which provide necessary survival factors and signals to ensure persistent humoral responses (Arbore et al., 2016; Cyster and Allen, 2019). Studies have shown that in terms of bacterial clearance, B cells play a less significant role than T cells as co-stimulation via CD28 of $CD4^+$ T cells are sufficient for clearing avirulent *Salmonella* in naïve hosts (McSorley and Jenkins, 2000). B-cell deficient mice have impaired resistance to oral challenge with STm that cannot be rescued by passive transfer of immune serum, suggesting that failure to control the infection is due to reduced B cell-dependent T cell responses (Mastroeni et al., 2000). This theory is further supported by the finding that susceptibility of B cell deficient mice correlated with reduced $CD4^+$

T cell IFNγ production (Nanton et al., 2012). suggesting a role for B cells in clearance of primary infections that is antibody independent. On the other hand, antibody-dependent mechanisms of B cell responses have been shown vital for secondary exposure to virulent *Salmonella*, either by re-infection or vaccination (McSorley and Jenkins, 2000; Mittrücker et al., 2000; Nanton et al., 2012). Antigen-specific antibody production can occur via two distinct pathways: the extrafollicular response or the germinal centre reaction.

1.8.3.1 Extrafollicular response

EF responses are critical for early control of infections, as the B cells that enter the extrafollicular (EF) reaction are responsible for rapid production of antibody after antigen encounter. B cells that encounter antigen either in the blood or marginal zone of the spleen migrate to splenic T cell zones, whereas B cells activated in the afferent lymph nodes migrate to T cell-rich regions of the same node (MacLennan et al., 2003). Local growth of both the B and T cell is induced; after 2 cell cycles the B blasts develop into plasmablasts. Some of these plasmablasts migrate from the T cell zone to the EF regions and enter a site of EF growth, at which point there is no further interaction with T cells (MacLennan et al., 2003). In some cases, the development of EF responses can occur without any interaction with T cells, where certain antigens are capable of inducing EF responses in a T-independent (TI) manner (Elsner and Shlomchik, 2020; MacLennan et al., 2003). Plasmablasts (PBs) produced by the EF reaction are short-lived, lasting only around 3 days before undergoing apoptosis in a Bcl2-dependent mechanism (Smith et al., 1996). EF responses peak around 4-6 days post antigen encounter (Elsner and Shlomchik, 2020). The antibody produced by plasmablasts can be both switched and non-switched but is generally low affinity due to low-level hypermutation and affinity maturation (Marshall et al., 2011; MacLennan et al., 2003).

In mice, primary infection with *Salmonella* results in induction of massive T-dependent (TD) extrafollicular response that impedes extracellular spread of infection in the absence of GC reactions (Cunningham et al., 2007). Additionally, further elucidation of the EF mechanisms in *Salmonella* infection showed that the EF response, whilst producing low-affinity antibody, is capable of somatically mutated, *Salmonella* specific antibody in the absence of GCs (Di Niro et al., 2015), perhaps as a means of producing protective function to fast-replicating pathogens such as *Salmonella*.

1.8.3.2 Germinal centre reaction

B cells that enter the GC reaction are activated in a similar way to EF responses, but ultimately have a different trajectory. Antibody produced from GC B cells are class-switched and high-affinity due to somatic hypermutation (SMH) in the immunoglobulin variable region genes and rigorous competitive selection by $CD4^+$ T cells (Crotty, 2015; MacLennan, 2005). Germinal centre responses peak 10-14 days and may be seen as early as 4 days post-immunisation (Jacob et al., 1991; Liu et al., 1991).

Activated B cells and T cells will migrate from the primary proliferative focus into the follicles, where they further proliferate and initiate the GC reaction (Elsner and Shlomchik, 2020). Resulting centrocytes of the GC reaction differentiate into memory B cells or long-lived plasma cells and exit the GC reaction (MacLennan, 1994; Boyce et al., 2010). Plasma cell differentiation is driven by B-lymphocyte-induced maturation protein 1 (BLIMP-1) (Shaffer et al., 2002). The GC reaction is governed by transcriptional regulator B-cell lymphoma 6 (Bcl-6) expression (Lin et al., 2003). Bcl-6 suppresses BLIMP-1 expression, ensuring plasma cell differentiation only occurs after a selected B cell has exited the GC reaction (Crotty et al., 2010).

Interactions between B and T cells are essential for GC maintenance (Cyster and Allen, 2019), as T cells will provide necessary survival and costimulatory signals to B cells. CD40-CD40L interactions are critical for formation of long-lived plasma cells from the GC reaction. Expression of inducible T cell co-stimulator (ICOS) has been found crucial in GC reactions, where engagement of ICOS on Tfh cells with ICOSL expressed on B cells augments GC B cell entanglement and promotes more cell-cell interactions (Mintz and Cyster, 2020). ICOSL expression on B cells is upregulated upon CD40 signalling, which is achieved through engagement of the CD40 receptor with CD40L expressed on activated Tfh cells in a positive feedback loop (Liu et al., 2014). Stimulation of the BCR and CD40 activation by T cells promotes survival of GC B cells, and it has also been found that complement signalling through C3aR and C5aR drives B cell selection, survival, and ultimately high affinity antibody production (Siniscalco and Eisenbarth, 2021).

It has been recently shown that systemic *Salmonella* infections can disrupt existing and new GC formation *in vivo* (Marcial-Juárez et al., 2023; Biram et al., 2022). This active disruption of GCs by *Salmonella* infections provide rationale for the use of OMVs in the context of immunisation, as STm-derived GMMA has shown to induce extensive GC reactions as quickly as day 4 post-vaccination (Schager et al., 2018). These suggest an effective role for GCs and the resulting antibody in secondary responses, in addition to potential host-evasion mechanisms exhibited by *Salmonella* in an effort to establish infection.

1.9 Aims of thesis

To summarise, *Salmonella* is a prevalent agent of infections and disease, particularly in low to middle income countries (LMICs). Treatment of *Salmonella* infections is

becoming increasingly limited due to rising frequency of AMR in *Salmonella* species, in addition to low accessibility of required treatments in endemic regions. Vaccines represent an attractive and necessary route of controlling the spread of *Salmonella* infections, and especially in lowering the severity of both NTS and iNTS-induced disease. This necessity for vaccines against NTS infections requires a greater understanding of the mechanisms that mediate vaccine immunity to STm. Others have previously identified that antibody raised against STm-OMVs play a significant role in protective responses to secondary infections with STm. However, other aspects of the immune response (e.g., complement) have not been thoroughly studied. Additionally, consequences of OMV-immunisation on the host have not been thoroughly explored. Therefore, the aim of this thesis was to explore how immunisation and protective immunity to OMVs is regulated using mouse models of iNTS infections.

CHAPTER 2: MATERIALS AND METHODS

For a detailed list of all reagents, kits and buffers used throughout this study, please see Appendix A.

2.1 Mouse strains

For *in vivo* studies, male and female mice typically between the age of 6-12 weeks were used. Wild-type (WT) C57BL/6 mice were sourced from Charles River Laboratories (UK). Genetically modified mice (table 2.1) were obtained and bred at the Biomedical Services Unit (BMSU) at the University of Birmingham. All mice were maintained under specific pathogen-free (SPF) conditions and were maintained on a regular 12:12 light:dark lighting cycle with *ad libitum* access to food and water. Litter mates of the same sex were housed together, with a maximum caging density of five mice. Animal experiments were conducted with approval from UK Research Animal Ethics Committees under PPL number P06779746.

Table 2.1 – Mouse strains

Mouse strain	Background	Phenotype	Reference
C57BL/6J Wildtype		No IgG2a, only 2b and 2c. Genetically identical within the strain.	Charles River Strain #027
$C1q^{-/-}$	C57BL/6J	Deletion mutation of the complement component 1q gene	(Botto et al., 1998)
$C3^{-/-}$	C57BL/6J	Deletion mutation of the complement component 3 gene	(Wessels et al., 1995)
$C5^{-/-}$ B10.D2-Hc^0 $H2^d$ H2-$T18^c$/oSnJ	C57BL/6J	Natural mutation of the complement component 5 gene resulting in non-functional protein	(Trendelenburg et al., 2005)
$Tbet^{-/-}$	C57BL/6J	Homozygous deletion of *tbet* gene, no gene product	(Finotto et al., 2002)

		detected in isolated lymph node T cells.	
IgHγ1μ/γ1μ Igα+/+	C57BL/6J	Genetic mutation of the IgH locus to produce B cells that only express the IgG1 antibody subtype	(Waisman et al., 2007)
NU/J Nude	Balb/c	Spontaneous mutation of the *Foxn1^{nu}* gene resulting in abnormal hair growth and defective development of thymic epithelium resulting in lack of T cells.	(Flanagan, 1966)
CD1	Crl:CD1(ICR)	No IgG2c, only 2a and 2b. Resistant to bacterial infection. Outbreeding system to manage random antigenic drift.	Charles River Strain #022
B6.Cg-Rag2tm1.1Cgn/J Rag2 KO	C57BL/6J	Produce no mature B or T cells. Phenotype can be described as 'non-leaky' immune deficiency.	(Hao and Rajewsky, 2001)

2.2 Bacterial strains

For *in vivo* and *in vitro* experiments, *Salmonella* Typhimurium strains were used. Both invasive and attenuated strains were used throughout the study. For a detailed list of all strains used, see table 2.2.

2.2.1 Glycerol stocks

All bacterial culture work was carried out aseptically in MSC Class II hoods. Stocks of bacterial strains were created for long-term storage. Solid or liquid cultures of bacteria were streaked to single colonies on Luria-Bertani (LB) agar plates and grown for 12-18 hours at 37°C. Single colonies were picked and resuspended in 5 mL LB broth ± antibiotics and grown 12-18 hours at 37°C without agitation. Meanwhile, 50% glycerol solution was made by mixing equal volumes of sterile 100% glycerol and sterile water. Once the bacterial culture had grown, it was diluted 1:5 in pre-warmed sterile LB broth and grown until it reached an OD_{600} 1.0. Once this OD was reached, 500 mL of culture was added to 500 mL of 50% glycerol solution, making a final glycerol concentration of 25%. Glycerol cultures were then frozen and stored at -80°C.

2.2.2 Bacterial culture for infections

Salmonella Typhimurium was used for animal studies. For infections, bacteria were streaked down to single colonies on LB agar (Sigma-Aldrich). One colony was used to inoculate 5 mLs of LB broth and grown static overnight at 37°C. Culture was diluted 1 in 5 and sterile LB broth and grown shaking at 37°C until OD_{600} 1.0 was reached. 1 mL of OD_{600} 1.0 was centrifuged and washed with sterile phosphate buffered saline (PBS). The OD_{600} 1 cell suspension was then diluted to achieve a final dose of either

5×10^5 CFU/200 µL or 2.5×10^5 CFU/200 µL in sterile PBS. See figure legends for specific doses used. 200 uL of bacterial suspension was injected by intraperitoneal route. The dose of the infection was confirmed using the Miles and Misra (Miles et al., 1938) method of determining colony forming units in suspension (**figure 2.1**).

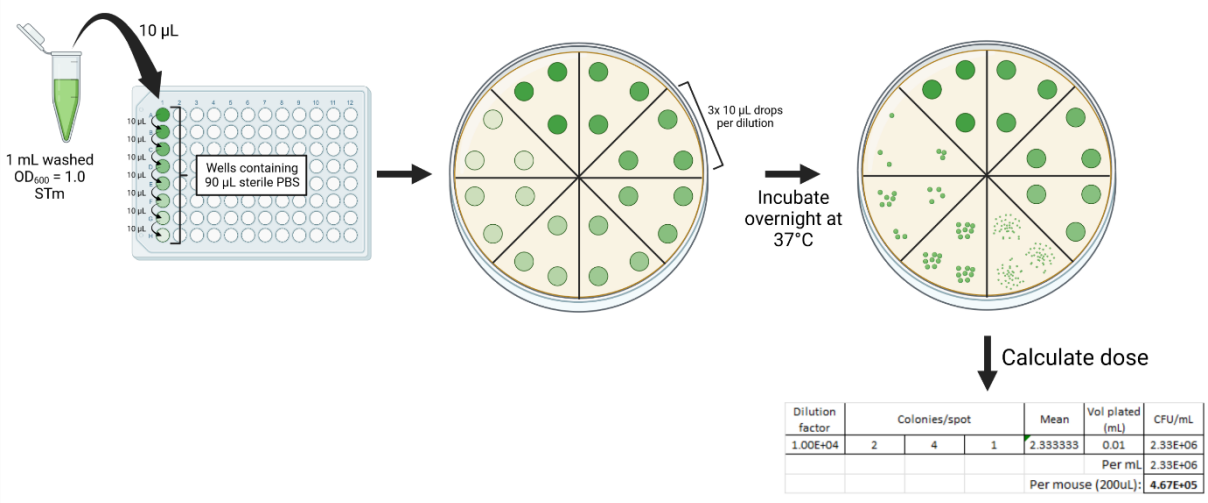

Figure 2.1 Miles & Misra method of calculating bacterial concentration in a solution. Figure created with BioRender.

Table 2.2 – Bacterial strains

Strain	Phenotype	Description	Reference
STm SL3261	*ΔaroA*	Deletion in *aroA* gene which hinders an aromatic pathway, attenuating growth of the bacteria	(Hoiseth and Stocker, 1981)
STm D23580	Virulent STm isolate	Isolate from Malawi/Kenya. Multiple drug resistant (MDR) ST313 with composite genetic element encoding MDR genes located on a virulence-associated plasmid	(Kingsley et al., 2009)
STm *ΔtolR*	*ΔtolR*	Hyper blebbing mutant used to produce STm outer membrane vesicles for vaccines with full LPS molecule (O-antigen of varying chain length)	Prof. Ian Henderson, (Schager et al., 2018)

STm *ΔtolR Δwzy*	*ΔtolR Δwzy*	Hyper blebbing mutant used to produce STm outer membrane vesicles for vaccines with a single O-antigen on its LPS molecile	Areej Alshayea, (Collins and Hackett, 1991)
STm *ΔtolR ΔwbaP*	*ΔtolR ΔwbaP*	Hyper blebbing mutant used to produce STm outer membrane vesicles for vaccines without O-antigen on its LPS molecule	Areej Alshayea, (Schager et al., 2018; Wang et al., 1996)
STm TH177	IR715 *rpoS*::Cm^r*phoN*::*viaB*	Salmonella Typhimurium strain expressing the Vi antigen	(Haneda et al., 2009)

2.3 Antigens

2.3.1 Antigens for *in vivo* immunisations

Outer membrane vesicles (OMVs). Three types of outer membrane vesicles (OMVs) were used for immunisations in mouse studies. These are the wild-type (WT) *ΔtolR* OMV, the *ΔwzyΔtolR* OMV, which only contains one O-antigen subunit on its LPS molecule, and the *ΔwbaPΔtolR,* which lacks any O-antigen subunits on its LPS molecule (**Figure 2.2**). *Salmonella* Typhimurium *ΔtolR*, a strain kindly provided by

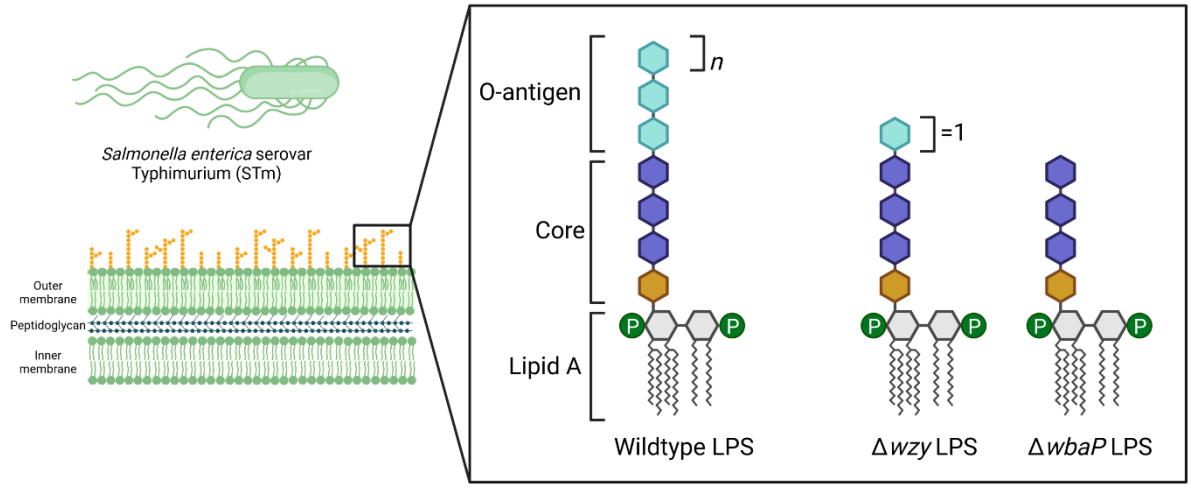

Figure 2.2 LPS structures of mutant *Salmonella* Typhimurium used for OMV generation. Figure created with BioRender.

Professor Ian Henderson, was used to produce OMVs. Bacteria were grown overnight at 37°C. The culture was used to inoculate 300 mL of sterile LB broth to an OD_{600} 0.05. The culture was then grown to an OD_{600} of 1.0 for WT *ΔtolR* OMVs, and OD_{600} of 1.5 for *ΔwzyΔtolR* and *ΔwbaPΔtolR* OMVs. The culture was spun down at 4000 g for 10 minutes at 4°C. The supernatant was collected and filtered from 0.22 μM filter bottle (Millipore), and pellet discarded. The filtrate was then centrifuged in an ultracentrifuge for 1.5 – 2 hours at 4°C at 18,600 g. Supernatant was discarded and pellet was resuspended in cold, sterile PBS. Suspension was then centrifuged again for 45-90 minutes under the same conditions. The supernatant was discarded, and pellet resuspended in a small volume (100-200 μL) of cold, sterile PBS. Suspension was filtered with a small 0.22 μM filter (Millipore) and stored at 4°C until use.

Soluble recombinant FliC (sFliC). The following was kindly performed by William Channel and Dr Maher Alwethaynani. Soluble recombinant FliC (sFliC) was generated and purified as previously described (Cunningham et al., 2004a). Briefly, FliC was amplified from *Salmonella* SL3261 and ligated into pET22b+ (Merck Chemicals, Nottingham, UK) to generate pET22b+ FliC Xho1 with a C-terminal His-tag, to produce an isopropyl β-D-thiogalactopyranoside- (IPTG) inducible beta-lactamase overexpression vector with ampicillin resistance. sFLiC-expressing *Escherichia coli* were grown overnight in LB media at 37°C, supplemented with ampicillin. The culture was used to inoculate fresh LB media at a 1:10 dilution an incubated for ~4 hours. IPTG (Promega, Southampton, UK) was added to the culture at mid-log phase (OD_{600} 0.6-0.8) to a final concentration of 1mM to induce sFliC expression and grown for a further ~2.5 hours. The culture was then centrifuged at 6000 g for 20 minutes at 4°C, supernatant discarded, and pelleted cells transferred to -20°C until protein purification step. To purify sFliC, pelleted cells were thawed and Bugbuster® (Merck, Nottingham,

UK) was added to the cells and left at room temperature for 20 minutes. Lysed cells were then centrifuged at 16000 g for 30 min 4°C. The supernatant was then incubated with Ni-NTA Sepharose Beads (Qiagen, Hilden, Germany) for 1 hour at room temperature. The flagellin was then passed through a disposable polypropylene column (Qiagen), twice, before purification by Ni-affinity chromatography. The column was washed three times with PBS and eluted using 100mM imidazole (Melford Laboratories) in 5 mL PBS. The eluted sFliC was then dialysed in PBS at 4°C overnight. After dialysis, sFliC was filtered-sterilised using 0.22 µM filter (Milipore). sFliC was flowed and purifies using an anti-FliC monoclonal antibody via affinity chromatography, with the assistance of Dr Margaret Goodall. The purified sFliC was again dialysed in PBS at 4°C overnight. SDS-PAGE was then used to visualise the expected molecular weight of ~56 kDa. Protein concentration was then determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific, MA, USA) and the sterile, purified sFliC was stored at -20°C until required.

Vi-polysaccharide/Vi-TT. In some experiments, Vi-polysaccharide and Vi-conjugate vaccines were used to look for antigen persistence (Chapter 4). Vaccine preparations were manufactured by GSK Vaccines for Global Health for Dr Siân Jossi (Jossi et al., 2023), and mice immunised as previously stated.

2.3.2 Antigens used *in vitro*

Antigens described here were used for Enzyme-linked Immunosorbent Assays (ELISAs). Total protein concentration of each antigen preparation was measured using Pierce™ BCA Protein Assay Kit, except in the case of LPS which was commercially available at a standard concentration of 1 mg/mL.

Bacterial strains. The SL3261 strain (**table 2.1**) was used as a coating antigen in ELISAs. Bacteria were grown as previously described in section 2.2.2 to an OD_{600} 1.0. 1 mL of OD_{600} 1.0 bacteria was centrifuged at 8,000 g for 5 minutes at 4°C. The pellet was then resuspended in PBS + 0.05% sodium azide and stored at 4°C for up to 6 months. A working stock of OMVs were made by diluting to 10 µg/mL.

OMVs. OMVs were prepared as described previously in section 2.3.1. OMVs were used for coating ELISA plates for detection of antigen-specific antibody. A working stock of OMVs were made by diluting to 2 µg/mL.

Porins (including purified OmpD). Porins were kindly provided by Prof Constantino Lopez-Macias at the Mexican Social Security Institute and were prepared as described (Gil-Cruz et al., 2009). Porins and OmpD were used in ELISAs at a concentration of 5 µg/mL

sFliC. FliC was purified as previously described by Mr William Channel or Dr Maher Alwethaynani. FliC was diluted to a working dilution of 5 µg/mL for coating of ELISA plates.

LPS. LPS was purchased from Enzo Life Sciences (Exeter, UK). LPS was diluted at a factor of 200 for a working concentration of 5 µg/mL, for coating ELISA plates.

2.4 Opsonisation of bacteria for infections

For opsonisation experiments, bacteria were opsonised prior to intraperitoneal injection in mice. First, sera from either naïve or OMV-immunised mice (previously confirmed to have OMV-specific antibody by ELISA) were pooled. The sera were then heat-inactivated at 56°C in a pre-heated water bath for 1 hour. Meanwhile, bacteria for infection were prepared as described in section 2.2.2 until the final dilution of bacteria was achieved. The washed bacteria were diluted to a final dose of 5×10^5 CFU/200

µL in sterile PBS + 10% heat-inactivated sera, to a final volume of 1 mL. The final dose was confirmed using the Miles and Misra method. The bacteria + sera were then incubated, rotating, at room temperature for 30 minutes. Opsonised bacteria were then injected into mice i.p.

2.5 Adoptive transfer of sera to mice

For adoptive transfer experiments, sera from either naïve or OMV-immune (previously identified by ELISA) were pooled. For groups that were to receive heat-inactivated sera, sera were incubated at 56°C in a pre-heated water bath for 1 hour. Sera that was to be used as a non-heat-inactivated comparison were incubated at room temperature (~25°C) for 1 hour. The sera were then injected into the mice i.p. 24 hours prior to infections. Mice were then infected as described in section 2.2.2.

2.6 Clodronate treatment

Clodronate-treatment was used to deplete macrophages from naïve or immunised mice as previously described (Hitchcock et al., 2015; Ikawa et al., 2005). In this system, liposomes are ingested by macrophages. Digestion of the liposomes leads to release of clodronate which accumulates within the macrophage. Apoptosis of macrophages is triggered at a specific intracellular concentration of clodronate. Specific macrophage subsets can be targeted depending on route of administration, for example intravenous administration can efficiently target Kuppfer cells in the liver and splenic red-pulp macrophages (Clodrosome® - Encapsula NanoSciences-Manufacturer of liposome based kits and formulations for research laboratories, n.d.; Hitchcock et al., 2015; Ikawa et al., 2005). At day 0, mice were immunised i.p. with either with 200 µL PBS or 0.5 µg Δ*tolR* OMVs. At day 12, mice were injected by intravenous injection with 200 µL of either empty liposomes or Clodrosomes®

purchased from Encapsula Nano Sciences (SKU# CLD-8909, Tennessee, USA) for 24 hours. Mice were then infected by i.p. injection as previously described in section 2.2.2 for 24 hours.

2.7 *In vivo* experiment endpoints

At the indicated endpoints, mice were anaesthetised and exsanguinated by cardiac puncture and Schedule 1 procedure, according to Home Office Regulation. Humane endpoints were considered when mice experienced more severe symptoms of disease; these include loss of >15% body weight within 24 hours of any procedure, loss of >20% body weight at any endpoint, lethargy, or piloerection.

2.8 Serum separation from blood

Blood harvested from mice by cardiac puncture were incubated at 37ºC for 1-3 hours to induce clotting. The blood was then centrifuged at 10,000 g for 7 minutes at 25ºC. Serum that had collected at the top of the blood was aliquoted and stored at -20ºC for future use. Sera was used for ELISA, and opsonisation or adoptive transfer *in vivo* experiments.

2.9 Bacterial culture from mouse tissues

Spleens and livers were weighed and segmented for bacterial culture. Segments for culture were mashed through a 0.2 µm cell strainer (Milipore) into ice cold, sterile PBS (Sigma-Aldrich). Cell suspensions were then serially diluted 10-fold up to a dilution of 1 in 10000, and a range of dilutions spread on LB agar (Sigma-Aldrich). Plates were incubated overnight at 37°C or over the weekend at room temperature. Colonies were counted and the colony-forming unit (CFU) per organ for each mouse was calculated using the following formula:

$$CFU = \frac{Organ\ mass\ (g)}{Culture\ mass\ (g)} \times \frac{Volume\ cultured\ (mL)}{Volume\ plated\ (mL)} \times Dilution \times No.Colonies$$

2.10 Gentamicin protection assay

Spleens and livers segments were prepared as for bacterial culture. To obtain both extra- and intracellular bacterial load data, homogenates were split into two Eppendorf tubes and centrifuged at 800 g for 10 minutes at 4°C. Supernatants were discarded and cells resuspended in RPMI ± 50 µg/mL gentamicin, and then incubated at 37°C for 1 hour. Cells were centrifuged again and washed twice with sterile PBS to remove all gentamicin. Washed cell suspensions were serially diluted and spread on LB agar. Plates were incubated overnight at 37°C and colonies counted. Cells treated with gentamicin indicated intracellular bacterial load, whereas those not treated with gentamicin indicated total bacterial content.

$$\%\ Extracellular = \frac{(log10(-Gn\ CFU) - log10(+Gn\ CFU))}{log10(-Gn\ CFU)} \times 100$$

$$\%\ Intracellular = \frac{log10(+Gn\ CFU)}{log10(-Gn\ CFU)} \times 100$$

2.11 Freezing mouse tissues

Segments of spleens and livers frozen via liquid nitrogen. Tissues that were kept on ice were partially dried on clean tissue. The tissue was then transferred onto a square of clean aluminium foil and lightly pressed down onto it. The foil piece was then picked up with forceps, and the edge of the foil dipped in liquid nitrogen. Once the tissue appeared to be completely frozen, they were submerged briefly into the liquid nitrogen multiple times with gradually increasing increments of time until the tissue did not produce bubbles when submerged. The foil was carefully folded around the tissue

using forceps and left in the liquid nitrogen until they could be stored at -80°C until needed.

2.12 Cryo-sectioning of frozen tissues

Frozen tissues were transferred on dry ice to a cryostat (Bright Instruments, Cambridge, UK) which was kept at ~-20°C. Tissues were mounted onto chucks using Tissue-Tek OCT compound (Sakura Finetek, California, USA). Tissues were then cut into 6 µm sections and collected on microslides for staining. Slides were stored at -20°C.

2.13 Immunohistochemistry

Slides were defrosted fully and submerged in Tris-buffered saline (TBS) pH 7.6 (Appendix 1). Slides were incubated with two purified anti-mouse primary antibodies for specific cell markers for up to 1 hour at room temperature in a humidity chamber. Slides were washed in TBS pH 7.6 and incubated with secondary antibody conjugated to either horseradish peroxidase (HRP) or biotin for up to 1 hour at room temperature. Slides were again washed and finally incubated a third time with Vectastain ABC Alkaline Phosphatase (AP) Kit (Vector laboratories) for 45 minutes at room temperature. Slides were developed using ,3′-Diaminobenzidine (Sigma) for 1-2 minutes and FastBlue Salts (Sigma) for 6-10 minutes. Slides were then washed, rinsed, and dried before mounting with Vectamount (Vector laboratories) and cover slides. A list of antibodies used for IHC can be found in **Table 2.3**. Images were taken using Zeiss AxioScan 7 and Zeiss Zen Blue version 3 software. TIFF images were exported and quantified using ImageJ version 1.54b.

2.14 Immunofluorescent staining

Slides were defrosted fully and submerged in PBS pH 7.6 + 0.05% Tween® 20 (Sigma-Aldrich, SKU# P1379, Missouri, USA) for 5 minutes. Sections on the slides were then kept hydrated throughout the rest of the protocol. Slides were incubated with up to four purified or fluorophore-conjugated primary anti-mouse antibodies for 1 hour in a humidity chamber, covered from the light. Slides were then washed in PBS pH 7.6 for 5 minutes. Slides then incubated with fluorophore-conjugated secondary antibodies as described above, for 45 minutes. Slides were washed for a final time and mounted with Prolong™ Gold Antifade Mountant (Thermo Fisher Scientific, Cat# P36930, Massachusetts, USA) and cover slides, being careful not to let the sections dry out. A list of antibodies used for IF can be found in **Table 2.4**. Scans were collected using the Zeiss Axio Scan 7 and Zeiss Zen Blue Software. TIFF images were exported and analysed using ImageJ version 1.54b.

2.15 Immunostaining quantification method

Quantification of stained tissues was achieved using ImageJ version 1.54b. TIFF files obtained in section 2.13 were imported into ImageJ. The DAB and FastBlue stains were separated using the ColourDeconvolution2 plugin. The images were processed to remove non-specific background staining, and the images converted to black and white images where the threshold to distinguish what was regarded as a positively stained cell was set between 195-215. Measurements of the image were then taken, including the area of the image (in pixels) and the mean grey area. The mean grey area was represented as both a direct value and a percentage of the area measured. For livers, 5 measurements at random were taken. For spleens, 5 measurements of red pulp, white pulp, and marginal zone areas were taken.

IHC Quantification method

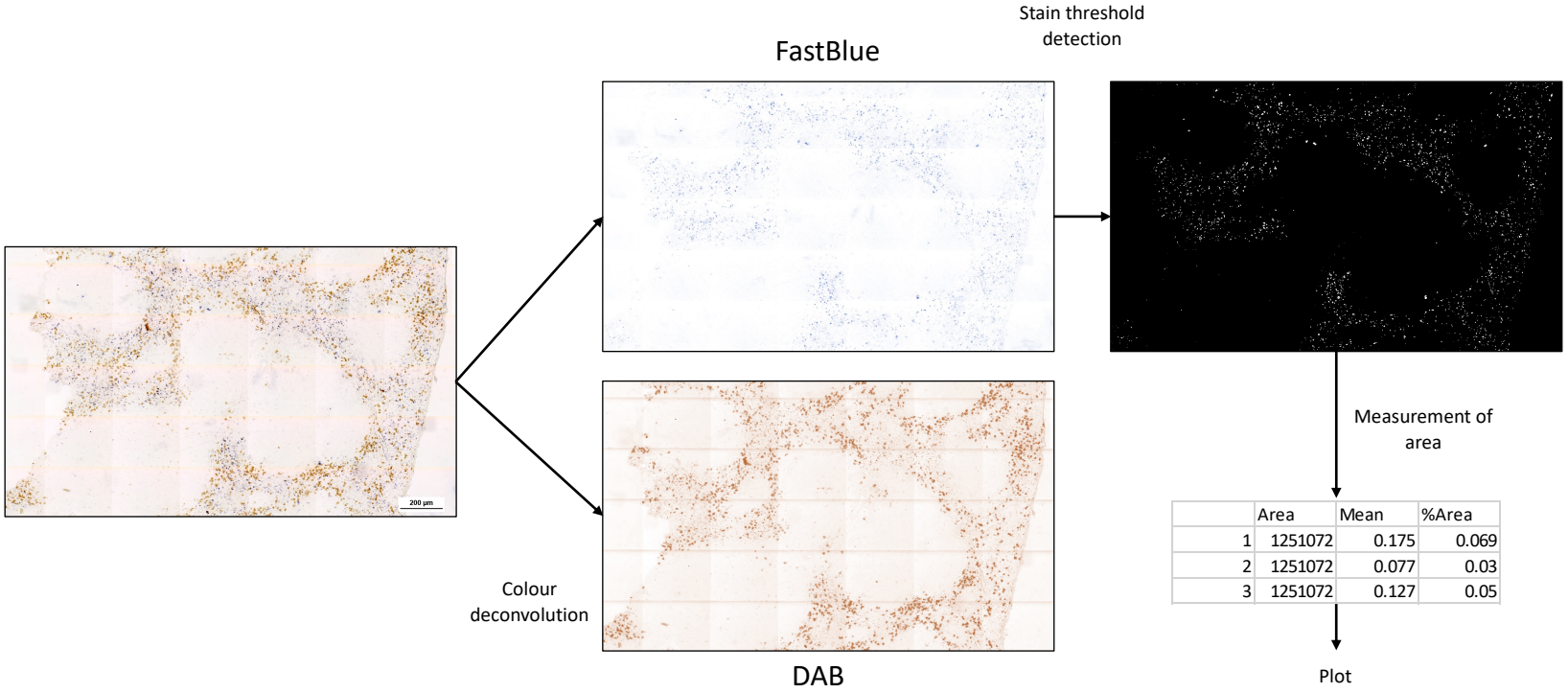

Figure 2.3 Illustrative representation of image quantification analysis.

2.16 Enzyme-linked immunosorbent assay (ELISA)

ELISAs were used to identify antigen-specific antibodies (Abs) post-immunisation and challenge. Ab specific was tested against whole STm bacteria, OMVs, LPS, porins or OmpD.

Flat-bottom 96-well NUNC plates (Thermo Fisher Scientific) were coated with 2 µg/mL, 5 µg/mL, or 10 µg/mL, with OMVs, proteins, or bacteria, respectively. These antigens had been diluted in 1X PBS pH 7.6 or carbonate coating buffer in the case of *Δwzy* and *ΔwbaP* OMVs. Plates were then incubated overnight at 4ºC. Plates were then washed with 1X PBS pH 7.6 and then blocked with 1X PBS + 1% Bovine Serum Albumin (BSA)(Sigma-Aldrich) for 1 hour at 37ºC. Plates were then washed with 1X PBS + 0.05% Tween® 20 Wash Buffer (Thermo Fisher Scientific). Sera were serially diluted 3-fold in dilution buffer (PBS + 1% BSA + 0.05% Tween® 20) at a starting dilution of 1:100 and incubated for 1 hour at 37ºC. Plates were then washed as previously mentioned. AP-conjugated goat anti-mouse secondary antibodies against IgM or IgG (Southern Biotech) were added and incubated for 1 hour at 37ºC. Plates were washed again before addition of colorimetric substrate SigmaFast *P*-nitrophenyl phosphate (pNPP) tablets (Sigma-Aldrich) dissolved in dH_2O. Plates were incubated at 37ºC for 1 hour and read on an Emax microplate reader (Molecular Devices, California, USA) at $OD_{\lambda 405}$ using SoftMax pro software version 5.4.5.

2.17 Fluorescence-activated cell sorting (FACS)

FACS was kindly performed by Dr. Edith Marcial-Juarez. Small segments of harvested spleens from mice were cut and weighed and kept in sterile, cold RPMI 1640 w/ Glutamine (Gibco). Segments were mashed gently through 0.2 µm cell strainers in up to 5 mL of RPMI + 5% FBS (Gibco) and 5mM EDTA (AppliChem GmbH, Darmstadt,

Germany) into 15 mL centrifuge tubes. The cell suspensions were then centrifuged at 300 g at 4ºC for 5 minutes. The supernatant was discarded, and the pellet resuspended in 1 mL of ACK lysing buffer (Gibco) for 1 minute to lyse red blood cells. 3 mL of RPMI media was added to stop the reaction, and cell suspensions were centrifuged as before. Cell pellet was resuspended in ~5 mL of RPMI media (depending on the size of the pellet), and cells counted using Fast-Read Plastic Counting Chambers (Kova International, California, USA) and Trypan Blue (Gibco) diluted at 1:10. Cell density was then calculated using the following equation:

$$Total\ cells\ =\ Cell\ count\ \times\ Dilution\ in\ trypan\ blue\ (1{:}10)$$
$$\times\ Square\ factor\ of\ hamocytometer\ \times\ Volume\ of\ sample$$

The volume of the cell suspensions was then adjusted for a final concentration of $1\ \times\ 10^7$ cells/mL. 2.5×10^6 cells were then harvested per well of a 96-well V-bottom plate (Thermo Fisher Scientific). Cells were pooled for unstained controls. The 96-well plate was then centrifuged at 1400 rpm at 4ºC for 5 minutes. Supernatants were discarded and cell pellets resuspended with 50 µL blocking solution (CD16/32, Cat #553142) diluted 1:150 in PBS pH 7.6 + 5mM EDTA + 5% FBS + 1% sodium azide FACS buffer (Appendix 1). Cells were incubated on ice for 15 minutes. The plate was centrifuged as before, and excess liquid decanted. 50 µL of extracellular Ab mix prepared in FACS buffer was added to each well and pellets resuspended. A list of antibodies used for FACS can be found in **table 2.5**. 50µL of FACS buffer was added to the unstained controls. Cells were incubated for 25 minutes on ice, covered from light. 180 µL of FACS buffer was added to each well before centrifugation as before. Supernatants were discarded and cell pellets resuspended in 50 µL ZombieViolet™ (Biolegend) or ZombieAqua™ prepared in PBS. Cells were incubated for 10 minutes on ice, covered from light. At this stage, if only extracellular staining was necessary,

cells were centrifuged as before and resuspended in 200 µL PBS + 1% Paraformaldehyde (PFA) and analysed the next day.

For intracellular staining, cells were centrifuged and resuspended in 100 µL FoxP3 Fixation/Permeabilization buffer (eBioscience, California, USA). Cells were then incubated for 90 minutes on ice, covered from light. 120 µL of 1X Permeabilization buffer (eBioscience) was added to each well before centrifugation as before. Supernatants were discarded and pellets resuspended in 50 µL of intracellular antibodies prepared in 1X permeabilization buffer. 50 µL of permeabilization buffer only was added to the unstained controls. Cells were incubated for 30 minutes on ice, covered from light. Finally, 180 µL of permeabilization buffer was added to each well before centrifugation as before. Supernatants were discarded and cells resuspended in PBS to be analysed the next day.

Single colour controls were made using UltraComp eBeads™ Plus Compensation Beads (Invitrogen). Samples were acquired using the BD LSRFortessa X-20 (BD Biosciences, New Jersey, USA) and FACSDiva software. Data was analysed using FlowJo version 10.

2.18 Statistical analysis

Statistical analysis of the data was performed using GraphPad version 9.0. Normality testing was performed using Shapiro-Wilk's test for CFU data or Kolmogorov-Smirnov for titre data. Non-parametric analysis was used where data was not normally distributed. Kruskal-Wallis with Dunn's multiple comparison test was used for more than 2 groups. For FACS data where normal distribution was assumed, one-way ANOVA with Bonferroni's correction for multiple comparison was used. For gentamicin-treatment data where normal distribution was assumed, two-way ANOVA

with Bonferroni's correction for multiple comparison was used. For CFU and quantification data in chapter 4, normal distribution of the data was assumed and unpaired t test was used for comparisons between 2 groups or one-way ANOVA for more than 2 groups. See figure legends for exact details of which statistical analysis was used.

Table 2.3 Antibodies used for IHC.

Reactivity	Host, isotype	Clone	Conjugate	Manufacturer	Cat #
CD3	Ham Arm IgG1, κ	145-2C11	**Purified**	eBioscience	MCA497GA
F4/80	Rat, IgG2b, κ	Cl:A3-1	**Purified**	Bio-rad	
F4/80	Rat, IgG2a, κ	BM8	**Purified**	eBioscience	14-4801-82
Salmonella	Rabbit	Polyclonal	**Purified**	Abcam	ab35156
Ly6G	Rat LEW, also known as Lewis IgG2a, κ	1A8	**Purified**	BD Biosciences	551459
IgD	Rat IgG2a, κ	11-26c.2a	**Purified**	BD Biosciences	553438
CD169	Rat, IgG2a, κ	3D6.112	**Purified**	Biolegend	142402
B220	Rat, IgG2a, κ	RA3-62B	**Purified**	Biolegend	103201
IgM	Rat, IgG2a, κ	RMM-1	**Purified**	Biolegend	406501
Rabbit Ig	Swine	Polyclonal	**Biotin**	Dako	E0353
Rat IgG (H+L)	Rabbit	Polyclonal	**HRP**	Southern Biotech	6180-05
Rat IgG (H+L)	Goat	Polyclonal	**HRP**	Southern Biotech	3030-05
Hamster Ig	Goat	Polyclonal	**Biotin**	eBioscience	13-4113-85
Avidin-Biotin complex			**Alkaline phosphatase**	Vector Laboratories	AK-5000

Table 2.4 Antibodies used for IF.

Reactivity	Host, isotype	Clone	Conjugate	Manufacturer	Cat #
CD3	Ham Arm IgG1, κ	145-2C11	**Purified**	eBioscience	MCA497GA
Salmonella	Rabbit	Polyclonal	**Purified**	Abcam	ab35156
Ly6G	Rat, IgG2a, κ	1A8	**Biotin**	Biolegend	127603
IgM	Goat, IgG	Polyclonal	**AMCA**	Jackson Immunoresearch	115-155-020
F4/80	Rat, IgG2a, κ	BM8	**FITC**	Invitrogen	11-4801-82

iNOS	Rat, IgG2a, κ	CXNFT	APC	eBioscience	
IgG1	Rat, IgG	M1-14D12	APC	Invitrogen	17-4015-82
IgA	Rat IgG1, κ	RMA-1	Biotin	Biolegend	407003
B220	Rat, IgG2a, κ	RA3-6B2	AF488	Biolegend	103228
Peanut Agglutinin (PNA)			Biotin	Vector Laboratories	B-1075-5
Rabbit Ig	Donkey, IgG	Polyclonal	Cy3	Jackson Immunoresearch	711-165-152
FITC	Rabbit	Polyclonal	AF488		
Armenian hamster Ig	Goat, IgG	Polyclonal	Alexa Fluor 647	Jackson Immunoresearch	127-605-160
Streptavidin			Brilliant Violet 421	Biolegend	405226

Table 2.5 Antibodies used for FACS.

Reactivity	Host, isotype	Clone	Conjugate	Manufacturer	Cat #
B220	Rat IgG2a, κ	RA3-6B2	Alexa Fluor 488	BioLegend	103225
CD138	Rat F344	281-2	Brillian Violet 650	BD Horizon	564068
CD19	Lewis IgG2a, κ	1D3	Brilliant Violet 786	BD Horizon	563333
CD21	Rat IgG2b, κ	76G	APC	BD Pharmingen	558658
CD23	Rat IgG2a, κ	B3B4	PE	eBioscience	12-0232-82
CD27	Armenian Hamster IgG1, κ	LG.3A10	PE-Cy7	BD Pharmingen	563604
CD3	Rat IgG2b, κ	17A2	Brilliant Violet 510	BioLegend	100234
CD38	Rat IgG2a, κ	90	Alexa Fluor 700	eBioscience	56-0381-82
CD4	Rat DA	RM4-5	PE-CF594	BD Horizon	562285
CD44	Rat IgG2b, κ	IM7	Brilliant Violet 510	BD Horizon	563114
CD5	Rat IgG2a, κ	53.7.3	PerCP-Cy5.5	Invitrogen	45-0051-82
Fas (CD95)	Armenian Hamster IgG2, λ2	Jo2	Brilliant Violet 605	BD OptiBuild™	740367
GL-7	Rat LOU	FL7	FITC	BD Horizon	562967

IgD	Rat IgG2a, κ	11-26c.2a	**V450**	BD Horizon	560869
IgG (H+L)	Goat F(ab')2 IgG: anti-Mouse	Polyclonal	**FITC**	Southern Biotech	1032-02
IgM	Rat IgG2a, κ	II/41	**APC-eFluor780**	eBioscience	47-5790-82
Ki-67	Mouse IgG1, κ	B56	**PE-Cy7**	BD Pharmingen	561283
TACI (CD267)	Rat WF	8F10	**Alexa Fluor 647**	BD Pharmingen	558453

CHAPTER 3: THE ROLE OF COMPLEMENT IN VACCINATION AGAINST *SALMONELLA* TYPHIMURIUM

3.1 Introduction

Effective clearance of *Gram*-negative infections from mammalian hosts often relies on the complement system. *Salmonella* Typhimurium (STm) is one such type of infection. The outer membrane of STm is densely populated with proteins (e.g., porins such as OmpD, OmpF, and OmpC (Gil-Cruz et al., 2009)), in addition to lipopolysaccharide molecules that are common in Gram-negatives (Schager et al., 2018). These antigens are potent activators of the three pathways of the complement system, resulting in rapid accumulation and activation of the downstream cascade. The cascade is a series of ~50 plasma-phase proteins that activate consecutively to provide rapid innate response and control of invading pathogens. Complement activation has a range of downstream effects, including opsonisation of foreign pathogens (Dunkelberger and Song, 2009) , immune cell recruitment leading to rapid inflammation and buying the adaptive immune response time to gain momentum (Vandendriessche et al., 2021; Ricklin et al., 2010; Shivshankar et al., 2020).

The complement system, as mentioned above, works through three pathways. This can be seen in **figure 3.1**. These pathways are called the classical, mannose-binding lectin (MBL) and alternative pathways. Each are distinct through their initial activation triggers but become intertwined further down the cascade. Briefly, the classical pathway is activated by the presence of immune complexes (ICs) of antibody and antigen (Hester and Frank, 2019). The MBL pathway is activated by carbohydrates

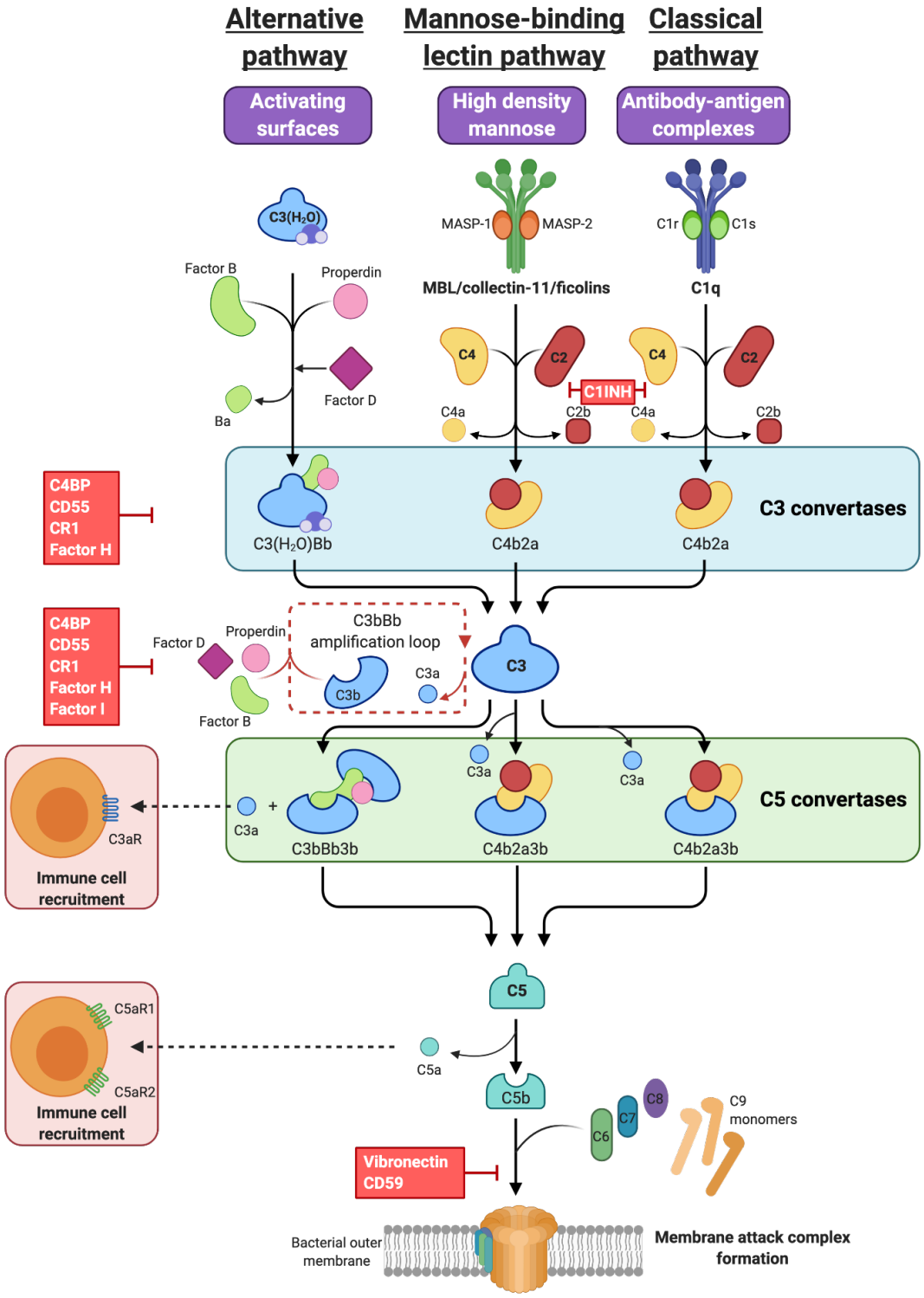

Figure 3.1 The complement cascade. Recreated from the following sources (Thurman and Holers, 2006; Kolev et al., 2014; Giang et al., 2018; Kumar, 2016; Nesargikar et al., 2012; Coulthard and Woodruff, 2015; Cochrane and Müller-Eberhard, 1968) using Biorender.

commonly found on bacterial and viral surfaces (Eisen and Minchinton, 2003; Mueller-Ortiz et al., 2004). Finally, the alternative pathway is also known as the 'tick over' pathway, in that this pathway is constitutively active. This provides a 'surveillance' mechanism, where its primary complement component 3 (C3) is consistently hydrolysed to an active form, only to be broken down by its relatively short half-life. In the presence of activating surfaces (e.g., that of a bacterial cell envelope), the active form of C3 is stabilised leading to rapid accumulation and positive feedback driving full activation of this pathway (Mueller-Ortiz et al., 2004).

The three pathways also have distinct initiator molecules. The classical pathway is initiated by activation of the C1q-C1r-C1s complex (Hester and Frank, 2019). The MBL pathway is initiated by structurally similar MASP-1 and MASP-2 and ficolin proteins (Matsushita and Fujita, 2001). Both complexes will catalyse the cleavage and activation of downstream C4 and C2 components, leading to production of the classical C4b2a C3 convertase. The alternative pathway, as mentioned above, is spontaneously activated by activating surfaces, activating a positive feedback loop to create the alternative C3bBb C3 convertase. These C3 convertases are an essential step to merging of the three pathways on the central C3 component of the complement cascade. These convertases, as the names suggest, convert C3 to its active form in a rapid fashion. Activated C3 can then bind the C3 convertases to create C5 convertases, which goes on to activate C5 and the terminal cascade components C6-9. This leads to formation of the C5b-9 MAC, which forms pores in foreign surface membranes leading to cell lysis (Kolb and Mueller Eberhard, 1975; Esser et al., 1979). *Gram*-negative bacteria are particularly susceptible to the innate bactericidal activity of the complement and the MAC. Furthermore, complement receptors 1 and 2 (CR1 and CR2, respectively) are expressed on B cells and other immune cells. CR1 (CD35)

is largely involved in internalisation of immune complexes, leading to antigen processing and presentation (Ahearn and Fearon, 1989; Leslie et al., 2003; Nielsen et al., 1997), whereas CR2 (CD21) largely acts as a B cell coactivator (Leslie et al., 2003; Ahearn and Fearon, 1989) by forming complexes with CD19 and CD81 (Sato et al., 1997). The complexed B cell receptor (BCR) lowers the threshold required for B cell activation leading to enhanced humoral and memory responses.

The complement system has been shown to act on STm through both antibody-dependent and -independent mechanisms (Gondwe et al., 2010; Galdiero et al., 1984). These studies have shown that both complement, and antibody are required to action optimal phagocytosis by peripheral blood cells (Gondwe et al., 2010). Other studies have demonstrated that LPS is able to interact with C1q in the absence of antibody, or by directly activating C3 via the alternative pathway and without consumption of earlier complement proteins (Galdiero et al., 1984). It has, however, been argued that antibody-dependent mechanisms contribute more to anti-*Salmonella* responses than those independent of antibody. For example, it was shown that healthy sera containing both antibody and complement was able to lyse *Salmonella* bacteria, whereas sera lacking the antibody component but containing functional complement was unable to affect the same level of lysis (Siggins et al., 2011; MacLennan et al., 2008). Taken in conjunction with the findings that children who were most vulnerable to NTS bacteraemia were those between 4 months and 2 years of age, a time where passive maternal immunity has waned but too early of an age to have personally experienced infection, this study suggested that whilst complement aided bacterial clearance, it was not essential in the role of protection.

The role of the complement system in protection against iNTS infection, as already briefly shown, has been heavily debated for years. As mentioned above, sera

containing functional complement, but no anti-*Salmonella* Ab was not effective at killing bacteria. Moreover, supplementation of Ab-deficient sera with specific Ab to STm restored its bactericidal capacity. Supplementation of Ab-sufficient sera with functional complement did not improve its ability to lyse bacteria, suggesting that Ab is more important than complement alone. In contrast, studies involving complement-deficient mice have challenged these findings. A 2002 study that used C1q-deficient mice demonstrated that an absence of C1q lead to heightened susceptibility to primary STm infections, in addition to higher intracellular bacterial loads (Warren et al., 2002). Additionally, a separate study showed using antibody receptor- and C3-deficient mice, complement activity is essential for IgG-mediated clearance of bacteria from tissues (Rossi et al., 2019). In this case, whilst both components are important for bacterial clearance, Ab could not affect bacterial clearance without complement. Gondwe *et al.* has suggested both antibody and complement are essential for oxidative burst and phagocytosis of iNTS by peripheral blood cells irrespective of their bactericidal activity alone (Gondwe et al., 2010). It could be suggested that complement may not be required for bacterial clearance due to the role of LPS in immune evasion, and its ability to mediate complement deposition (Spitzer et al., 2007; Murray et al., 2006). Further, complement activity is often measured by its activation or consumption. A study conducted by Murray *et al.,* highlighted that heightened complement activity does not necessarily translate to complement sensitivity of the bacterium (Murray et al., 2006), and therefore studies that have reported an activation of complement in response to infection may not reflect its actual usage and significance in clearance.

Despite its clear role in pathogen clearance, the relationship between antibody and complement is unclear in murine systems. Studies have argued a more essential role of one over the other, where others have suggested that both are important in

protection from infection. Additionally, this relationship has rarely been explored in the context of vaccination with a complex vaccine antigen such as outer membrane vesicles (OMVs).

Therefore, despite a plethora of knowledge on complement and its role in humoral immune responses a gap exists regarding the relationship between these two facets of the immune response in the context of vaccination and challenge. I hypothesise that the two systems are intimately intertwined, with the complement system playing an integral role in the ability to produce protective immune responses after successful vaccination and challenge.

In this chapter, mice deficient in several specific components of the complement system (C1q-/-, C3-/-, C4-/-, C5-/-) were used to examine the nature of successful vaccine-mediated protection. This was achieved by analysing bacterial burden of the spleens and livers of vaccinated and challenged animals and the accompanying immune responses.

3.2 Results

3.2.1 Complement-deficiency in mice does not impair ability to control bacterial burden following primary STm infections.

It has been shown previously that absence of complement results in an impaired ability to control bacterial numbers in *Salmonella* infections in humans (Gondwe et al., 2010; MacLennan et al., 2008). However, in mice, the role of complement in bacterial clearance is less significant, where mice are unable to clear bacteria unless supplemented with a human complement source (Siggins et al., 2011). To identify this phenotype, wildtype (WT) and complement-deficient mice were infected i.p. with STm SL3261, spleens and livers were harvested, and bacterial burdens enumerated at 7 days post-infection (**Fig. 3.2A**). The bacterial burdens of complement-deficient mice in spleens (**Fig. 3.2B**) and livers (**Fig. 3.2C**) did not significantly differ from those of the WT mice. Therefore, an absence of complement does not affect a mouse's ability to control bacterial burdens after 7 days of primary infection.

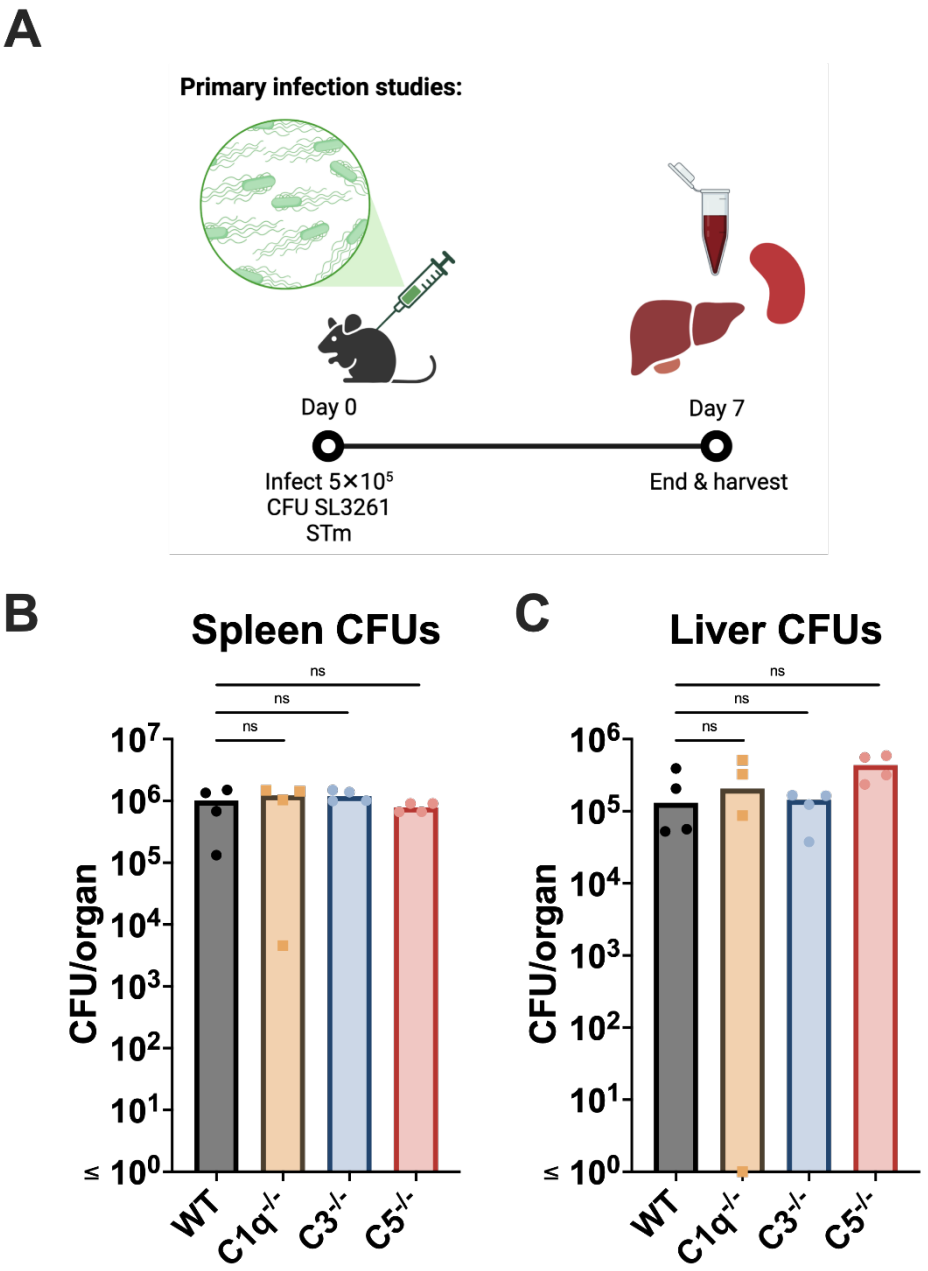

Figure 3.2 Complement deficiency does not affect bacterial burden of spleens and livers in primary infections. WT or complement-deficient mice were infected i.p. with 5×10^5 CFU SL3261 STm for 7 days (A). Spleens and livers were weighed, and bacterial burden quantified (B and C). Data is representative of one experiment, where each dot represents one mouse (n = 4 mice). Bars represent the median of each group. Data were analysed by Kruskal-Wallis test with Dunn's multiple comparison.

3.2.2 Complement-deficient mice show Ab responses comparable to wild-type mice following primary STm infection.

To test if mice with complement-deficiencies were able to induce Ab responses following primary infection, sera from mice were tested by ELISA for antigen-specific IgM or IgG (**Fig 3.3**). Across most antigens and Ab isotypes, serum Ab of complement-deficient mice did not significantly differ from WT responses. However, anti-LPS IgG was significantly higher in the C5-deficient mice ($P \leq 0.05$). Overall, these results show that an absence of complement does not impair, and in some cases bolsters, antibody responses against various STm antigens.

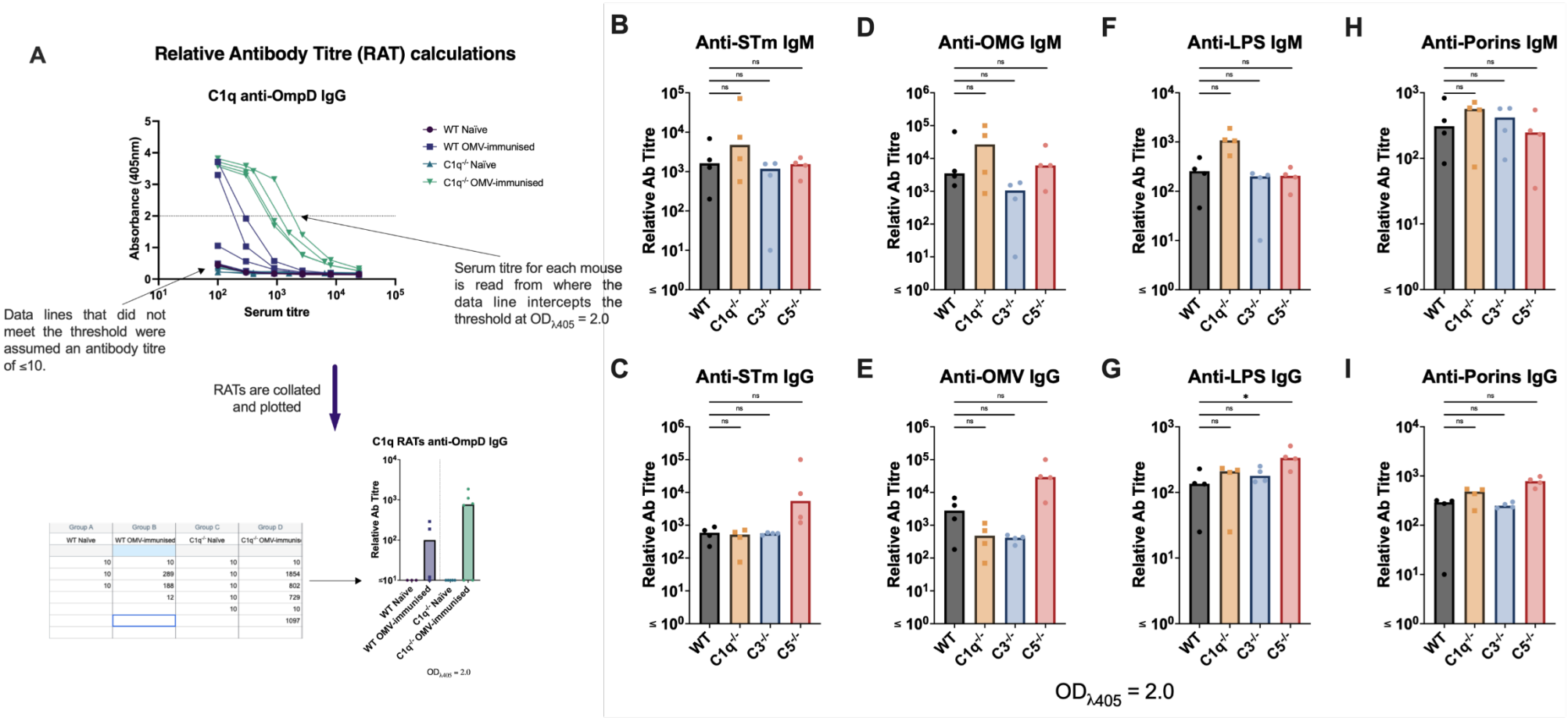

Figure 3.3 Complement-deficiency does not significantly affect a mouse's ability to induce antibody responses in primary infections. WT or complement-deficient mice were infected i.p. with 5×10^5 CFU SL3261 STm for 7 days. Sera was collected at day 7 and measured for antigen-specific antibody. Antibody titres were enumerated as shown in (A). Sera was measured for anti-whole cell IgM (B) and IgG (C), anti-OMV IgM (D) and IgG (E), anti-LPS IgM (F) and IgG (G), and anti-porins IgM (H) and IgG (I). Data is representative of one experiment, where each dot represents one mouse (n = 4 mice). Bars represent the median of each group. Data were analysed by Kruskal-Wallis test with Dunn's multiple comparison; ns = non-significant, * = $P \leq 0.05$.

3.2.3 Internalisation of STm bacteria is not impaired by an absence of complement following primary infection.

It is well established that complement will opsonise pathogens and tag them for destruction by phagocytosis (Tosi et al., 1990; Radbruch et al., 2006; Ricklin et al., 2010). Opsonophagocytosis is an initial stage in the process of bacterial killing, in which bacterial cells are internalised following opsonisation by antibodies or complement fragments (Gratz et al., 2015). Eukaryotic cell membranes are impermeable to the antibiotic gentamicin (Sharma and Puhar, 2019). Gentamicin treatment of spleens and liver cultures would enable killing of only extracellular bacteria. Enumeration of these cultures will only represent bacteria that have been internalised by phagocytes. Therefore, in the absence of complement, internalisation of bacteria may be affected due to the lack of opsonins and impaired opsonophagocytosis. To test this hypothesis, we incubated mashed spleens and livers ± gentamicin for 1 hour at 37ºC, before washing and serially diluting the cultures and enumeration on LB agar (**Fig. 3.4A**). In all complement-deficient strains, the level of intracellular bacteria was not significantly different compared to the WT in either spleen (**Fig. 3.4B**) or liver (**Fig. 3.4C**). Therefore, an absence of complement does not significantly impair bacteria internalisation at 7 days post primary infection.

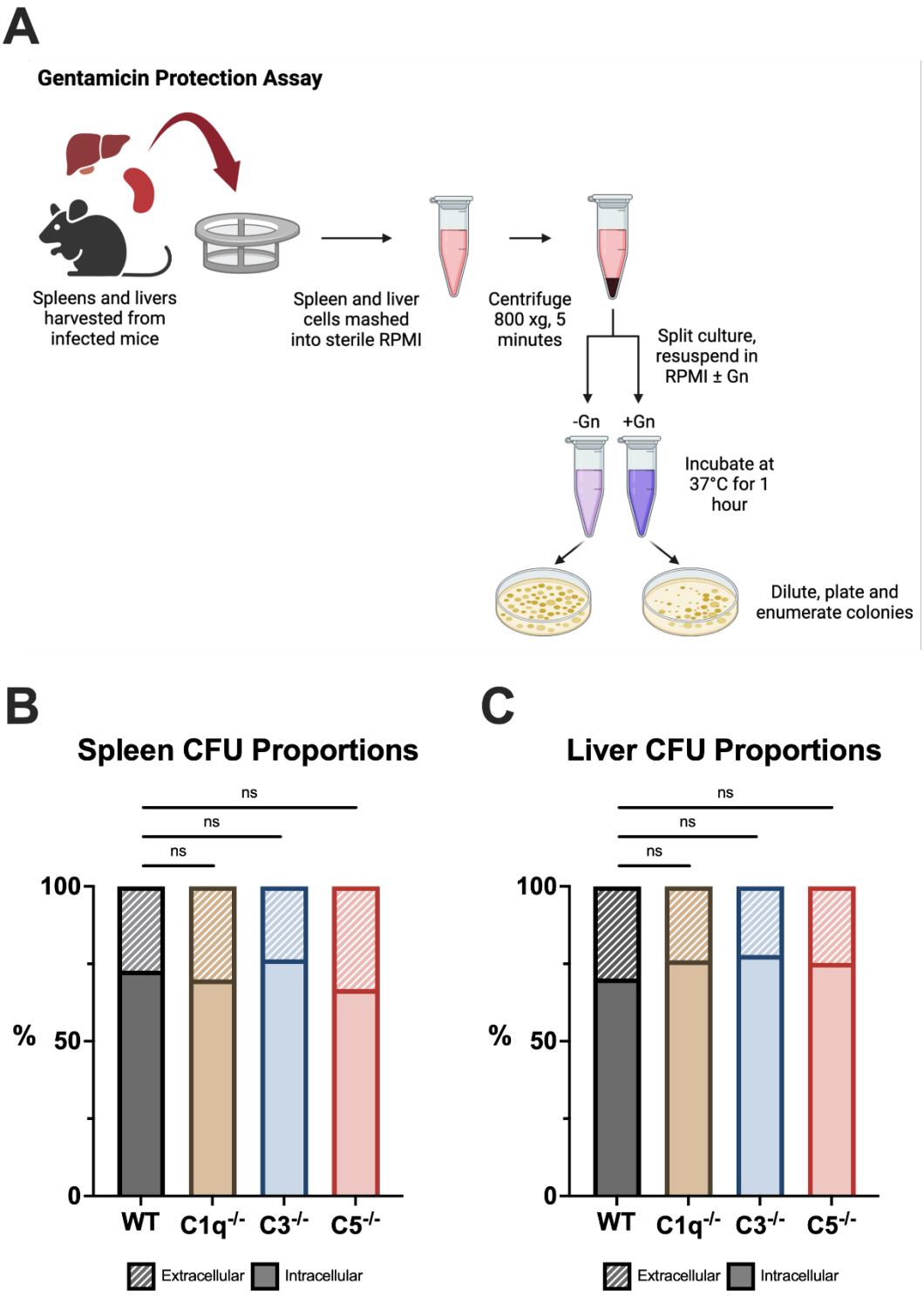

Figure 3.4 Complement-deficiency does not affect internalisation of bacteria in the spleens and livers of infected mice in primary infections. WT or complement-deficient mice were infected i.p. with 5×10^5 CFU SL3261 STm for 7 days. Cells from tissues were gentamicin-treated, bacterial burdens quantified, and proportions calculated for spleens (B) and livers (C). Data is representative of one experiment, where each bar represents one group (n = 4 mice). Data were analysed by two-way ANOVA with Bonferroni's multiple comparison.

3.2.4 Spleen or liver pathology are not markedly affected by either complement deficiency nor vaccination status following 24 hours of infection.

As a blood borne infection, invasive *Salmonella* infection is often accompanied by changes in organ pathology, particularly in the spleen and liver. Splenomegaly has been observed in mice infected with *Salmonella* (Jackson et al., 2010), and hepatomegaly has been observed in infections where high levels of bacterial endotoxin are present (Shao et al., 2011). To identify if pathology of these organs were affected by either vaccination status or complement deficiency, spleens and livers were weighed and assessed for gross pathology during dissection (**Fig. 3.5A and B**). The results show that spleen and liver weights are not greatly affected by prior vaccination when compared to naïve controls in infected mice. In general, spleens from immunised mice displayed a more pigmented and reddish colour, whereas spleens from naïve mice were notably paler in comparison. Livers from naïve mice displayed small lesions and increased signs of inflammation, compared to the clean and healthier appearing livers from immunised mice. Both spleens and livers from naïve mice were larger by size compared to immunised mice (data not shown). Additionally, absence of specific complement components did not considerably affect the weights of organs when compared to that of the WT controls in either organ. Therefore, vaccination status, nor an absence of complement, influences organ weight after 24 hours of infection.

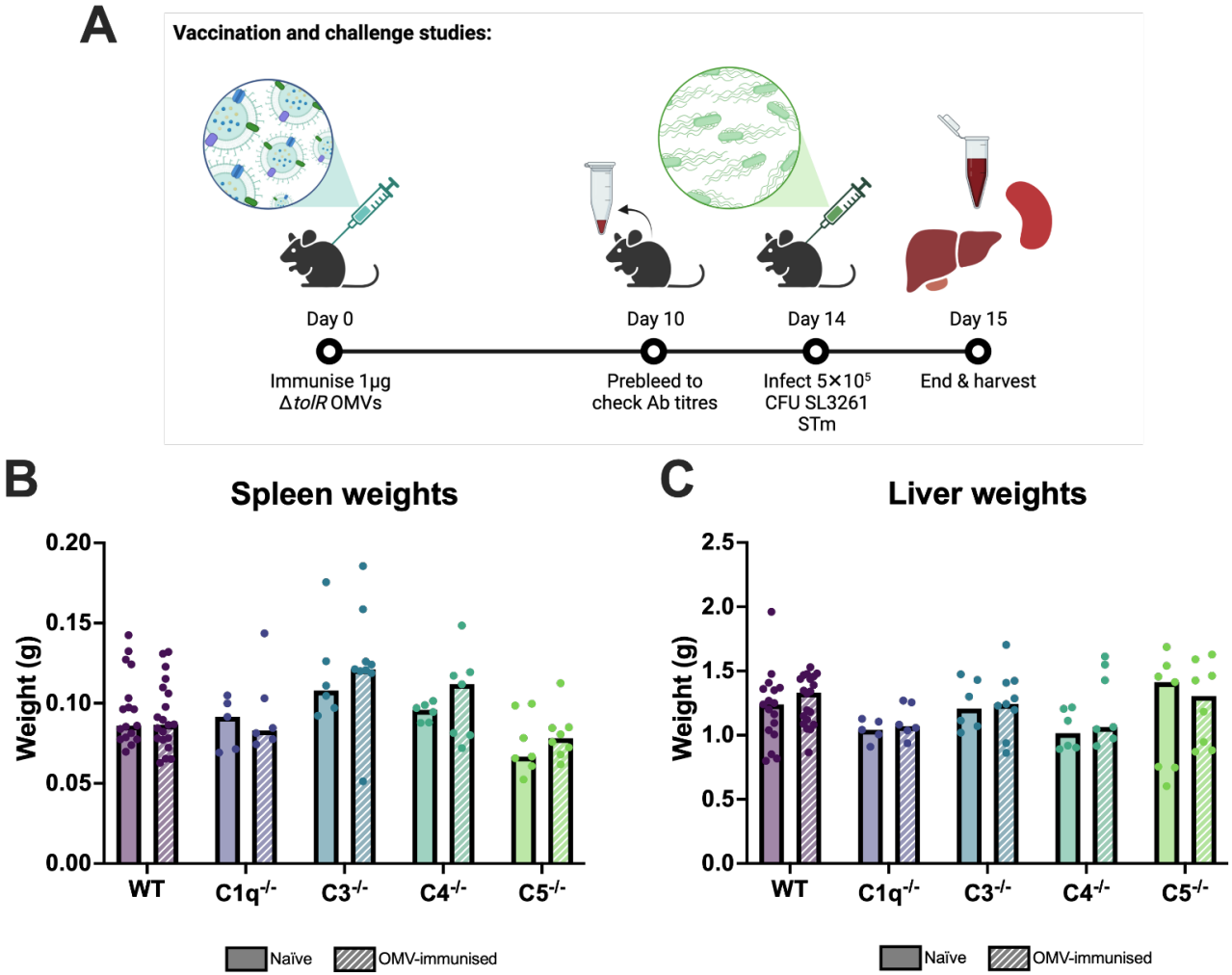

Figure 3.5 Prior vaccination with OMVs does not affect organ weights of complement-deficient mice challenged with STm. WT or complement-deficient mice were vaccinated i.p. with 200 µL PBS or 1 µg ΔtolR OMVs for 14 days before challenge with 5×10^5 CFU SL3261 STm for 24 hours (A). Spleens (B) and livers (C) were harvested and weighed. Data are pooled from 7 independent experiments and each dot represents one mouse. Bars represent the median of each group. For WT naïve n = 17 mice, immunised n = 21 mice, for C1q-/- naïve n = 8 mice, immunised n = 10 mice, C3-/- naïve n = 6 mice, immunised = 9 mice, for C4-/- naïve n = 6 mice, immunised n = 7 mice, for C5-/- n = 6 mice, immunised n = 8 mice.

3.2.5 C3-deficiency impairs the ability to control bacterial burden in spleens and livers of mice following vaccination and challenge for 24 hours.

Despite a lesser role in bacterial clearance, it has been shown that complement, specifically C3, is essential for vaccine-mediated antibody responses against STm infection (Rossi et al., 2019). In this study, however, mice were only passively immunised with anti-O-Ag IgG2a. Other studies have shown no role for complement in vaccine-mediated protection (Mastroeni et al., 1993), but again these studies were conducted by adoptive transfer of antigen-specific Ab. Therefore, the role of antibody in vaccine-mediated protection remains vague, specifically in place of direct immunisation with complex bacterial antigens such as OMVs. To identify whether complement plays a role in the successful vaccination of mice with OMVs against STm infection, mice were immunised with PBS or *ΔtolR* OMVs for 14 days before infection for 1 day with STm SL3261. Bacterial burdens of spleens and livers were then quantified (**Fig. 3.6**). When immunised with OMVs prior to infection, WT mice show a significant 2-3 log reduction in the bacterial burdens of the spleen (**Fig. 3.4A**) and liver (**Fig. 3.4B**) compared to the naïve controls. This magnitude of control was also shown in the $C1q^{-/-}$, $C4^{-/-}$, and $C5^{-/-}$ mice. However, for $C3^{-/-}$ mice, there was no significant reduction in bacterial CFU in either organ, remaining at approximately10^5-10^6 CFU/organ regardless of vaccination status. Vaccination of mice in the context of C3-deficiency does therefore not result in a protective immune response capable of controlling bacterial burden in infected organs of challenged mice. These results suggest that C3 is a necessary component of vaccine success against invasive STm infections in mice.

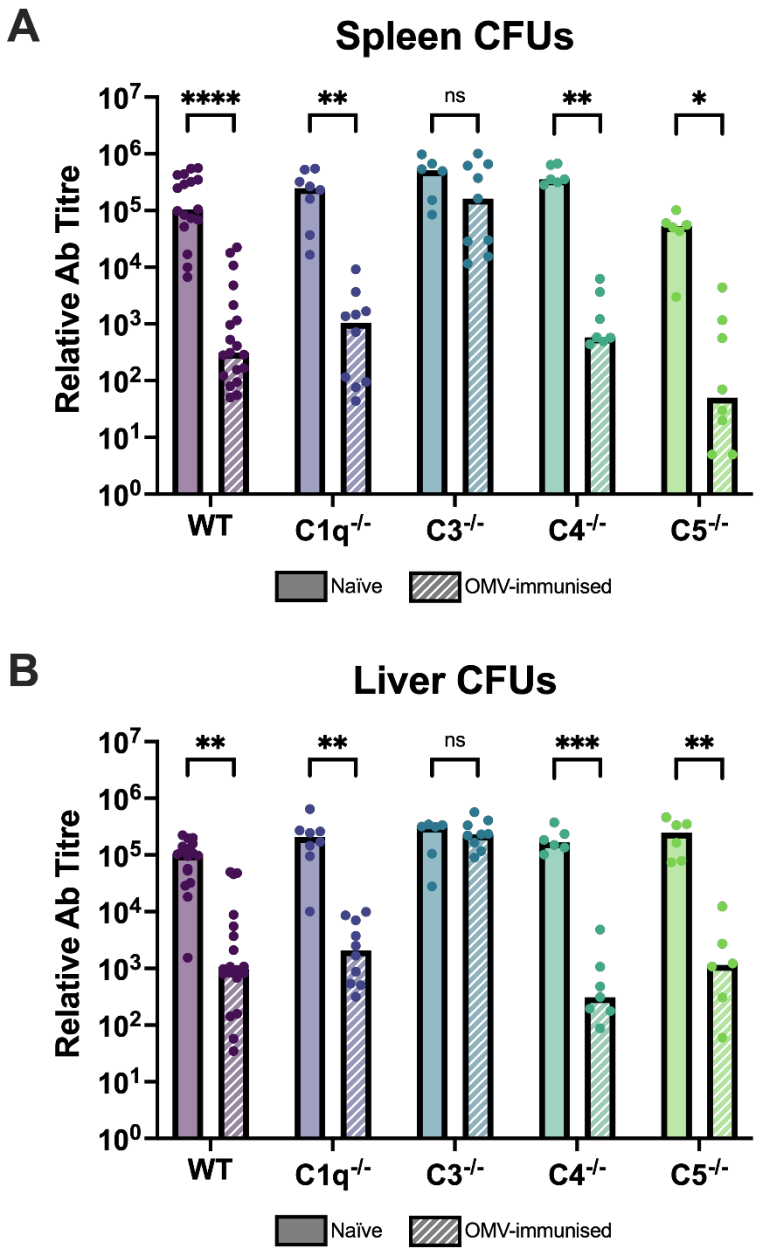

Figure 3.6 Complement-deficient mice show reduced bacterial burdens of their spleens and livers following vaccination with OMVs, except those deficient in C3. WT or complement-deficient mice were vaccinated i.p. with 200 µL PBS or 1 µg ΔtolR OMVs for 14 days before challenge with 5×10^5 CFU SL3261 STm for 24 hours. Bacterial burdens of spleens (A) and livers (B) were quantified. All strains except C3-/- showed a reduction in bacterial CFU in both organs. Data are pooled from 7 independent experiments, where each dot represents one mouse. Bars represent the median of each group. For WT naïve n = 17 mice, immunised n = 20 mice, for C1q-/- naïve n = 8 mice, immunised n = 10 mice, C3-/- naïve n = 6 mice, immunised = 9 mice, for C4-/- naïve n = 6 mice, immunised n = 7 mice, for C5-/- n = 6 mice, immunised n = 8 mice. CFU data were tested for normality using the Shapiro-Wilk test. Differences in bacterial burden between naïve and vaccinated mice of each strain was analysed using the Kruskal-Wallis test with Dunn's multiple comparison; * = $P \leq 0.05$, ** = $P \leq 0.005$, *** = $P \leq 0.0005$, **** = $P < 0.0001$.

3.2.6 Antigen-specific Ab responses in vaccinated and challenged C3-deficient mice are impaired.

It has been shown previously that Ab against STm is protective (Schager et al., 2018; Cunningham et al., 2007; Gil-Cruz et al., 2009; Dominiguez Medina, 2017). To test if the observed inability of C3-deficient mice to mount protective vaccine responses was caused by impaired Ab production, I tested sera for IgM and IgG against two abundant STm antigens, LPS and OmpD (**Fig. 3.7**). Mice were immunised with PBS or *ΔtolR* OMVs for 14 days before infection for 1 day with STm SL3261. IgM and IgG against LPS (**Fig. 3.7A and 3.7B**) were detected in all mouse strains, except those deficient in C3, which showed minimal response for LPS-specific Ab. The same was also true for anti-OmpD IgM, where C3-deficient mice showed no response to this antigen (Fig. 3.8C). For anti-OmpD IgG, heightened levels of Ab could be detected in all vaccinated groups, for all strains, albeit responses for C3-deficient mice were on average lower than other strains (Fig. 3.6D). These results suggest that absence of C3 diminishes STm-specific IgM responses in mice following vaccination and challenge, which may be protective. However, C3-deficient mice seem to be able to produce lower-levels of OmpD-specific IgG, meaning that total Ab responses are not entirely diminished in the absence of C3.

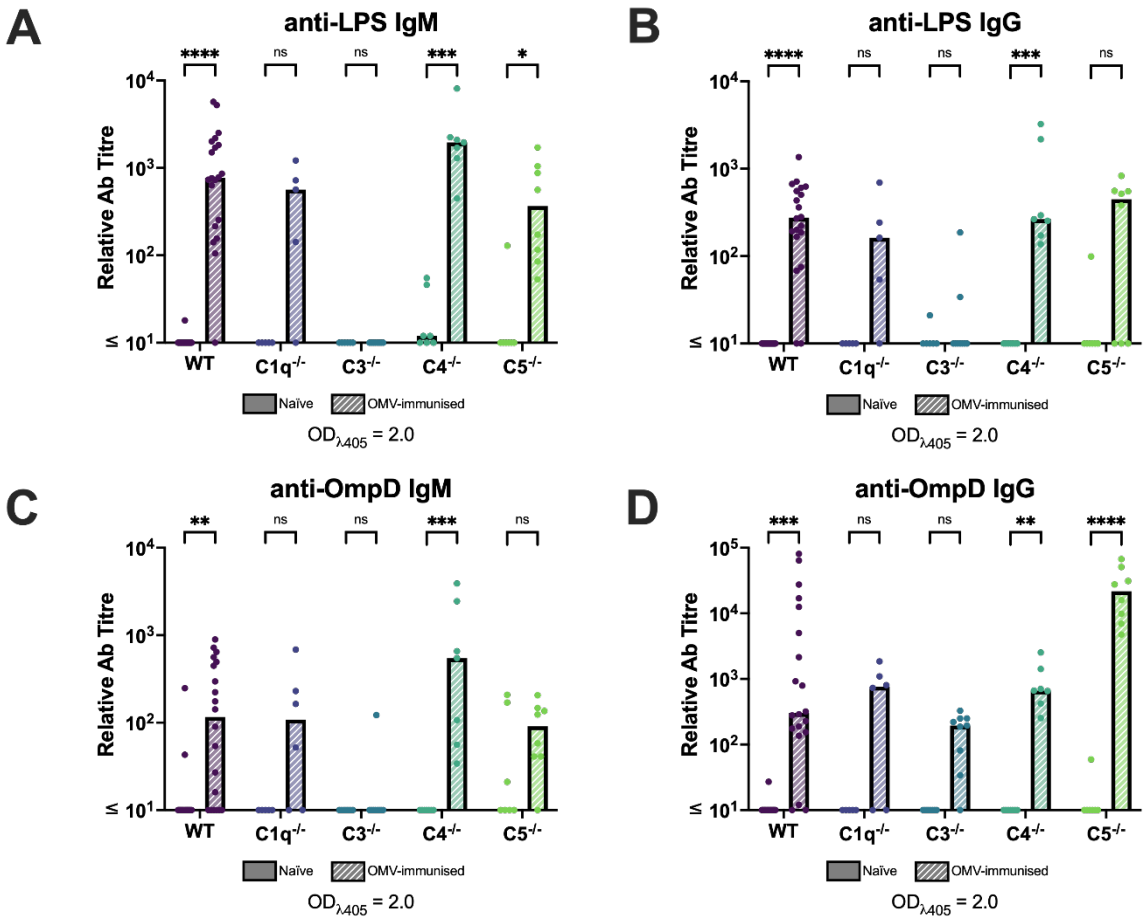

Figure 3.7 Complement deficient mice, except C3-deficient, are able to induce antigen-specific serum Ab following vaccination and challenge. WT or complement-deficient mice were vaccinated i.p. with 200 µL PBS or 1 µg ΔtolR OMVs for 14 days before challenge with 5×10^5 CFU SL3261 STm for 24 hours. Anti-LPS IgM (A) and IgG (B), and anti-OmpD IgM (C) and IgG (D) were quantified by ELISA. RATs were obtained as illustrated in figure 3.1. Data are pooled from 7 independent experiments, where each dot represents one mouse. Bars represent the median of each group. For WT naïve n = 17 mice, immunised n = 20 mice, for C1q-/- naïve n = 5 mice, immunised n = 6 mice, C3-/- naïve n = 8 mice, immunised = 9 mice, for C4-/- naïve n = 7 mice, immunised n = 7 mice, for C5-/- n = 7 mice, immunised n = 8 mice. Titre data were analysed for normality using Kolmogorov-Smirnov test. Differences in Ab titres was analysed using Kruskal-Wallis test with Dunn's multiple comparison; * = P ≤ 0.05, ** = P ≤ 0.005, *** = P ≤ 0.0005, **** = P < 0.0001.

3.2.7 STm antigen localises to the red pulp of spleens in infected mice, regardless of immunisation or complement status

Cells within secondary lymphoid organs such as the spleen are intricately organised into a microarchitecture that allows for functionally efficient and consistent cell-cell interactions (Mebius and Kraal, 2005). The organisation of zones in the spleen allows for thorough surveillance and effective action by the immune system of pathogens, such as iNTS (Junt et al., 2008; Mebius and Kraal, 2005). Monocytes and granulocytes, such as neutrophils, reside primarily in the red pulp, where they effectively act as a first line of defence against pathogens (Mebius and Kraal, 2005). They therefore can be used to visualise the distinct red and white pulp zones in the spleen. The above data indicated that C3-deficient mice are unable to control bacterial burden in the spleen. To visualise this lack of control, we stained spleen sections from WT and complement deficient mice that were immunised according to **Fig 3.1B**. Sections were stained for neutrophil marker Ly6G (Deniset et al., 2017), and STm antigen. All antibodies used for IHC can be found in Table 2.3. Staining for Ly6G would also provide insight to STm bacterial localisation, and how this is affected by immunisation status.

Spleen sections for naïve and immunised mice can be seen in **Fig. 3.8** for (A) WT, (B) $C1q^{-/-}$, (C) $C3^{-/-}$, (D) $C4^{-/-}$, and (E) $C5^{-/-}$ mice. The microarchitecture of the spleens did not greatly differ between naïve or immunised mice of any strain regarding the distinction of red pulp (RP) vs white pulp (WP). Interestingly, despite previous studies indicating that STm is largely engulfed by neutrophils, co-staining of Ly6G and STm was not evident in any of the spleen sections. STm antigen largely localised to the RP of the spleen, regardless of immunisation and complement status. These data suggest

that at 24-hours post infection, STm bacteria localises in the red pulp and therefore is interacting with cells of the innate immune system.

A notable difference evident in the $C3^{-/-}$ mice (**Fig. 3.8C**) was the apparent neutrophilia observed in the immunised group. Immunisation increased the levels of brown $Ly6G^{+}$ stain in all groups, however this increase was visually stronger in the immunised $C3^{-/-}$ group. This suggests that in the absence of C3, immunisation increases neutrophil cell recruitment.

A final observation noted from the IHC staining of these spleen sections was the apparent heightened level of blue STm antigen staining in all immunised groups, regardless of mouse strain. This can be seen in the rightmost panels of each group in **Fig 3.8**. Despite lower cultivable CFUs being obtained from mouse spleens as shown in Fig. **3.6A**, the level of STm antigen visible in the spleen sections was markedly greater than that seen in the naïve groups. This phenotype of antigen persistence will be further explored in Chapter 4.

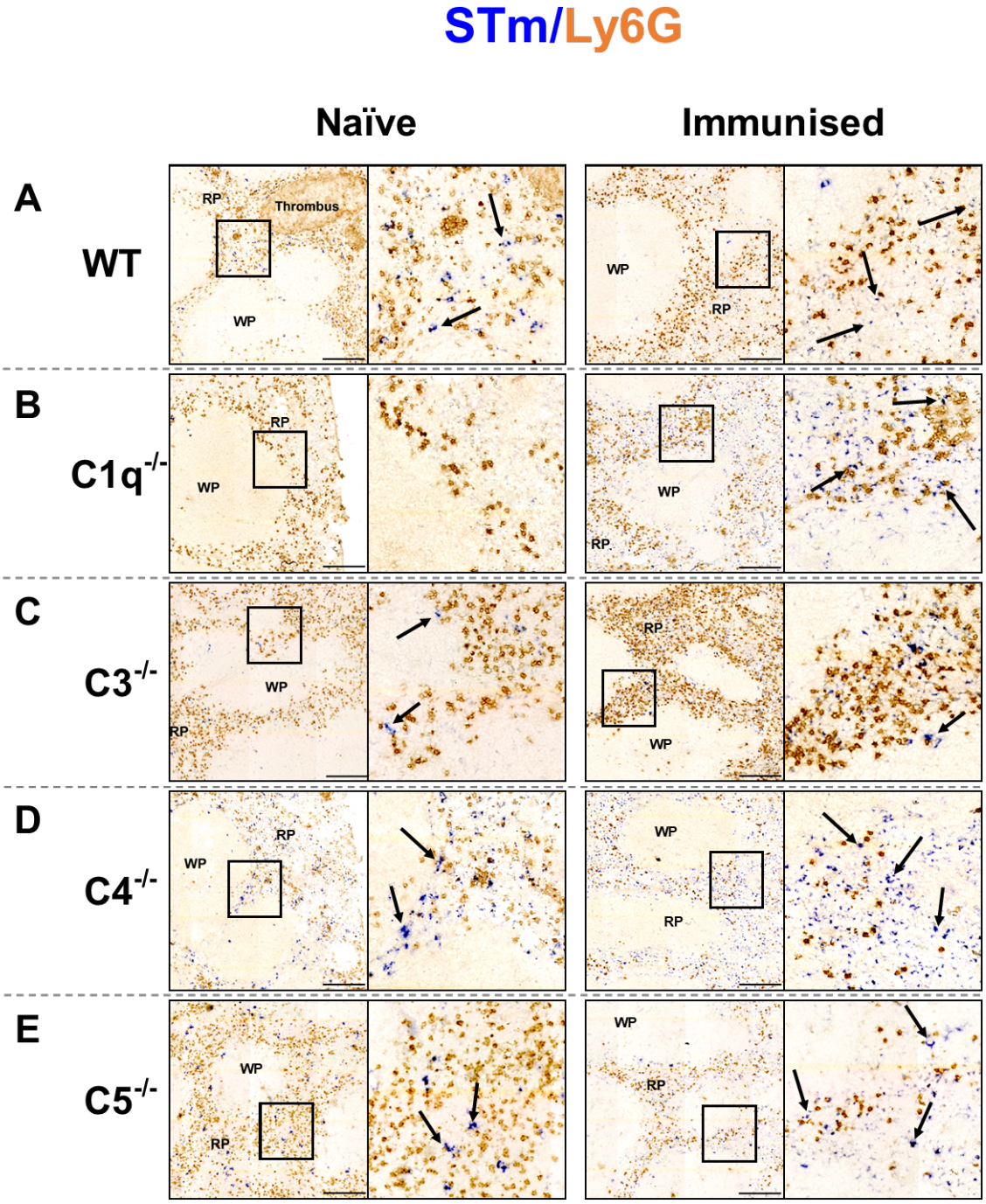

Figure 3.8 Complement-deficient mice do not differ greatly in their splenic microarchitecture following immunisation and challenge. WT or complement-deficient mice were vaccinated i.p. with 200 µL PBS or 1 µg ΔtolR OMVs for 14 days before challenge with 5×10^5 CFU SL3261 STm for 24 hours. (A) WT, (B) $C1q^{-/-}$, (C) $C3^{-/-}$, (D) $C4^{-/-}$, (E) $C5^{-/-}$. Spleens were snap frozen and sectioned and stained by IHC where Ly6G is brown and STm antigen is blue. Images are representative of each group (WT naïve n = 4 mice, immunised n = 5 mice, for C1q-/- naïve n = 4 mice, immunised n = 4 mice, C3-/- naïve n = 6 mice, immunised = 9 mice, for C4-/- naïve n = 6 mice, immunised n = 7 mice, for C5-/- n = 6 mice, immunised n = 8 mice). WP = white pulp. RP = red pulp. Scale bars denote 200 µm.

3.2.8 C3-deficient mice have less B220$^+$ cells than WT mice post-immunisation

B220 is a tyrosine phosphatase that participates in the modulation of lymphocytes (Cascalho et al., 2000). It is known to be expressed abundantly on mature resting B cells (Matthias and Rolink, 2005; Thomas, 1989; Coffman and Weissman, 1981; Brynjolfsson et al., 2018). The previous data have alluded to an impairment in humoral responses in C3$^{-/-}$ mice following immunisation with OMVs. As all antibody secreting cells derive from mature B cells, I sought to identify how absence of C3 affected B220$^+$ cell populations in the splenic microarchitecture, and if STm antigen was commonly associated with these B220$^+$ cells. Spleens of either WT or C3$^{-/-}$ mice were frozen and sectioned and stained for B220 (brown) or STm antigen (blue).

The results shown in **Fig. 3.9** indicate that in WT mice, immunisation with OMVs prior to challenge results in an expansion of B220$^+$ cells, which is particularly noticeable in the red pulp (upper rightmost panel). This expansion of the B220$^+$ cell population makes distinction of the white pulp (B cell follicle and T cell zone) from red pulp more difficult. White pulp areas were also generally larger in the immunised WT spleens. In comparison, these phenotypes were not observed to the same extent in immunised C3$^{-/-}$ mice compared to the naïve controls. Indeed, both naïve and immunised C3$^{-/-}$ spleens were similar to the naïve WT mice, with smaller more distinct white pulp areas. These results indicate that C3$^{-/-}$ mice are unable to produce a rapid expansion of B220$^+$ cells following immunisation and challenge, as WT mice are able to. Further, in all groups, STm antigen rarely co-stained with the B220$^+$ marker. Therefore, STm antigen and B220$^+$ cells were not associating with each other.

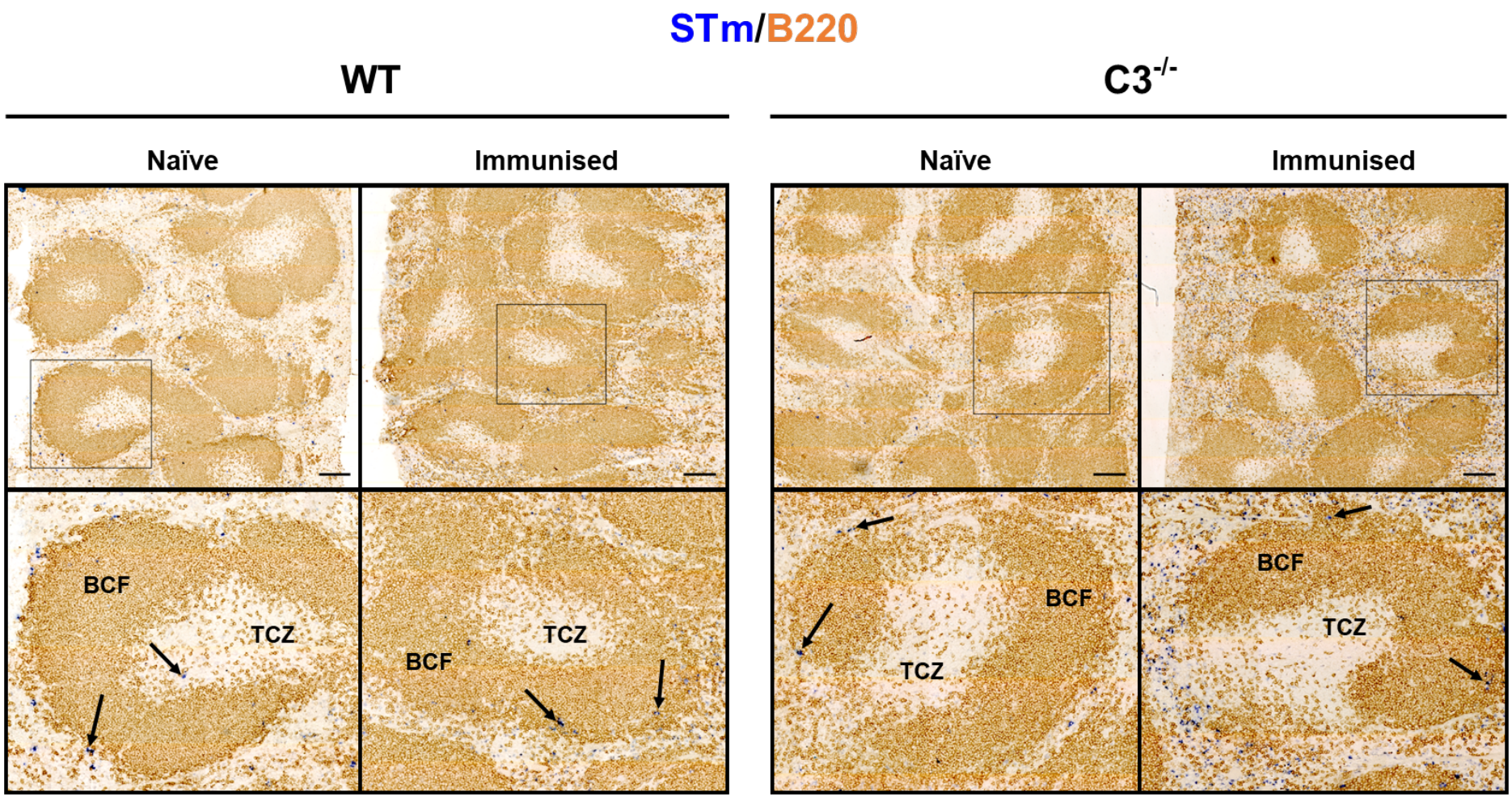

Figure 3.9 Expansion of B220+ cells in immunised WT mice is not observed in immunised mice with C3-deficiency. WT or complement-deficient mice were vaccinated i.p. with 200 µL PBS or 1 µg ΔtolR OMVs for 14 days before challenge with 5×105 CFU SL3261 STm for 24 hours. Spleens were snap frozen and sectioned and stained by IHC where B220 is brown and STm antigen is blue. Images are representative of each group (WT naïve n = 4 mice, immunised n = 5 mice, for C1q-/- naïve n = 4 mice, immunised n = 4 mice, C3-/- naïve n = 6 mice, immunised = 9 mice). BCF = B cell follicle, TCZ = T cell zone, individual STm cells are highlighted by arrows in naïve groups. One white pulp is highlighted by dotted line in each panel. Scale bars denote 200 µm.

3.2.9 B cell populations, but not total T cells, are markedly affected by an absence of C3 in vaccinated mice

The data thus far suggests that an absence of C3 impairs Ab responses. To test whether this impairment affects only antibody production/secretion, or whether the immune cell populations themselves are affected, we analysed spleen cell populations more broadly. Mice were immunised as according to **Fig. 3.5A**. Spleen cells were processed and sorted by FACS. Gating strategies for each cell type can be found in Appendix B. Both B cell populations and T cells were investigated, to identify if both T-dependent and T-independent responses were affected.

During STm infection, professional antigen presenting cells (APCs) will engulf the pathogen, process the foreign peptides, and migrate to the lymphoid tissues where they will present the antigen to other immune cells, such as B and T cells. B cells are activated in T cell-independent (TI) or T-dependent (TD) mechanisms (Pérez-Toledo et al., 2017). B cells express unique B cell receptors (BCR) which allows them to directly recognise antigen in its native form (Dorshkind and Rawlings, 2018). B cells presented with STm antigen, either directly or through APCs, will either develop through extrafollicular pathways in TI responses, or will enter the germinal centre reaction with T cell help (MacLennan, 1994; MacLennan et al., 2003; Cunningham et al., 2007). T cells that are $CD4^+$ will mature into T helper cells, whereas those that are $CD8^+$ will mature into cytotoxic T killer cells (Seder and Ahmed, 2003). These $CD4^+$ T helper cells, after activation, will proliferate and secrete cytokines that assists other arms of the immune response such as B cell activation (Hamel et al., 2012). This can be observed in **Fig. 3.10A and B**, which show, total B cell and T cell populations were significantly larger in immunised mice than naïve (**Fig 3.10A and B, respectively**). I would expect that the proliferating B cell population would be greater in those animals

that are actively responding to antigen, as this would indicate the induction of either extrafollicular or germinal centre B cell expansion. The number of proliferating B cells were assessed by presence of the marker Ki67 (Gerdes et al., 1984), and indeed, the number of proliferating B cells were greater in WT mice that had received OMVs prior to infection (**Fig 3.10C**).

In the spleen, marginal zone (MZ) B cells accumulate around the marginal sinus and are pre-activated to rapidly respond to bloodborne pathogens (Balázs et al., 2002; Lo et al., 2021) , and can respond without T cell help. Numbers of MZ B cells will increase rapidly within the first few hours of infection, however these populations drop 24-hours post infection (Lo et al., 2021). Follicular (FO) B cells, on the other hand, take up residence in the B cell follicles of the spleen, where they are poised to respond to antigen and interact with T cells at the initial stages of germinal centre responses (Pillai and Cariappa, 2009). FO B cell populations were significantly higher in the immunised group (**Fig. 3.10D**), yet MZ B cell numbers remained similar regardless of vaccination status. This is concurrent with previous findings.

Upon immunisation or infection, activated B cells that receive survival signals from T cells will remain in the B cell follicle and enter the germinal centre (GC) reaction and undergo various rounds of somatic hypermutation and selection (Hamel et al., 2012; MacLennan, 1994) and develop into plasma cells (PCs) which produce highly specific, high affinity antibody (Khodadadi et al., 2019; Nutt et al., 2015). GC B cell populations (**Fig. 3.10E**) were significantly higher in the immunised group compared to the naïve, indicating an induction of the GC response in response to vaccine antigen.

In $C3^{-/-}$ mice, however, the picture is vastly different. $C3^{-/-}$ mice do not show a significant increase in either total B cell (**Fig 3.10A**) nor T cell (**Fig. 3.10B**) populations yet did show a significantly smaller population of B cells post-immunisation and

infection compared to the WT mice. The number of proliferating B cells (**Fig 3.10C**) did not significantly increase in immunised $C3^{-/-}$ mice following vaccination compared to the naïve. MZ B cells, on the other hand, were greatly increased in both $C3^{-/-}$ groups, regardless of vaccination status, when compared to the WT controls (**Fig 3.10D**). The opposite was true of FO B cells, where both naïve and immunised $C3^{-/-}$ mice showed lower FO B cell populations compared to the WT, and this population did not expand following vaccination of $C3^{-/-}$ mice. Finally, the number of GC B cells (**Fig. 3.10E**) in immunised $C3^{-/-}$ mice was similar to that of naïve mice, and both $C3^{-/-}$ groups displayed significantly lower numbers of GC B cells compared to the WT controls.

In conclusion, these data suggest that C3 is important for B cell subset and T cell expansion following vaccination and challenge, including total, FO, and GC B cells. However, these data also suggest that there is a skew towards the MZ B cell phenotype in the absence of C3.

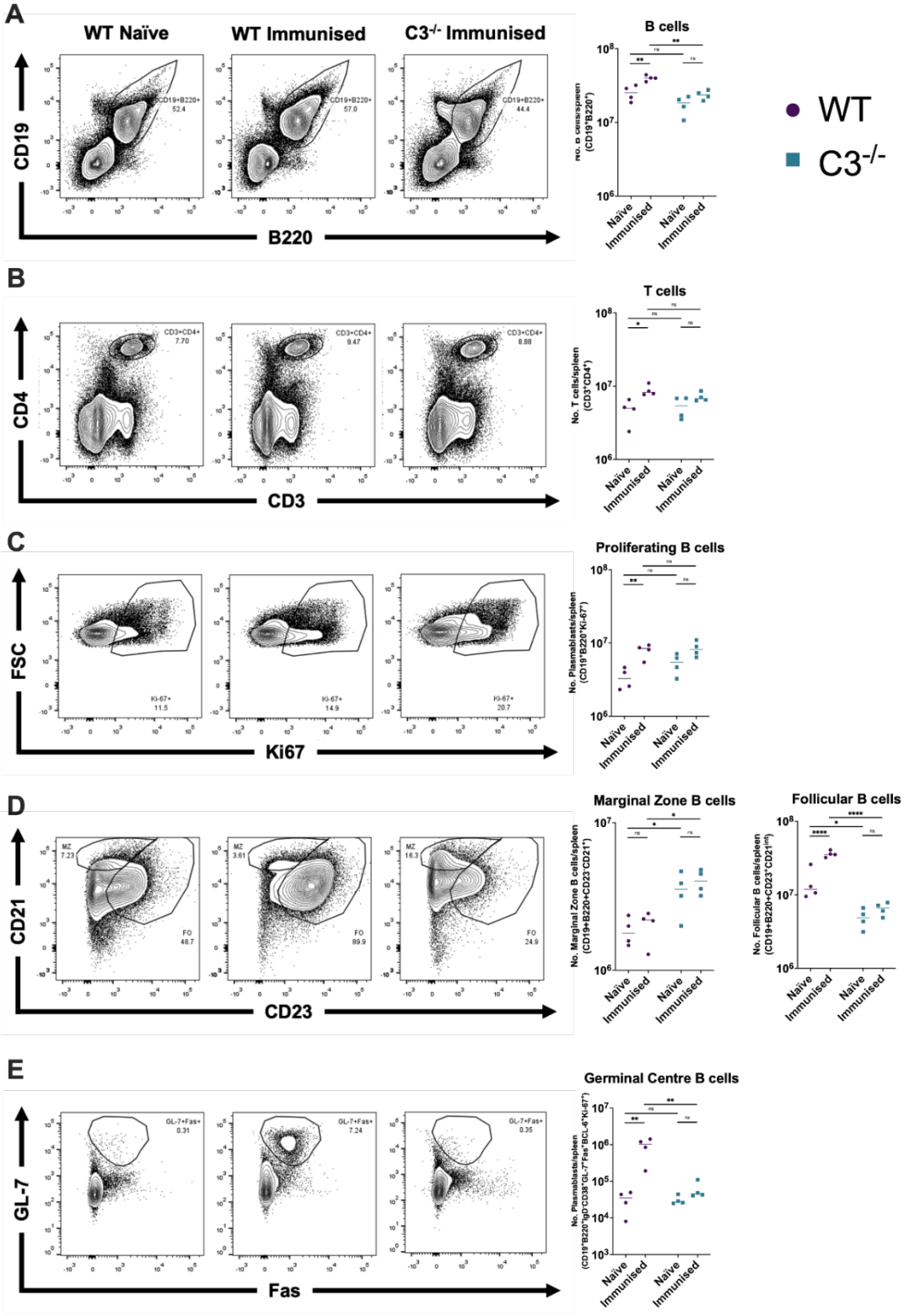

Figure 3.10 C3-deficiency affects B cell populations following vaccination and challenge, but not T cells. WT or C3-deficient mice were vaccinated i.p. with 200 µL PBS or 1 µg ΔtolR OMVs for 14 days before challenge with 5×10^5 CFU SL3261 STm for 24 hours. Spleens were harvested and (A) Total B cell, (B) Total T cell, (C) Proliferating B cell, (D) Marginal zone and follicular B cell, (E) Germinal centre B cell cell populations analysed by flow cytometry on day 15. Data is representative of one experiment, where each dot represents one mouse (n = 4 mice per group). Data was analysed by one-way ANOVA with Bonferroni's multiple comparison; ns = non-significant, * = P ≤ 0.05, ** = P ≤ 0.005, *** = P ≤ 0.0005, **** = P < 0.0001.

3.2.10 Absence of C3 presents a block in the development progression of transitional B cells.

Due to the aberrations in specific B cell populations, I sought to investigate if B cell development was affected by an absence of C3. In mice, a fraction of immature B cells successfully passes negative selection checkpoints in the bone marrow and are transferred to the spleen as immature transitional B cells to develop into mature B cells (Loder et al., 1999; Zhou et al., 2020).

Transitional B cell populations were analysed by their CD23, and IgM expression as discussed previously; transitional B cells develop through 3 distinct 'checkpoints' (T1, T2, and T3), which can be distinguished through differential expression of these markers (Allman et al., 2001). T1 B cells are the transitional state between migration from the bone marrow to spleen, whereas T2 B cells are largely localised to the spleen, before development into a mature B cell (Petro et al., 2002). T3 B cells, on the other hand, whilst develop from the T2 B cell stage, have been shown to not develop into mature B cells and are likely involved in regulating autoimmunity (Teague et al., 2007). General transitional B cells have been shown to respond to Toll-like receptor (TLR) 9 stimulation, progressing through an IgM^+ B memory cell phenotype followed by terminal differentiation into antibacterial antibody secreting PCs (Capolunghi et al., 2008). This suggests that transitional B cells play a role in first-line defence against infection.

Representative population dot plots for each transitional B cell population can be observed in **Fig. 3.11A**. An increase in total transitional B cells (**Fig. 3.11B**) in WT mice can be observed, where this population was significantly larger in mice that had received immunisation, suggesting an ongoing response to vaccine antigen. Further,

immunised WT mice demonstrate a larger population of each T1, T2, and T3 stages of transitional B cell development (**Fig 3.11C, D and E, respectively**), suggesting a larger proportion of B cells are progressing through the transitional stages in an ongoing response to immunisation and infection.

Immunised $C3^{-/-}$ mice showed similar levels of overall transitional B cells to wildtype mice (**Fig. 3.11B**), suggesting that B cells are able to actively progress through transitional stages to become mature B cells. However, naïve $C3^{-/-}$ mice showed a significantly larger population of total transitional B cells compared to naïve WT mice, suggesting a larger proportion of $C3^{-/-}$ B cells are in this transitional stage. Moreover, $C3^{-/-}$ mice showed larger populations of T1 B cells compared to WT mice, regardless of vaccination status (**Fig 3.11C**), and immunisation of $C3^{-/-}$ mice resulted in a larger T1 population as with WT responses. However, $C3^{-/-}$ mice did now show a significant increase in the T2 B cell population following vaccination and challenge, and the observed increase is much smaller compared to the WT group (**Fig. 3.11B**). Indeed, the T2 population of immunised WT mice was significantly larger than immunised $C3^{-/-}$ mice. Finally, immunisation of $C3^{-/-}$ mice resulted in a significant increase of T3 B cells, however the magnitude of increase was markedly smaller than that of the WT mice (**Fig. 3.11E**). In fact, both naïve and immunised $C3^{-/-}$ mice showed significantly smaller T3 populations compared to the WT counterparts.

These data suggest that in the absence of C3, B cells cannot advance into mature B cells of the spleen and are 'developmentally blocked'. This would further suggest that the absence of C3 has crucial cellular roles regarding successful vaccine immune responses, when taken with the previous results, has a knock-on effect on humoral responses.

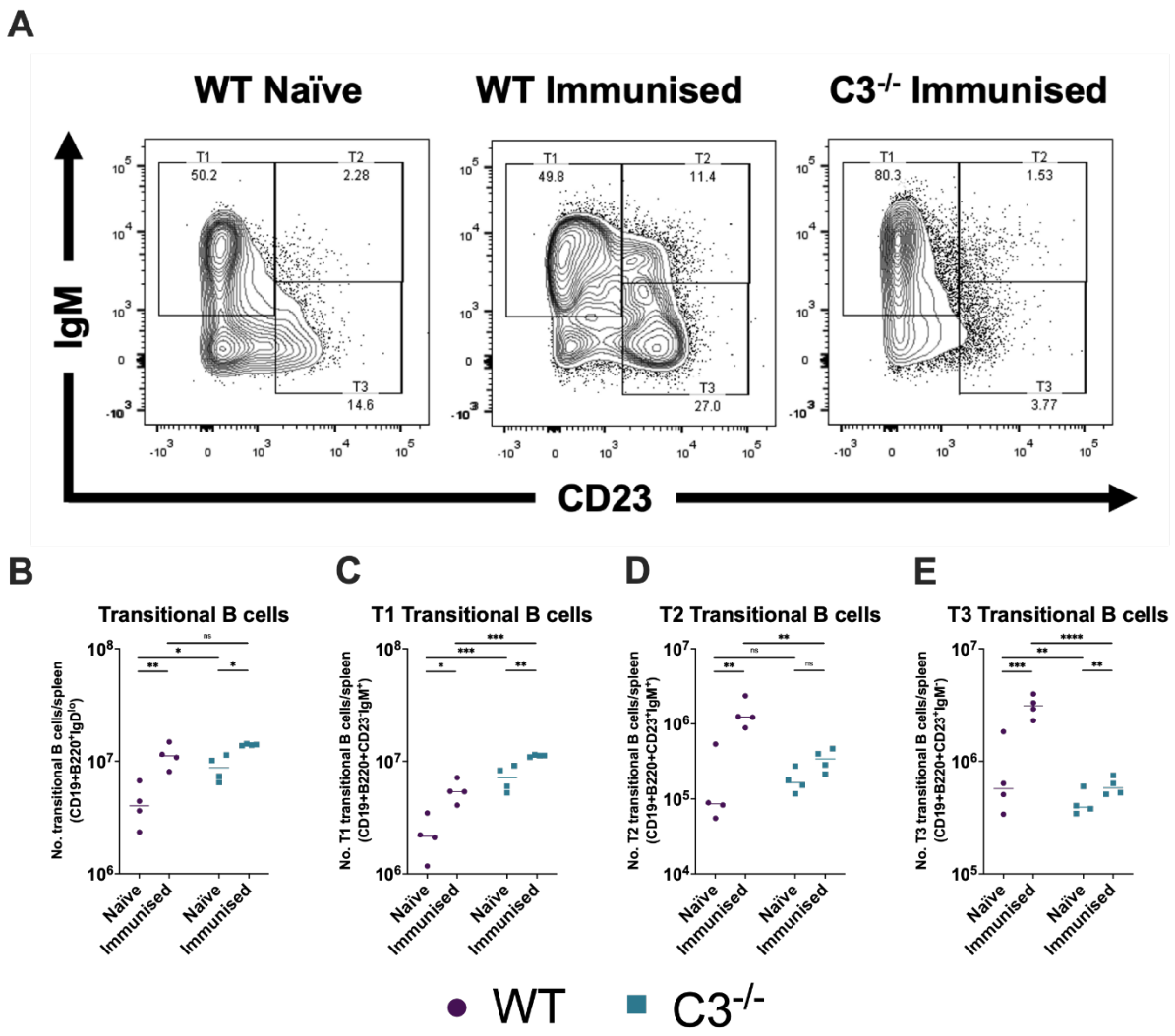

Figure 3.11 C3-deficiency affects transitional B cell populations following vaccination and challenge, but not T cells. WT or C3-deficient mice were vaccinated i.p. with 200 µL PBS or 1 µg ΔtolR OMVs for 14 days before challenge with 5×10^5 CFU SL3261 STm for 24 hours. Spleens were harvested and transitional B cell populations analysed by flow cytometry on day 15. Data is representative of one experiment, where each dot represents one mouse (n = 4 mice per group). Data was analysed by one-way ANOVA with Bonferroni's multiple comparison; ns = non-significant, * = P ≤ 0.05, ** = P ≤ 0.005, *** = P ≤ 0.0005, **** = P < 0.0001.

3.2.11 Antibody secreting cell populations are affected by an absence of C3 in immunised and challenged mice

Following diminished Ab titres in $C3^{-/-}$ mice, I investigated the populations of cells responsible for antibody production to identify if this phenotype was affected at the cellular level or was solely humoral. Both plasmablasts (PBs) and plasma cells (PCs) are considered antibody secreting cells (ASCs), and the development of a mature B cell into either of these cell types is well established. PBs and PCs differ in that they produce immediate or persistent protective antibody responses (respectively) to T cell-dependent antigen presentation (Nutt et al., 2015). PBs are short-lived cycling cells and produce rapid humoral responses, however this antibody is generally low-affinity due to the lack of somatic hypermutation (Tellier and Nutt, 2019). PCs, on the other hand, are long-lived cells canonically derived from follicular B cells in the germinal centre reaction (Tellier and Nutt, 2019). For this reason, they produce highly specific, high-affinity antibodies in response to infection. Few PBs have the potential to become long-lived PCs, should they receive necessary survival signals (Khodadadi et al., 2019). PBs are also suggested to dominate secondary infections, but in these cases are derived from germinal centre memory B cells and produce high-affinity antibodies (Tellier and Nutt, 2019; Khodadadi et al., 2019).

General ASCs can be sorted by expression of both CD38 and TACI cell surface markers (Pracht et al., 2017). Mice were immunised as according to **Fig. 3.5A**. My results demonstrate that in WT responses, ASC populations are significantly larger in primed mice that have previously received vaccination (**Fig. 3.12A**), as expected. PB populations were larger in immunised WT mice, however this was not statistically significant (**Fig. 3.12B, middle panel**), though I may have expected to see a significant increase at an earlier timepoint post-immunisation due to the short lifespan of PBs (4-

6 days) (Elsner and Shlomchik, 2020). PC numbers were significantly higher in immunised WT mice (Fig. 3.11B, rightmost panel) compared to naïve, indicating a robust PC-producing B cell response. Finally, isotype-specific ASCs for IgM and IgG were both significantly larger in immunised WT mice (**Fig. 3.12C**). As expected, the increase observed in IgG^+ ASCs is larger than that for IgM^+ ASCs, which would be concurrent with previous knowledge of isotype switching and somatic hypermutation experienced in animals that are primed against infection.

In $C3^{-/-}$ mice, however, ASC populations are overall smaller than that of WT, regardless of vaccination status (**Fig. 3.12A**). PB populations are similar to WT, however PC populations are markedly reduced in both naïve and immunised groups when compared to WT controls (**Fig. 3.12B**), suggesting a skew towards the short-lived, low affinity ASC phenotype. Isotype-specific ASC populations were also smaller in both naïve and immunised $C3^{-/-}$ mice when compared to WTs (**Fig. 3.12C**). In all of these populations, immunisation of $C3^{-/-}$ mice resulted in small increases in their ASC populations, but these were not significantly larger.

These results indicate that an absence of C3 has a negative effect on the expansion and development of ASC populations in vaccinated and challenged animals.

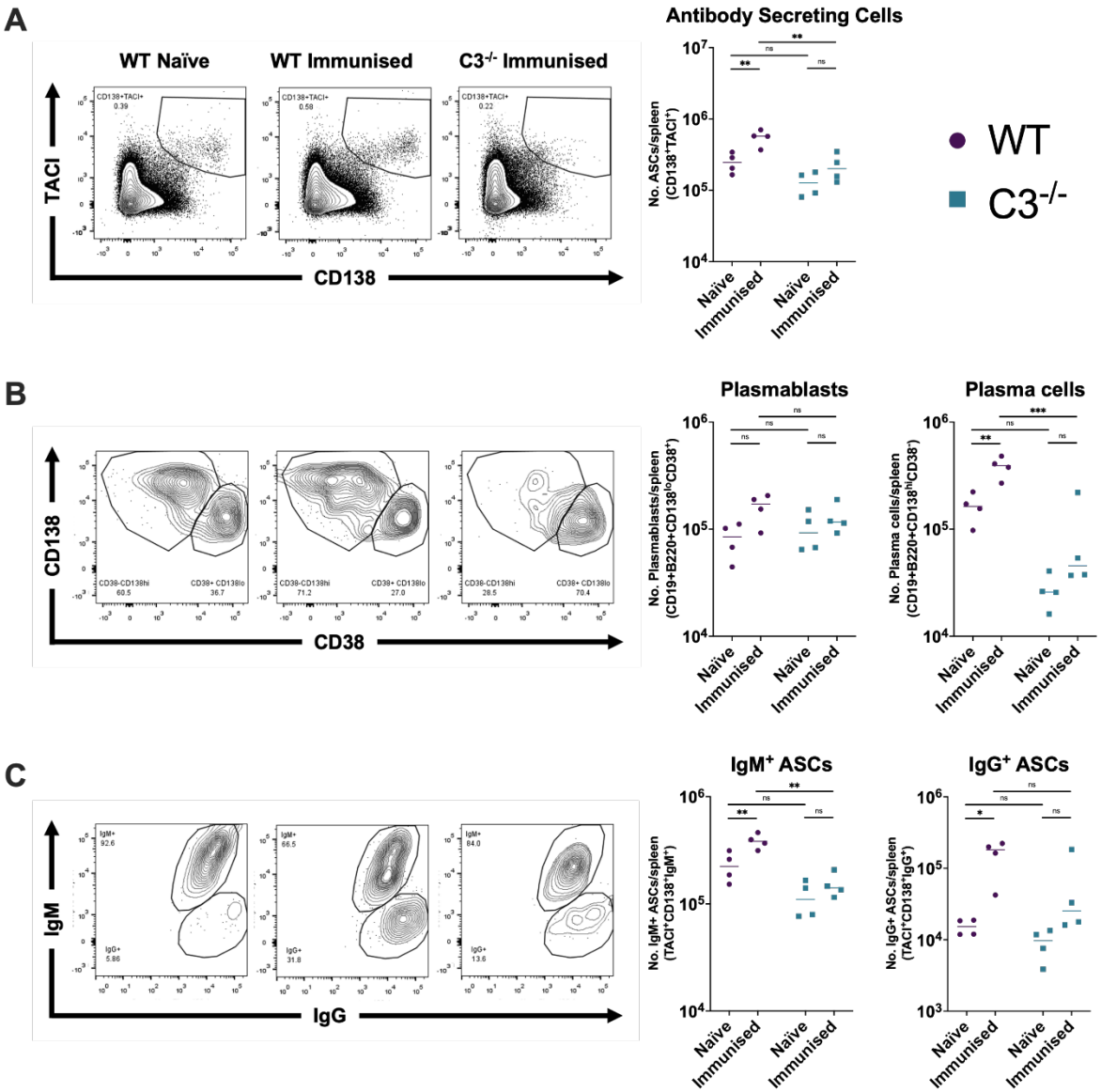

Fig 3.12 C3-deficiency affects antibody secreting cell populations following vaccination and challenge, but not T cells. WT or C3-deficient mice were vaccinated i.p. with 200 µL PBS or 1 µg Δ*tolR* OMVs for 14 days before challenge with 5×10^5 CFU SL3261 STm for 24 hours. Spleens were harvested and (A) Total antibody secreting cell, (B) Plasmablasts and plasma cells, (C) Isotype-specific ASC populations analysed by flow cytometry on day 15. Data is representative of one experiment, where each dot represents one mouse (n = 4 mice per group). Data was analysed by one-way ANOVA with Bonferroni's multiple comparison; ns = non-significant, * = $P \leq 0.05$, ** = $P \leq 0.005$, *** = $P \leq 0.0005$.

3.2.12 Opsonisation of STm does not rescue the non-protective phenotype of C3-deficient mice.

We have established that C3-deficient mice are unable to generate protective immune responses that control bacterial burden in the spleen and livers of vaccinated and challenged mice (Fig. 3.5). Further, we have recognised that C3-deficient mice show stunted Ab responses to STm-specific antigens, following vaccination and infection. To test if the non-protective phenotype of C3-deficient mice is solely due to a lack of antibody, we infected WT or $C3^{-/-}$ with STm SL3261 opsonised with heat-inactivated sera for 24 hours and quantified bacterial burdens in the spleens and livers (**Fig. 3.13A**). We have previously shown that opsonisation of bacteria with immune sera (i.e. high level of protective antibodies) is able to markedly reduce bacterial burden in organs compared to mice that received bacteria opsonised with naïve sera (i.e. minimal protective antibodies present) (Schager et al., 2018). WT mice that received STm opsonised with immune sera showed a 2-log reduction in CFU in spleens (**Fig. 3.13B**) and livers (**Fig. 3.13C**) compared to those that received STm opsonised with naïve sera. In contrast, $C3^{-/-}$ mice that received bacteria opsonised with immune sera showed a smaller (< 1-log), non-significant reduction in their bacterial burdens compared to those that received STm opsonised with naïve sera. These results suggest that replacement of antibody alone, in the form of pre-opsonised bacteria, is not sufficient to rescue the non-protective phenotype of C3-deficient mice. This also suggests that C3 could be directly involved in the protective responses that make a successful vaccine.

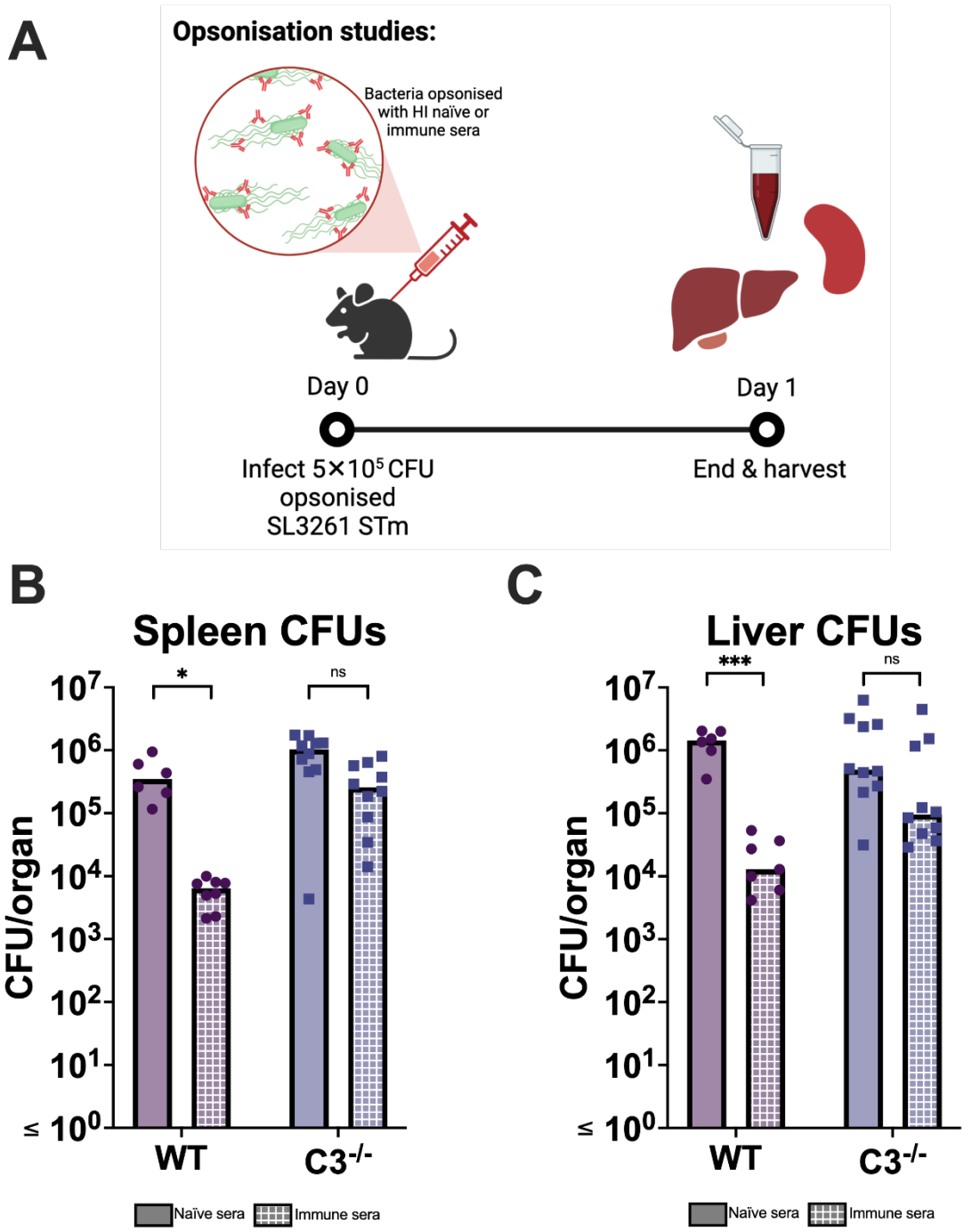

Figure 3.13 Opsonisation of bacteria prior to infection into C3-deficient mice does not significantly reduce bacterial burden in the spleen or liver. WT or C3-/- mice were infected i.p. with pre-opsonised 5×10^5 CFU SL3261 STm for 24 hours. Sera used for opsonisation was heat-inactivated. Weights and bacterial burdens of the spleen (A and C) and liver (B and D) were quantified. Data are representative of 3 independent experiments. Bars are representative of the median for each group. For WT with naïve sera, n = 6 mice, immune sera n = 8 mice, for C3-/- with naïve sera n = 10, immune sera n = 10. Data were tested for normality using the Shapiro-Wilk test. Differences in CFU data were analysed using Kruskal-Wallis test with Dunn's multiple comparison; ns = non-significant, * = $P \leq 0.05$, ** = $P \leq 0.005$, *** = $P \leq 0.0005$.

3.2.13 Supplementation of a complement source prior to infection does not rescue the non-protective phenotype of C3-deficient mice.

To test whether supplementation of complement would aid Ag-specific Ab in reducing bacterial burden of spleens and livers, $C3^{-/-}$ mice were infected i.p. with STm SL3261 opsonised with immune sera + 200 µL heat-inactivated immune sera or non-heat-inactivated immune sera (**Fig. 3.14A**). The heat-inactivation of sera denatures proteins (excluding antibodies) such as complement found in sera (Soltis et al., 1979). Through this experiment, we could interrogate the contribution of complement directly on the mouse's ability to generate protective immune responses. Bacterial burdens of spleens (**Fig. 3.14B**) and livers (**Fig. 3.14C**) were quantified after 24 hours of infection. Mice that received non-heat-inactivated sera + opsonised bacteria (i.e., +complement) did not show reduced bacterial burden in either spleen or liver, compared to those that received heat-inactivated sera + opsonised bacteria (i.e. – complement). Therefore, reintroduction of complement and antibody at the same time as infection is not adequate in rescuing the phenotype of C3-deficient mice.

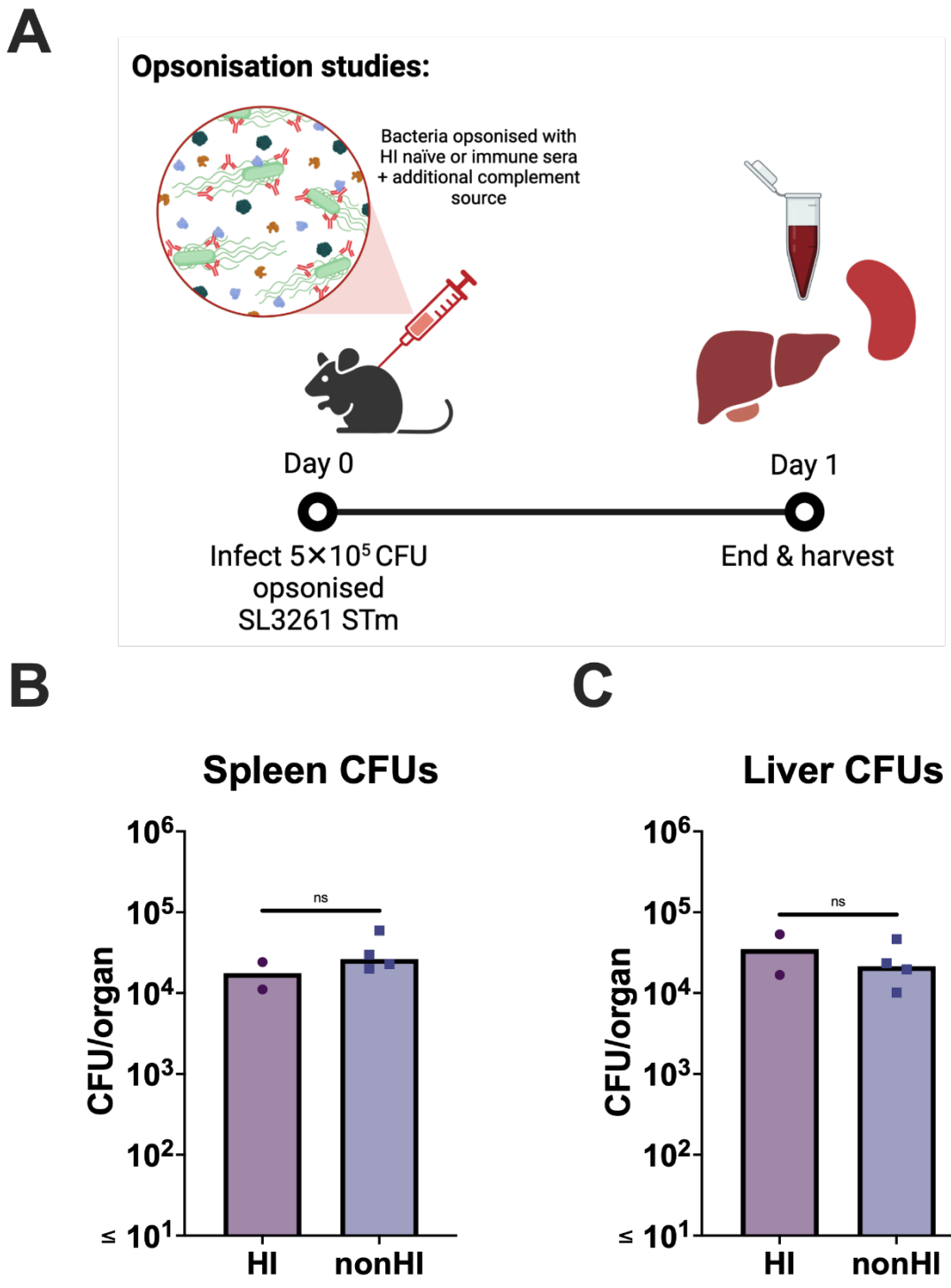

Figure 3.14 Supplementation of complement into C3-deficient mice does not significantly reduce bacterial burden in tissues. C3-/- mice were infected i.p. with pre-opsonised with immune sera 5×10^5 CFU SL3261 STm + a heat-inactivated (HI) or non-heat-inactivated (nonHI) complement source for 24 hours. Weights and bacterial burdens of the spleen (A and C) and liver (B and D) were quantified. Data are representative of one experiment, where each dot represents one mouse. Bars represent median of each group (n = 2 mice for heat-inactivated, n = 4 mice for non-heat-inactivated). Data were analysed by Mann Whitney U; ns = non-significant, * = P ≤ 0.05.

3.2.14 Adoptive transfer of sera 24-hours prior to infection reduces bacterial load in tissues of C3-deficient mice.

The above data indicate the supplementation of STm-specific Ab and complement into C3-deficient mice does not reconstitute the protective phenotype afforded by vaccination, when supplemented at the time of infection. However, the time points analysed after these interventions are relatively short. Consequently, we may have seen a difference in the $C3^{-/-}$ mice ability to control bacterial numbers in the organs, had we examined time points later than 24 hours.

To test whether a temporal component is also important for protective responses, we performed an adoptive transfer of serum into $C3^{-/-}$ mice. Mice received 200 µL of sera 24 hours prior to infection with STm SL3261. Sera was either heat-inactivated naïve, heat-inactivated immune, or non-heat-inactivated immune (**Fig. 3.15A**). These groups would provide insight into whether Ab alone would induce better control of bacterial numbers in tissues, or whether both Ab and complement is required.

These results show that the adoptive transfer of immune sera prior to infection reduced the bacterial burden in both spleens (**Fig. 3.15B**) and livers (**Fig. 3.15C**) compared to those mice that received heat-inactivated naïve sera. Further, supplementation of this immune sera with complement by lack of heat-inactivation can further reduce the bacterial burden in both organs compared to both mice that received heat-inactivated naïve sera and mice that received heat-inactivated immune sera. Therefore, antibody can partially rescue the protective immune response in vaccinated $C3^{-/-}$ mice when administered prior to infection, and this is further facilitated by providing an additional complement source.

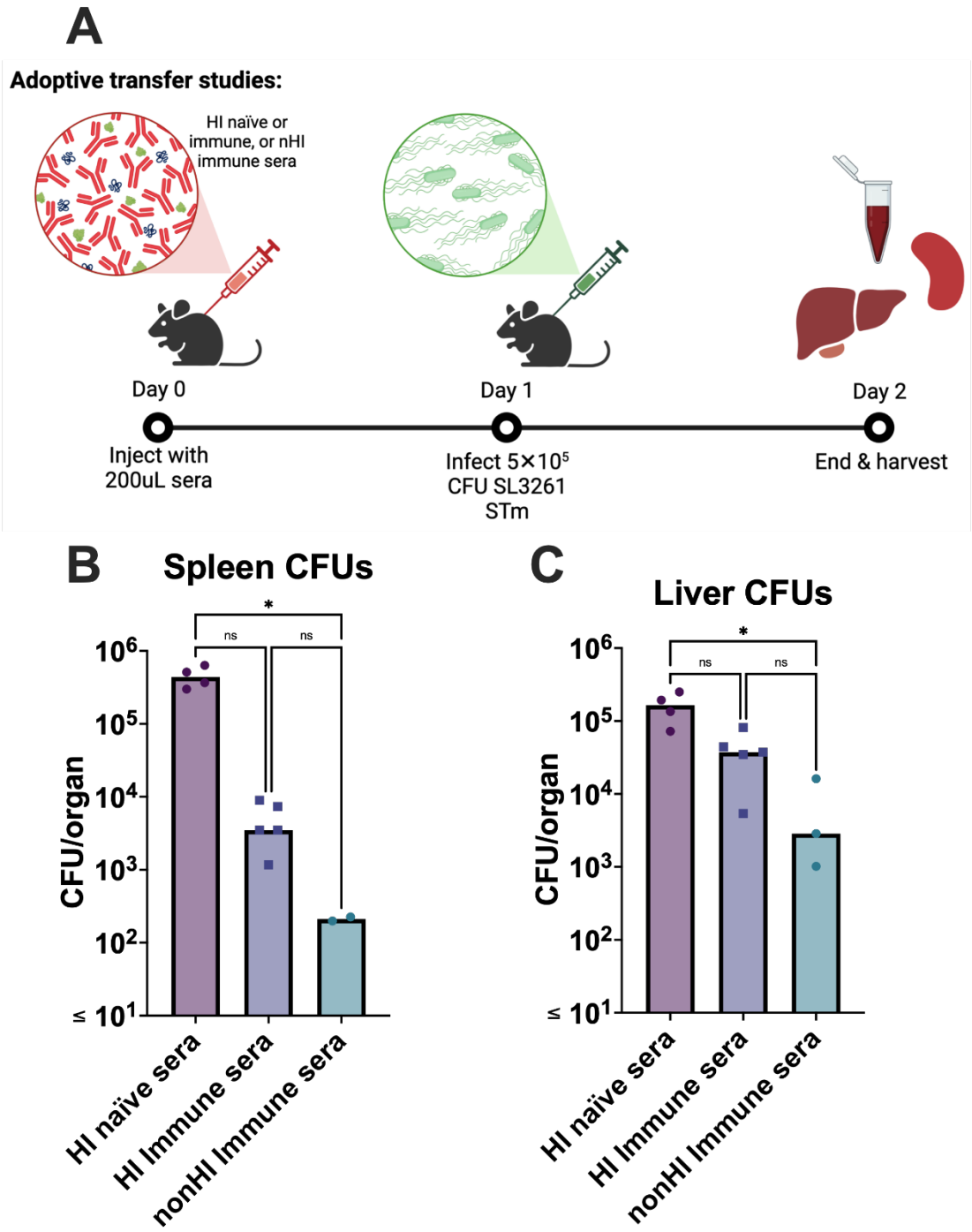

Figure 3.15 Adoptive transfer of antigen-specific antibody and complement 24 hours prior to infection reduces the bacterial burden in tissues of C3-deficient mice. C3-/- mice were injected i.p. with 200 µL heat-inactivated (HI) or non-heat-inactivated (nonHI) sera for 24 hours prior to i.p. infection with 5×10^5 CFU SL3261 STm. Weights and bacterial burdens of spleens (A and C) and livers (B and D) were quantified. Data are representative of one experiment, where each dot represents one mouse (n = 4 mice HI naïve, n = 5 mice HI immune, n = 3 mice nHI immune). Data were tested for normality using the Shapiro-Wilk test. Differences in CFU data were analysed using Kruskal-Wallis test with Dunn's multiple comparison; ns = non-significant, * = $P \leq 0.05$.

3.3 Discussion

My work has demonstrated an essential role for C3 in protective immune responses against invasive *Salmonella* Typhimurium (STm) infections, using outer membrane vesicles (OMVs) as a vaccine antigen. The classical and terminal pathways of complement activation is not essential for vaccine-mediated protection, as demonstrated by the ability of $C1q^{-/-}$, $C4^{-/-}$, and $C5^{-/-}$ mice to protect themselves against STm infection following prior immunisation. The results show that C3-deficiency results in abrogated antibody responses to STm antigens, especially IgM responses, and that this abrogation may be rooted in diminished cell populations responsible for antibody production. Finally, my results show that supplementation of antibody alone is not sufficient to rescue the non-protective phenotype of $C3^{-/-}$ following STm infection, however supplementation of both complement and antibody 24 hours prior to infection produces enhanced control of bacterial numbers in infected organs, indicating a temporal aspect of rescuing $C3^{-/-}$ phenotype.

Numerous studies have investigated the role of complement in infections (Bottermann et al., 2019; Price et al., 2015; Wessels et al., 1995; Brookes et al., 2018; Galdiero et al., 1984; Griffioen et al., 1991; Alvarez-Dominguez et al., 1993; Behet et al., 2018; Yuste et al., 2008; Mueller-Ortiz et al., 2004). Further, the role of complement in the control of *Salmonella* Typhimurium infections has specifically been discussed (Galdiero et al., 1984; Gondwe et al., 2010; Murray et al., 2006). Few of these studies have addressed the role of complement in protective immune responses following immunisation (Rossi et al., 2019; Siggins et al., 2011). Here, we have demonstrated a critical role for complement component 3 (C3) in successful vaccination against iNTS infections in mice, and its intertwined relationship with the protective humoral response.

Examination of primary immune responses to initial STm exposure revealed that absence of C1q, C3, nor C5 did not greatly affect the ability to control bacterial numbers in the spleen or livers of infected mice after 7 days of infection. Further, internalisation of bacteria as tested by gentamicin protection assays was also not significantly affected by any of the complement deficiencies tested. Differences in antigen-specific IgM or IgG titres were negligible in all mouse strains. Previous findings have demonstrated an essential role for complement in clearance of STm in humans (Gondwe et al., 2010; MacLennan et al., 2008), but not in mice (Siggins et al., 2011). These results concur that complement-deficiency did not affect control of STm infections in mice. Thus, any differences we would identify in vaccination and challenge experiments would be specific to secondary immune response to STm.

Vaccination and challenge of several complement-deficient mice has revealed a key component for successful protective immunity against STm. Absence of either C1q, C4, or C5 did not affect a mouse's ability to control bacterial numbers following vaccination as compared to the WT. However, vaccinated $C3^{-/-}$ mice did not demonstrate this control, showing similar bacterial burdens as their naïve controls. It has been previously suggested that an absence of C3 can affect antibody production (O'Neil et al., 1988; Alper et al., 1976; Pepys, 1974). It has been previously shown that antibodies against two abundant STm antigens, LPS and porins, develop in response to vaccination with OMVs (Schager et al., 2018). Further investigation of C3-deficient mice showed a distinct lack of IgM and low levels of IgG against LPS and OmpD, whereas other complement-deficient strains all demonstrated an ability to produce antibodies against these antigens. These results indicate $C3^{-/-}$ mice are unable to protect themselves following vaccination and challenge, and this inability is likely due to the severely limited levels of detectable antigen-specific antibody in the sera.

Identification of impaired humoral responses in the unprotective phenotype of $C3^{-/-}$ mice raised questions of a cellular defect, or if this phenotype is strictly antibody-dependent. Previous studies have suggested a role for C3 in immune cell development and function, including B cells (Ghannam et al., 2008; Kremlitzka et al., 2019; Werner et al., 2020). Further studies have discussed a role for C3 and its cleaved fragment's interaction with CD21 in humoral immune responses (Carroll, 1998; Roozendaal and Carroll, 2007; Meng and White, 2017; Carroll, 1999; Leslie et al., 2003; Kozono et al., 1998). Considering this, it is possible that the absence of C3 influences the humoral response by affecting the cells responsible for antibody production.

Using FACS, we demonstrated that $C3^{-/-}$ mice have diminished B cell populations, particularly those that are primarily responsible for antibody production. Notably there was a significant lack of follicular B cells in immunised $C3^{-/-}$ compared to WTs, yet MZ B cells were significantly higher in $C3^{-/-}$ mice. It is commonly accepted that B cell receptor (BCR) signal strength can influence the fate of a mature B cell and its differentiation into a follicular B cell, or MZ B cell. Stronger BCR signalling generally pushes a B cell to become a follicular, whereas a weaker signal produces MZ B cells (Cariappa et al., 2001). B cells are also known to express complement receptor 2 (CR2), or CD21, which is a potent co-activator of the BCR (Ahearn and Fearon, 1989; Kovács et al., 2021). CD21 ligands include fragments of C3, such as C3b, iC3b, C3d, and C3dg (Ahearn and Fearon, 1989; Kovács et al., 2021). It therefore stands to reason that in the absence of its ligands, CD21 would receive less stimulation. This lack of stimulation would in turn result in a lack of coactivation of the BCR, weaker BCR signalling, and a disproportionate development of mature B cells into MZ B cells rather than FO B cells.

Transitional B cells represent the intermediate stage in the development of bone marrow-derived immature B cells to mature B cells that reside in the secondary lymphoid organs (Suryani et al., 2010; Bemark, 2015; Zhou et al., 2020). Murine T1/T2 B cells are both precursors to MZ B cells, whereas T2 B cells are also capable of development into follicular B cells (Bemark, 2015). T3 B cells were once thought to be a subsequential stage of the mature B cell development, however it has been suggested that rather these T3 cells play other roles in the regulation of autoimmunity (Merrell et al., 2006; Teague et al., 2007). We identified that a developmental block between the T1 and T2 stages of B cell development occurs in immunised and challenge mice that lack C3. Maturation of T1 to T2 B cells in mice requires a range of signalling factors, such as interleukin-4 (IL-4), B cell activating factor (BAFF), ST6Gal-1, and Syk (Zhou et al., 2020). Human B cells were shown to internalise serum derived C3, which bound multiple genomic regions such as Syk, a signalling molecule that involved in lymphocyte differentiation and development (Kremlitzka et al., 2019). Therefore, B cell development signalling pathways may not be triggered in a C3-deficient environment, leading to smaller mature B cell compartments and impaired humoral responses upon vaccination.

Immunised $C3^{-/-}$ mice also demonstrated a depleted GC B cell compartment, in addition to lower numbers of ASCs, particularly in the PC populations. B cells activated by T cell signalling migrate to the follicular dendritic cell (FDC) networks within the B cell follicle, receiving further antigen stimulation and undergoing various rounds of clonal expansion and somatic hypermutation (MacLennan, 1994; Hamel et al., 2012). The GC B cells produce high-affinity and highly specific antibody secreting cells over the course of infection, in addition to long-lived plasma cells and memory B cells (Slifka et al., 1998; Hamel et al., 2012; MacLennan, 1994). Previous studies have

demonstrated a role of C3 in GCs through histological analysis (Zwirner et al., 1989), or by showing that interruption of the C3-CD21 interaction can significantly impair antigen-specific Ab production (Kopf et al., 1998). Recently, Cumpelik *et al.* have presented that GC B cells repress expression of C3 convertase regulator, decay acceleration factor (DAF) whilst promoting MAC-inhibitor CD59 expression through a BCL6-dependent mechanism. This permitted C3a and C5a signals necessary for GC B cell positive selection in the absence of MAC formation, and transgenic DAF overexpression or deletion or C3a/C5a signalling resulted in limited activation of mTOR and the premature collapse of the GC, leading to impaired affinity maturation and humoral responses (Cumpelik et al., 2021; Robinson et al., 2021). Therefore, an absence of C3 in mice impairs the GC reaction to vaccine antigens. The lack of GC reaction would not only imply poorly developed humoral responses, but also defective memory responses to secondary infection. This would therefore explain the inability of C3-deficient mice to control secondary systemic *Salmonella* infections.

So far, the results have indicated that in the absence of C3, mice experience minimal GC reactions, a lack of B cell development and differentiation, and thus limited humoral responses to STm antigen. These results would explain why we see naïve-like responses in the immunised $C3^{-/-}$ mice, as no long-lasting memory B cells have been formed following vaccination, and thus no fast-acting secondary response to STm encounter is formed. However, in the context of primary infections, $C3^{-/-}$ did not behave very differently to WT, or either other complement-deficient mice, regarding their ability to control bacterial numbers nor their antigen-specific humoral responses. In this regard, we must consider that when we investigated ASCs in immunised and challenged mice, the PB numbers observed were not significantly lower than those observed in WT mice. PBs are short-lived proliferating ASCs, with short lifespans (3-

5 days) and a constantly replenishing population that arise from various mature B cell subsets (Tellier and Nutt, 2019; Khodadadi et al., 2019). PCs on the other hand are post-mitotic long-lived ASCs with lifespans of anywhere from several months to a lifetime (Hammarlund et al., 2017; Slifka et al., 1998). PCs arise from the GC reaction (or sometimes from PBs that receive the correct PC survival niche signals (Khodadadi et al., 2019)), and for this reason are key players in the memory response to infection. As the short-lived PB population was not significantly smaller in the $C3^{-/-}$ mice, the initial stages of the humoral response may not significantly rely on C3. Therefore, in the context of primary responses, $C3^{-/-}$ mice are not anymore hindered in their ability to control infection compared to their WT counterparts. Further, the sustained/growing levels of bacteria and therefore bacterial antigen in mice that had only experienced a primary infection may provide the necessary threshold of signalling required for B cell activation, even without interaction of C3 with CD21 and the BCR complex. However, in the context of vaccination and challenge, a longer amount of time has elapsed between the initial vaccine dose and secondary exposure to bacterial antigen, leading to diminished PB repertoires and, in conjunction with an impaired GC response, an absence of long-lived memory PCs that are able to respond to secondary exposure rapidly.

These findings are supported by the results from both the opsonisation and preliminary adoptive transfer studies. Indeed, where only an exogenous source of Ab was supplied, $C3^{-/-}$ mice were unable to control the bacterial burden in spleens and livers as competently as WT mice who received the same antibody. Further, addition of an exogenous complement source through pre-opsonisation of bacteria also did not afford a greater control of bacterial burden in $C3^{-/-}$ mice. Yet when both exogenous antibody and complement were received by adoptive transfer of sera, $C3^{-/-}$ mice

gained an enhanced ability to control bacterial burden in tissues than those mice that received no additional Ab nor complement, or those that only received additional Ab. This would suggest a temporal component of C3 to aid in bacterial control, where adoptive transfer of complement and antibody affords the time to effectively prime the host's B cells, ready to challenge incoming infection. This may be achieved through C3-CD21 interactions and co-activation with the BCR, which would further lower the threshold of activation required for B cell development and differentiation.

Whilst these data are promising in deciphering the exact role of C3 in vaccine-mediated immunity against STm, some gaps still exist. In the first instance, repeated experiments of the adoptive transfer studies must be considered to confirm the results found in preliminary data. Further, these mice would be examined for their serum Ab responses, to see if the ability of ASCs would be restored following adoptive transfer of both complement and antigen-specific Ab. It would also be of use to include a control group that would receive only an exogenous complement source in the absence of antigen-specific Ab. This would provide insight into the relative contributions of Ab and complement in the C3-deficient context.

Additionally, some investigation into the temporal effects of C3-deficiency should be considered. These data have already indicated that differences in primary infection are not evident between $C3^{-/-}$ mice and WT, yet the discrepancies of these two strains following vaccination and challenge are obvious. Future experiments may include time-course studies, where WT and $C3^{-/-}$ mice are observed under three conditions over several days. These conditions would include a primary infection, immunisation only, and immunisation + challenge, to be observed at days 1, 3-, 7-, 10-, and 14-days post-infection. In this suggested experiment, it would be critical to observe the development of different B cell populations, specifically ASCs, over the course of

immunisation or infection to provide insight into how these cell populations develop over time, how they persist, and how this may relate to antigenic burden in tissues either as vaccine antigen or live bacterial cells.

In conclusion, successful immunity against *Salmonella* elicited by immunisation with complex OMV antigen is mediated by a C3-dependent mechanism. These results indicate that C3 has a widespread impact on protective humoral responses to *Salmonella* that begin at the cellular level. These results are important to consider in the design and use of vaccines in immunocompromised individuals. Further, the results also signify an importance of innate immune components such as the complement system as adjuvants on the development of robust adaptive responses, which should be considered during the design of complex vaccines against invasive infections and disease.

CHAPTER 4: EXPLORING DEVELOPMENT OF ANTIGEN PERSISTENCE IN OMV-IMMUNISED MICE

4.1 Introduction

Full registration of new vaccines into the clinical setting requires rigorous testing. Initially, pre-clinical testing is performed in laboratory and animal studies that largely identify novel vaccine targets for infectious diseases.

Clinical trials are performed for promising vaccine candidates and are separated into distinct phases. Phase I trials are small trials, often using a handful of healthy volunteers to initially demonstrate safety and determine dosage. Phase II trials increase their group sizes to hundreds of participants, and these are usually a more targeted group of the intervention being studied. These studies continue to assess safety of the vaccine, whilst also measuring the immunogenicity. Phase III trials are larger scale trials (thousands of participants) that assess safety and efficacy of a vaccine, such as the ability of the vaccine to prevent infections and reduce disease burden over a defined period. Vaccines that successfully graduate from Phase III trials may be granted provisional registration and licensure. Finally, Phase IV trials are conducted after a vaccine's licensure and clinical availability, collecting data over a wide range of populations and surveillance of rare adverse effects and long-term efficacy. To enter clinical assessment in humans, a vaccine must show reasonable pre-clinical proof-of-concept, safety, immunogenicity, and efficacy in relevant models. These models may at first involve *in vitro* testing of vaccine compounds before assessment in animal models such as mice or non-human primates (NHPs).

Whilst several vaccines are available clinically against typhoid fever, several NTS vaccines are either in preclinical or early phase clinical development (**Fig. 4.1**). Before 2019, the only candidate vaccine that was being tested in humans was the live attenuated WT05 vaccine, however assessment of this halted due to prolonged fecal shedding (World Health Organisation, 2022; Hindle et al., 2002). Glycoconjugate vaccines involving conjugation of NTS O-antigen to carrier proteins have been

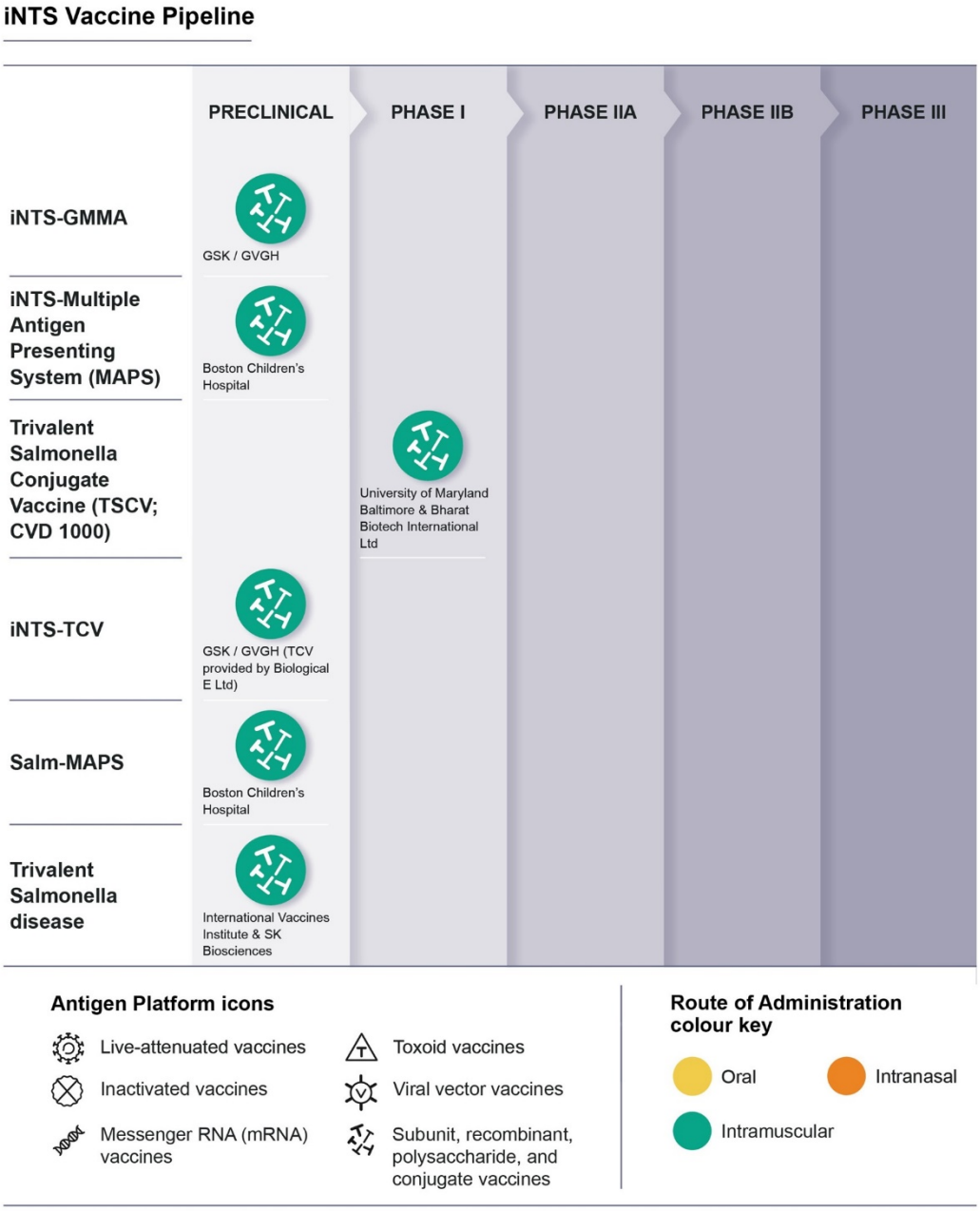

Figure 4.1: Current pipeline of iNTS vaccines. Infographic available from WHO website https://www.who.int/teams/immunization-vaccines-and-biologicals/diseases/nontyphoidal-salmonella-disease

explored in several animal models and were showed to elicit protective antibodies (Watson et al., 1992; Svenson and Lindberg, 1981). A trivalent iNTS-typhoid conjugate vaccine called CVD 1000 is currently in phase I clinical trials (Chen, 2021). Generalised modules for membrane antigens (GMMA)-based bivalent vaccines against both *S.* Typhimurium and *S.* Enteritidis are soon to enter Phase I clinical trials and have so far shown favourable immunogenicity and efficacy in mouse models (Micoli et al., 2018).

Experimental drugs and vaccines require stringent monitoring during their development in case of reactogenicity or adverse events. This is particularly true in preclinical trials before human testing, as evidence of pathological consequences to either drug treatment or vaccination can inform of harmful side effects that would occur in volunteers during early clinical trials. Therefore, identification of artefacts of successful vaccination not only highlight potential mechanisms of protective immunity, but also could give indication of an experimental vaccine's safety which are necessary for informing candidacy for clinical trials.

Histological analysis of spleens from immunised mice in chapter 3 alluded to a persistent antigen phenotype in mice that were immunised with OMVs prior to infection. Previous studies have reported incidence of persisting pathogenic antigen following acute infections. Persistent viral antigen has been reported in various models of viral infection in mice, and the persistence of this antigen has been suggested to influence the quality and memory of T cell responses post-infection (Kim et al., 2010; Schwarze et al., 2004; Zammit et al., 2006). In a study of vaccination against malarial liver stage parasites, development of robust protective $CD8^+$ T cell responses required prolonged antigen presentation in a $CD11c^+$ cell-dependent mechanism, and antigen was found to persist 8-weeks post-immunisation despite the vaccine candidate's non-

replicating and liver-stage-restricted nature (Cockburn et al., 2010). In a study of vaccine use as cancer therapies, researchers reported that persistent antigen in the vaccine site microenvironment induced by repeated same-site delivery may lead to persistent stimulation of immune pathways, causing beneficial specific T cell expansion and improved immunogenicity (Meneveau et al., 2022). In each of these cases, persisting antigen was viewed as an immunostimulatory benefit of infection or immunisation. On the other hand, persistent viral antigen has been identified in multiple organs of SARS-CoV-2 patients despite testing negative for active infection and has been correlated with chronic disease symptoms and long-COVID (Cheung et al., 2022; Swank et al., 2023), demonstrating the potential of pathological outcomes in antigen persistence phenotypes. However, these studies relate to viral or parasitic infections and rarely address persistent bacterial antigen.

Review of the literature regarding persisting bacterial antigen indicates that most articles and reviews focus on the persistence of live infections as opposed to the persistence of antigen in the absence of infection (Rhen et al., 2003). In a specific example, persistence of bacteria in the stool of volunteers immunised with live attenuated STm strain WT05 was observed up to 23 days post-immunisation (Hindle et al., 2002). Alternatively, the term ‘persistence’ in the context of vaccine research often refers to the persistence of induced antigen-specific humoral or cellular responses (Gilca et al., 2013; Martinón-Torres et al., 2019; Leuridan and Van Damme, 2007). Rarely, if ever, has the persistence of vaccine-dependent bacterial antigen been explored, nor its consequence on successful immunisation and protection of the host. Here, I describe my observation of antigen persistence that is only found in OMV-vaccinated and challenged animals. I determined the host-driven and vaccine-specific

factors that led to the development of this phenotype by using a range of knockout and depletion models, in addition to alternative vaccine antigens.

4.2 Results

4.2.1 Persistent STm^{+} antigen is observed in infected OMV-immunised mice spleens, despite low recoverable CFUs

Invasive *Salmonella* infections disseminate through the blood and lymph to colonise secondary sites, including the spleen and liver, and so protection afforded by vaccination can be assessed by measuring the bacterial burden within these organs. WT C57BL/6 mice were immunised with WT OMVs and then infected 35 days later with 2 x 10^5 CFU of attenuated STm strain SL3261, prior to analysis at 1- or 35-days post-infection to model acute and chronic infection. These results show that bacterial burden in the spleen is significantly reduced ~1000-fold in mice that received OMVs prior to infection (**Fig. 4.2A**). Therefore, protective immune responses following OMV vaccination persist for up to 35 days post-immunisation. At 35 days post-infection, bacterial burden of mice was ~10-fold lower in those that received OMVs compared to naïve controls, but this was not statistically significant. Therefore, protection afforded by OMVs is evident within he first 24 hours of infection, but this effect waned over time to be less significant during chronic infection.

Histological analysis of spleen tissues in the previous chapter (**Fig. 3.8**) indicated a phenotype of antigen persistence in mice that were OMV-immunised prior to infection with STm. This phenotype was unexpected due to the low recoverable CFU from organs of these mice. In light of this result, I wanted to confirm whether this was a true antigen persistence and its nature with further experiments. To replicate this finding and identify the longevity of this phenotype, spleens from immunised only, immunised

+ D1 infection, and immunised + D35 infection were assessed by histology and IHC. Representative immuno-stained spleen sections can be seen in **Fig. 4.2B**, with $Ly6G^+$ in brown and STm^+ in blue. Quantification of the STm^+ staining can be seen in **Fig. 4.2C**. Ly6G was used as a counterstain as it provided distinction between red and white pulp (RP and WP, respectively) regions of the spleen whilst not masking the blue stain of the STm. To identify if antigen persistence was present in vaccinated mice that had not experienced infection, mice immunised with WT OMVs for 35 days were analysed by histology and the STm^+ antigen quantified. Immunisation with OMVs in the absence of infection did not show a significant amount of STm^+ antigen in the spleen (**Fig 4.2B & C**). However, at 1-day post-infection there was a striking difference between naïve and OMV-immunised groups, since the frequency of blue STm^+ staining is markedly increased in the immunised group (**Fig. 4.2B,** highlighted by black arrows**)**. When quantified, the fraction of the area measured with STm^+ signal was approximately 12-fold higher in the RP of the immunised spleens, and ~4-fold higher in the marginal zones (**Fig. 4.2C**). This effect was largely restricted to the RP and marginal zone (MZ) of the spleen and did not occur in the WP. Spleens were also counterstained for marginal zone macrophage marker CD169 (Grabowska et al., 2018) (data not shown). STm^+ stain did not localise specifically to $CD169^+$ cells and thus the MZ, correlating with quantification data that antigen persistence was most commonly observed in the splenic RP. Antigen persistence was also observed at day 35 post-infection, albeit at a lower level than at day 1 post-infection **Fig. 4.2B and C**). Therefore, STm^+ antigen persists in spleens of mice that receive an OMV vaccination, but only after infection and may persist for up to 35 days.

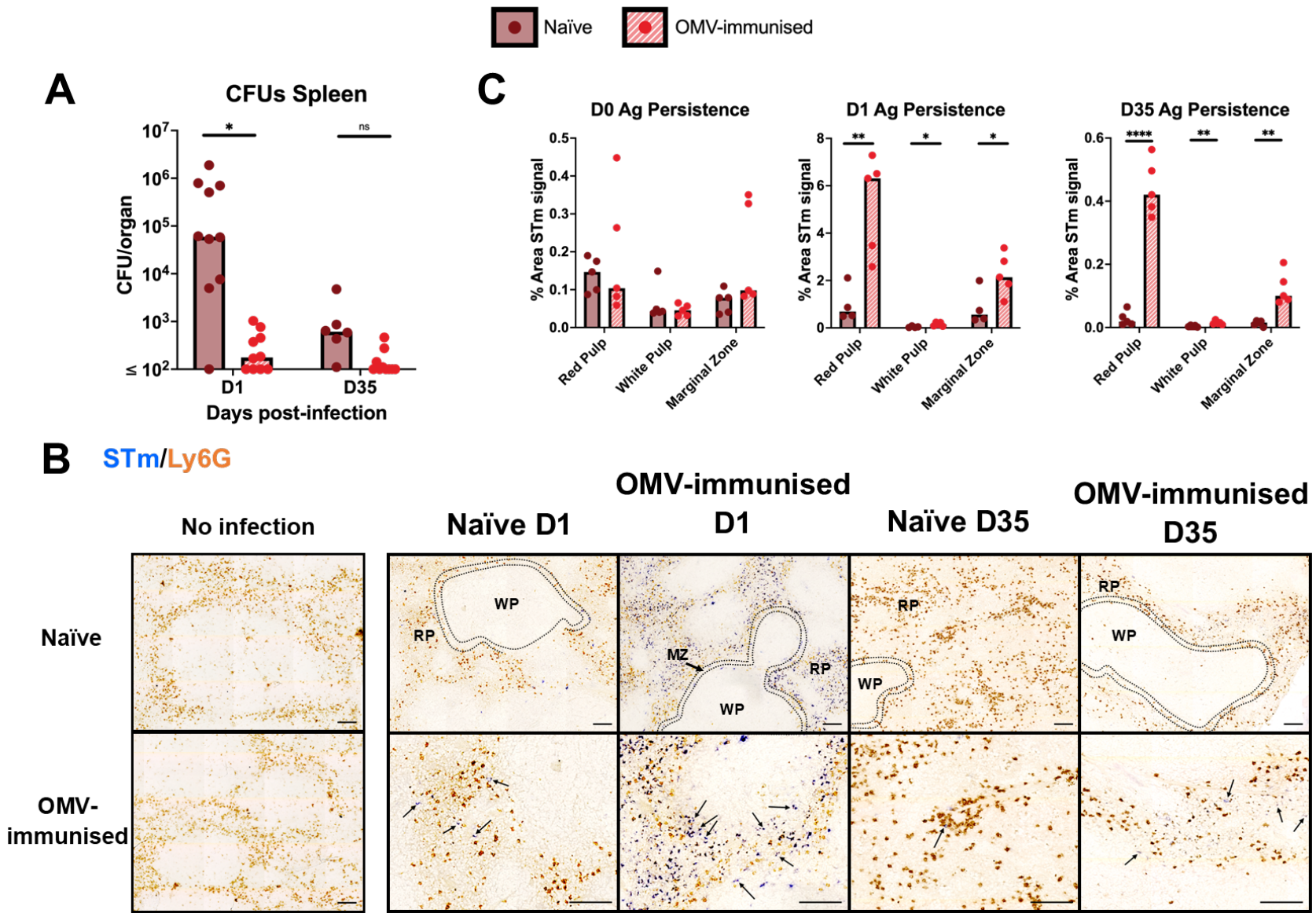
Naïve
OMV-immunised
A
CFUs Spleen
CFU/organ
Days post-infection
D1
D35
ns
C
D0 Ag Persistence
D1 Ag Persistence
D35 Ag Persistence
% Area STm signal
Red Pulp
White Pulp
Marginal Zone
B
STm/Ly6G
No infection
Naïve D1
OMV-immunised D1
Naïve D35
OMV-immunised D35
Naïve
OMV-immunised
WP
RP
MZ

Figure 4.2: OMV-immunised and infected mice show greater STm+ staining despite lower viable CFUs in spleens. C57BL/6 mice were immunised i.p. with with 1 µg *ΔtolR* OMVs for 35 days before i.p. infection with 2 x 10^5 CFU SL3261 STm. Spleens were harvested and weighed after 24 hours or 35 days post-infection. (A) spleens were mashed, and bacterial burden enumerated; (B) spleens were snap-frozen, sectioned and stained using IHC, imaged with Zeiss AxioScan 7 and processed using Zeiss Zen Blue software v3.7; (C) frequency of STm+ signal in IHC stained spleens was quantified using ImageJ v1.54d with ColorDeconvolution2 plugin. In (B), STm is stained in blue and Ly6G in brown, RP = red pulp, MZ = marginal zone, WP = white pulp, arrows highlight STm^+ staining and scale bars denote 100 µM. CFU data are collated from two independent experiments (*n* = 5), where each dot represents one mouse. Antigen persistence histology images and data are representative of 2 independent experiments (n = 5). Bars represent the median of each group. CFU data was statistically analysed using Kruskal-Wallis test with correction for multiple comparisons, persistence data was analysed using unpaired *t*-test; * = p ≤ 0.05, ** = p ≤ 0.005, *** = p ≤ 0.0005, **** = p ≤ 0.0001.

4.2.2 Persistent STm^+ antigen is also observed in infected OMV-immunised mice livers

As mentioned previously, invasive *Salmonella* infections can disseminate outside of the GI tract to colonise secondary sites such as the liver. The liver, whilst possessing its own repertoire of immune cells, is not considered a secondary lymphoid organ. Therefore, we may expect to see an increased level of antigen in the spleen as it is a major site of antigen processing and presentation, but this phenotype may not be present in non-lymphoid tissues such as the liver. To identify if the persistent phenotype occurs in non-lymphoid organs, the livers of immunised mice were assessed for bacterial burden and antigen persistence. These data are presented in **Fig. 4.3**. Prior immunisation with OMVs significantly reduced the viable bacterial burden of livers ~100-fold in mice that were infected for one day, and non-significantly reduced <100-fold for those infected for 35-days (**Fig. 4.3A**).

Histological analysis of liver sections shows antigen persistence is present in the liver in mice that were immunised prior to infection (**Fig. 4.3B**). This phenotype was notable

at both day 1 and day 35 post-infection (highlighted by black arrows) and is reflected in the quantified percentage area with STm^+ stain in **Fig. 4.3C.** In contrast with the spleen, livers from immunised mice infected for 35 days showed an overall higher percentage area with STm^+ stain compared to those infected for 1 day (compare Fig 4.2C with Fig 4.3C). Therefore, STm^+ antigen persists in livers of mice that receive an OMV vaccination prior to infection, and this phenotype persists and increases for up to 35 days post-infection.

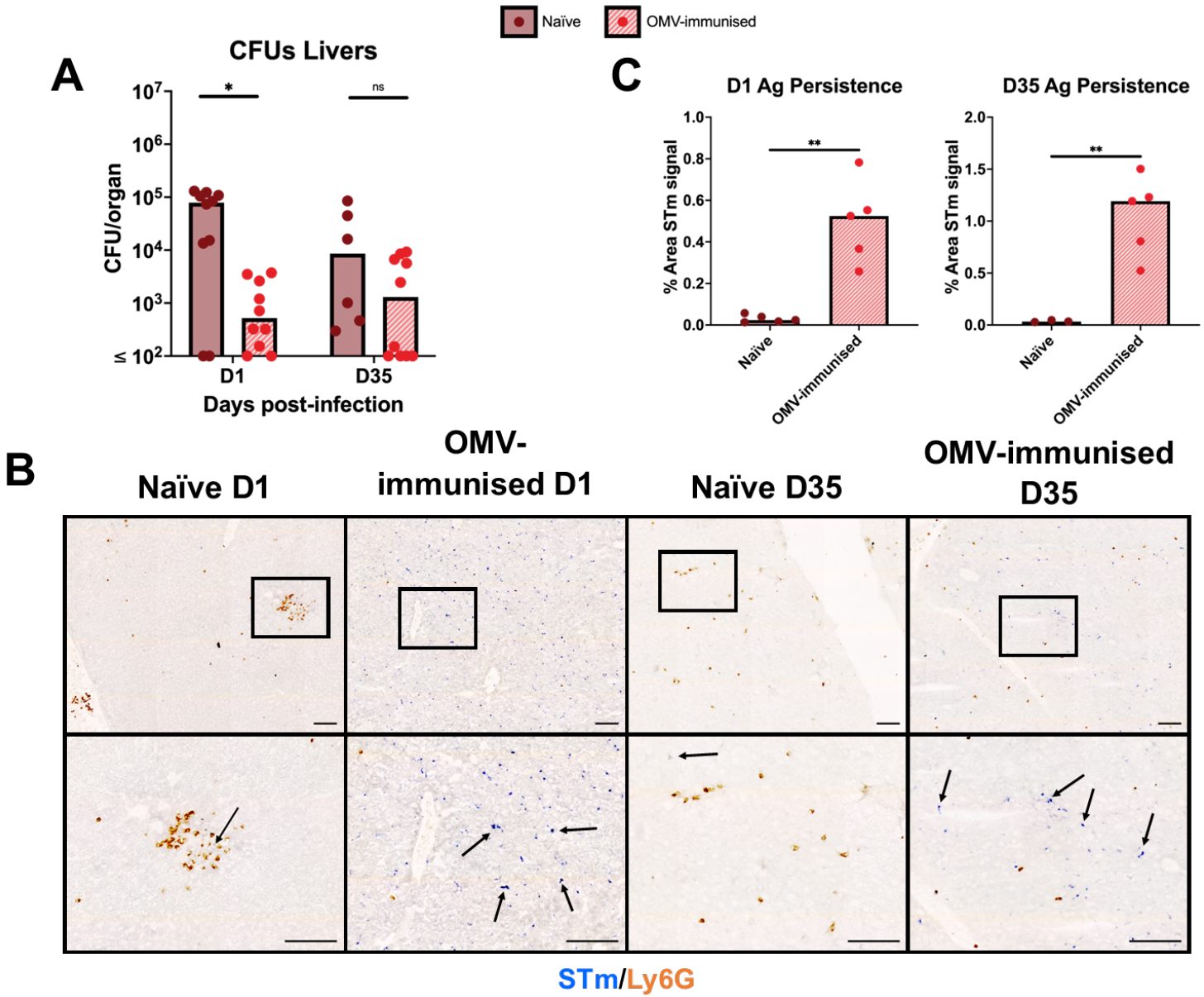

Figure 4.3: OMV-immunised and infected mice show greater STm+ staining despite lower viable CFUs in livers. C57BL/6 mice were immunised i.p. with with 1 µg *ΔtolR* OMVs for 35 days before i.p. infection with 2 x 10^5 CFU SL3261 STm. Livers were harvested and weighed after 24 hours or 35 days post-infection. (A) livers were mashed, and bacterial burden enumerated; (B) livers were snap-frozen, sectioned, and stained using IHC, imaged with Zeiss AxioScan 7 and processed using Zeiss Zen Blue software v3.7; (C) frequency of STm^+ signal in IHC stained livers was quantified using ImageJ v1.54d with ColorDeconvolution2 plugin. In (B), STm is stained in blue and Ly6G in brown, arrows highlight STm+ stain and scale bars denote 100 µM. CFU data are collated from two independent experiments (*n* = 5) where each dot represents one mouse. Histology images and data are representative of 2 independent experiments (n = 5). Bars represent the median. CFU data was statistically analysed using Kruskal-Wallis test with correction for multiple comparisons, persistence data was analysed using unpaired *t*-test; * = $p \leq 0.05$, ** = $p \leq 0.005$.

4.2.3 *Salmonella*-specific antibody titres in sera increases in WT OMV-vaccinated mice and are maintained during chronic infections

Antigen persistence and dysregulation of humoral immunity have been correlated with sufferers of long-COVID disease following SARS-CoV-2 infection (Opsteen et al., 2023). OMVs are potent inducers of humoral immunity (Schager et al., 2018; Zhang et al., 2017), thus, to determine if a correlation exists between antibody responses and antigen persistence following OMV-immunisation, I measured sera of mice for specific anti-*Salmonella* IgM, IgG, and IgA by ELISA (**Fig. 4.4**). Antigens tested for include whole cell STm, whole OMVs, LPS, and OmpD.

Humoral immune responses to primary challenge with antigen are dominated by IgM (Sathe and Cusick, 2022), and in some cases of non-protein antigen, the main isotype induced. IgM are generally low-affinity antibodies, but their pentameric structure improves their overall avidity for cognate antigen (Burrell et al., 2017), and substantial levels of IgM can be detected within 1 week of primary challenge. In these experiments, IgM was consistently higher in the OMV-immunised groups than the naïve, but usually only in those that were experiencing active infection (**Fig. 4.4, leftmost panels**). The only exception in this case was anti-OMV IgM, where uninfected OMV-immunised mice showed elevated levels of anti-OMV IgM compared to the naïve. Anti-OMV IgM, however, was higher than expected in the naïve, uninfected group which may be a result of high background measurements during this ELISA. Overall, IgM was higher in OMV-immunised groups experiencing active infections, suggesting an accelerated response to antigen with prior exposure.

IgG are a highly specific, high affinity isotype generated following class-switching recombination (CSR) and maturation of the antibody response, typically only rising to

detectable levels at later time points of infection (Muramatsu et al., 2000). I would therefore expect to see substantial IgG titres in those mice that had received immunisation, or in mice with no prior immunisation but infected for an extended length of time. IgG titres against all antigens tested (**Fig. 4.4, middle panels**) were elevated in all immunised groups, regardless of infection status. IgG titres measured in naïve mice only approached similar levels as those immunised when infection had continued until day 35. Therefore, production of substantial anti-*Salmonella* IgG titres required prior exposure via prior immunisation or lengthened infections, and immunisation with OMVs 35 days prior to infection provided these substantial levels against all anti-*Salmonella* antigens tested.

IgA is produced after CSR and somatic hypermutation in germinal centres (Muramatsu et al., 2000), is the main humoral contributor to immunity in mucosal membranes and is the most abundant antibody isotype produced in mammals/humans (Bonner et al., 2009). Despite being a primarily muscoal isotype, serum IgA is capable of potent effector functions against both bacterial and viral infections (Davis et al., 2020). When immunised with OMVs and/or infected with STm, anti-*Salmonella* IgA titres were typically lower compared to either IgM or IgG (**Fig. 4.4, rightmost panels**). IgA levels against OMVs and OmpD were negligible regardless of immunisation or infection status. However, IgA against both whole cell STm and LPS were detectable in OMV-immunised mice at day 1 post-infection, in addition to both groups of mice at day 35 post-infection. This suggests an advanced LPS-directed IgA response following immunisation, but only in those mice that are experiencing active infection.

Overall, immunisation with WT OMVs prior to infection induce a robust humoral response against common anti-*Salmonella* antigens. IgM and IgA are typically elevated in immunised mice in the event of active infection, whereas IgG persists in

the serum of primed, non-infected mice. Therefore, induction of robust IgM or IgG responses correlate with groups of mice that exhibit the antigen persistence phenotype.

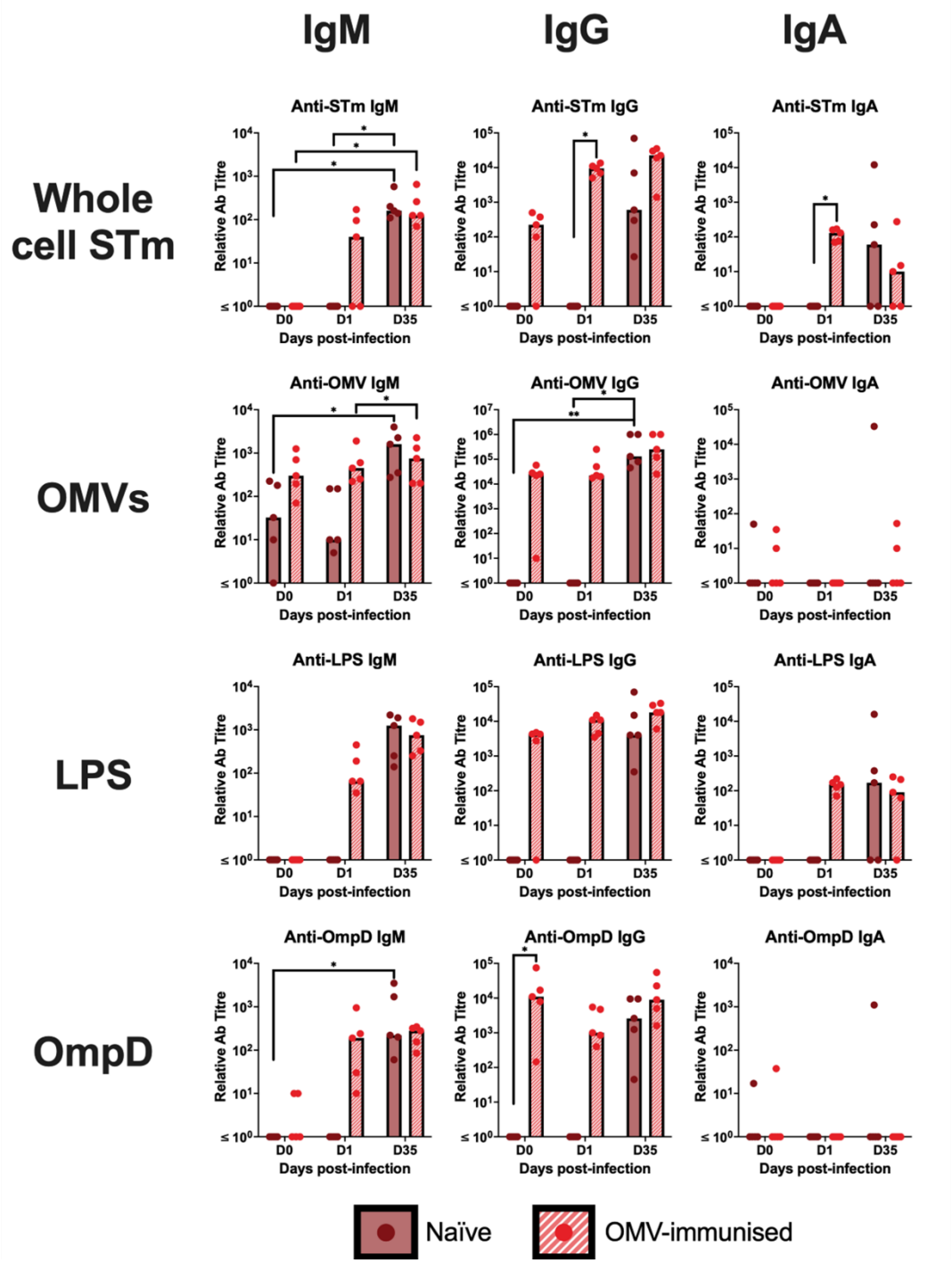

Figure 4.4: Antibody titres in OMV-immunised and infected mice. C57BL/6 mice were immunised i.p. with with 1 µg *ΔtolR* OMVs for 35 days before i.p. infection with 2 x 10^5 CFU SL3261 STm. sera were harvested and weighed after 24 hours or 35 days post-infection and analysed for STm-specific antibody titres by ELISA. Anti-STm IgM, IgG, and IgA were measured. Data are representative of two independent experiments (*n* = 5). Each dot represents one mouse, bars represent the median of each group. Data was statistically analysed using Kruskal-Wallis test with correction for multiple comparisons; * = $p \leq 0.05$, ** = $p \leq 0.005$.

4.2.4 Subcutaneous administration of WT OMVs also produces antigen persistent phenotype in spleens of mice infected with *Salmonella* Typhimurium

The intraperitoneal route of drug or vaccine administration is commonly used in rodent studies due to its procedural ease and relatively lower level of discomfort and stress induced on the animals (Al Shoyaib et al., 2019). However, this route of vaccine administration is rarely used in the clinic, with most vaccines being intramuscular, subcutaneous, intranasal, or intradermal. Therefore, i.p. injection of OMVs may not accurately reflect the development of antigen persistence in the clinic. To address this, I examined antigen persistence in mice immunised with the more clinically relevant subcutaneous route. Mice were immunised 14 days prior to infection by subcutaneous (s.c.) injection in the scruff of C57BL/6 mice with WT OMVs, followed by analysis at 1 day post-infection with SL3261 STm. Spleens and livers were assessed for bacterial burden to identify protective immunity, and spleens were examined by histology for antigen persistence (**Fig. 4.5**).

S.c. delivery of WT OMVs significantly reduced the bacterial burden in both spleens and livers of mice compared to naïve controls (**Fig. 4.5A**). Immunohistochemical analysis showed that persistent antigen is also present in mice that are immunised by s.c. injection, but this is fainter than that seen in mice immunised i.p (**Fig. 4.5B**). However, quantification of this antigen did not show a significantly higher amount of antigen in immunised mice compared to naïve controls (**Fig. 4.5C**). These results may be affected by less intensity of the persistent antigen stain, which may obscure subtle differences and additional methods may be needed to examine lower levels of antigen more accurately. Therefore, antigen persistence is a phenotype that occurs through

both i.p. and s.c. injection of OMVs, but the intensity of this STm^+ antigen is reduced following s.c. immunisation.

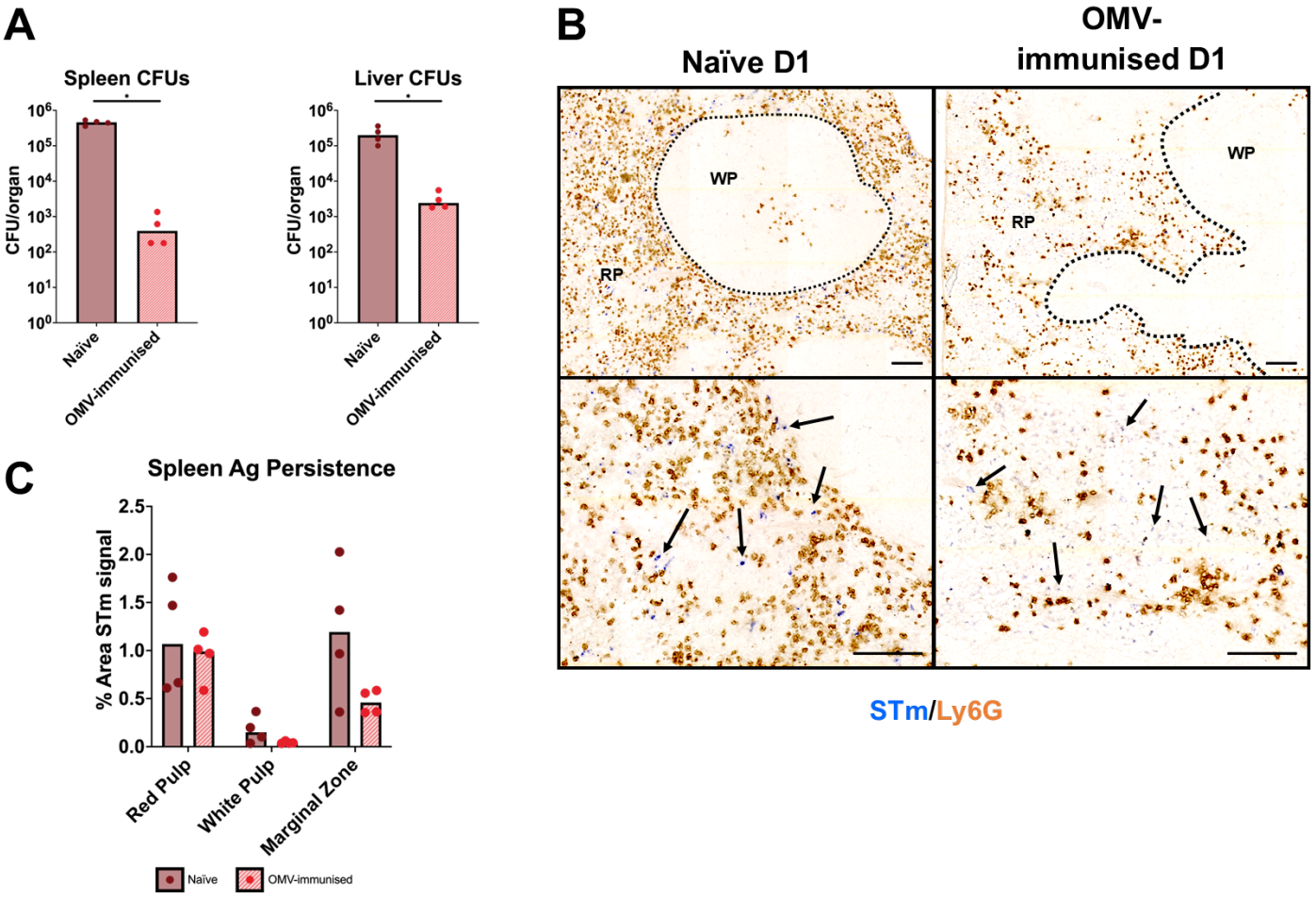

Figure 4.5 Mice immunised by subcutaneous injection of OMVs show antigen persistence in tissues following infection. C57BL/6 mice were immunised with with 1 µg ΔtolR OMVs s.c. in the scruff for 14 days prior to infection with 5 x 105 CFU SL3261. Spleens and livers were harvested and weighed 24 hours post-infection. Spleens and livers were segmented and processed: (A) organs were mashed and bacterial burden enumerated; (B) spleens were snap-frozen and sectioned into 6 µM slices, stained using IHC, imaged with Zeiss AxioScan 7 and processed using Zeiss Zen Blue software v3.7; (C) frequency of STm+ signal in IHC stained spleens was quantified using ImageJ v.1.54d with ColorDeconvolution2 plugin. In (B), STm is stained in blue and Ly6G in brown, RP = red pulp, MZ = marginal zone, WP = white pulp. Arrows highlight STm+ stain and scale bars denote 100 µM. Data are representative of two independent experiments (n = 4). Each dot represents one mouse, bars represent the median of each group. Data were statistically analysed using unpaired *t* test; * = $p \leq 0.05$.

4.2.5 Antigen persistence occurs in resistant mouse strains infected with a virulent STm isolate

Use of laboratory-adapted strains of STm such as SL3261 present concerns regarding their adequacy for the study of "real-world" pathogenesis. Years of sub-culturing bacteria from their first isolation may result in mutations and resulting in loss of physiological characteristics and adaptation to *in vitro* conditions (Fux *et al*., 2005). To address this concern in the context of the antigen persistence phenotype identified above, resistant CD1 mice were immunised with WT OMVs prior to infection with virulent, multidrug resistant clinical STm isolate D23580 (Yang et al., 2015), as outlined in **section 4.3.1**.

As shown in **Fig. 4.6A**, immunisation with OMVs protected against bacterial infection with this strain in spleens and livers, as evident by the significantly reduced bacterial burden in both organs at 1-day post-infection. At 35-days post-infection, bacterial burden was not significantly different between the naïve and immunised group, indicating that the bacteria are largely cleared from these organs without prior protection. As seen with the attenuated strain, STm^+ antigen was more frequent in OMV-immunised mice, particularly in the RP and marginal zone of infected spleens (**Fig. 4.6B** and **C**). Therefore, OMVs provide protection against and induces the antigen persistence phenotype during infection with clinically relevant strains of *Salmonella*.

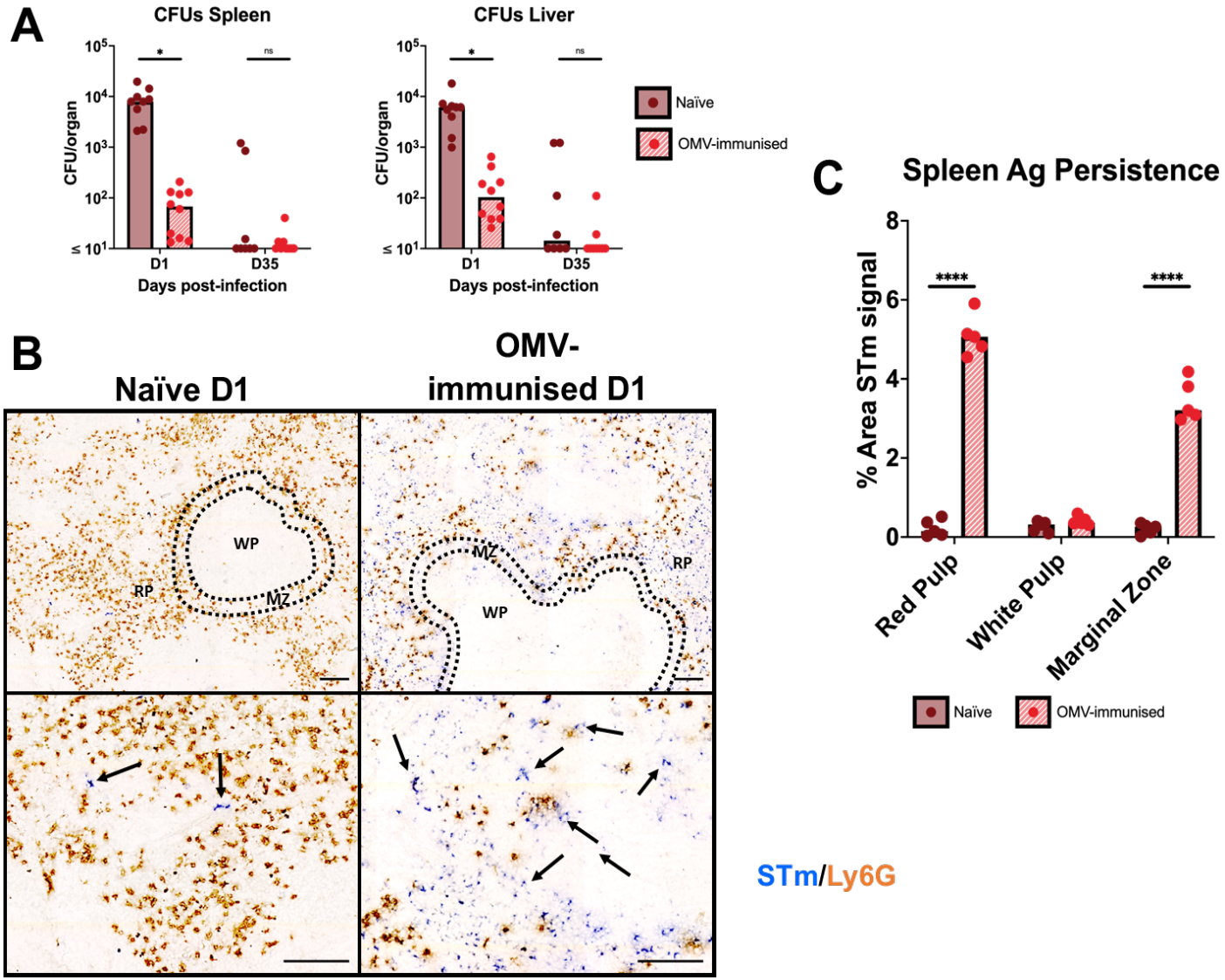

Figure 4.6 Antigen persistence is present in OMV-immunised resistant mice infected with MDR virulent STm. CD1 mice were immunised with with 1 µg ΔtolR OMVs i.p. for 35 days prior to i.p. infection with 2.5 x 10^5 CFU virulent STm strain D23580. Spleens and livers were harvested and weighed 24 hours or 35 days post-infection. Organs were segmented and processed: (A) organs were mashed and bacterial burden enumerated; (B) spleens were snap-frozen and sectioned into 6 µM sections, stained using IHC, imaged with Zeiss AxioScan 7 and processed using Zeiss Zen Blue software v3.7; (C) frequency of STm+ signal in IHC stained spleens was quantified using ImageJ v.1.54d with ColorDeconvolution2 plugin. In (B), STm is stained in blue and Ly6G in brown, RP = red pulp, MZ = marginal zone, WP = white pulp. Arrows highlight STm+ stain and scale bars denote 100 µM. Data are representative of two independent experiments (n = 4). Each dot represents one mouse, bars represent the median of each group. CFU data was statistically analysed using Kruskal-Wallis test with correction for multiple comparisons, persistence data was analysed using unpaired *t*-test; * = $p \leq 0.05$, **** = $p \leq 0.0001$.

4.2.6 Antigen persistence following vaccination with other antigens

4.2.6.1 Antigen persistence does not occur in spleens of sFliC-immunised mice

I next wanted to explore whether other antigens could also induce antigen persistence. To do this, I examined the soluble flagellin (sFLiC) vaccine. Flagellin has frequently been used as an adjuvant and can induce potent Th1 and Th2 B and T responses (Skountzou et al., 2010; Huleatt et al., 2007). Specifically, to *Salmonella* infection, immunisation with sFLiC provokes strong Th2 responses, though subsequent infection of sFLiC-primed animals results in augmented Th1 responses (Bobat et al., 2011). Antigen-specific antibody is produced in response to sFLiC-immunisation; however, it is functionally redundant due to the ability of STm to phase-switch (Bobat et al., 2011). By investigating if antigen persistence occurs in sFLiC-immunised mice, I could identify if antigen persistence is vaccine-specific. Additionally, these experiments would allude to the contribution of Th1 responses in antigen persistence.

To determine whether sFLiC could induce protection in my experiment models, mice were immunised with sFLiC, infected 35 days later with 2 x 10^5 CFU SL3261 and analysed at either 21- or 35-days post-infection. Later timepoints of infection were used in these experiments as significant reduction of STm in spleens does not appear until after the first week of infection in sFliC-immunised mice (Bobat et al., 2011).

Unlike previous studies, immunisation with sFliC did not induce reduced bacterial burden in immunised and infected mice compared to naïve controls (**Fig. 4.7A**). Persistent antigen was not observed in immunised mice at 21-days post-infection (**Fig. 4.7B**), and quantification of these images did not indicate any differences in STm^+ antigen between naïve and sFliC-immunised tissues (**Fig. 4.7C**). Therefore, prior

immunisation with sFliC did not induce protective immune responses against STm infections, and these immunised tissues did not display persistent antigen as seen with WT OMV-immunised tissues. This may indicate that antigen persistence occurs only during protective immune responses, and thus may be a positive consequence of immunisation with WT OMVs.

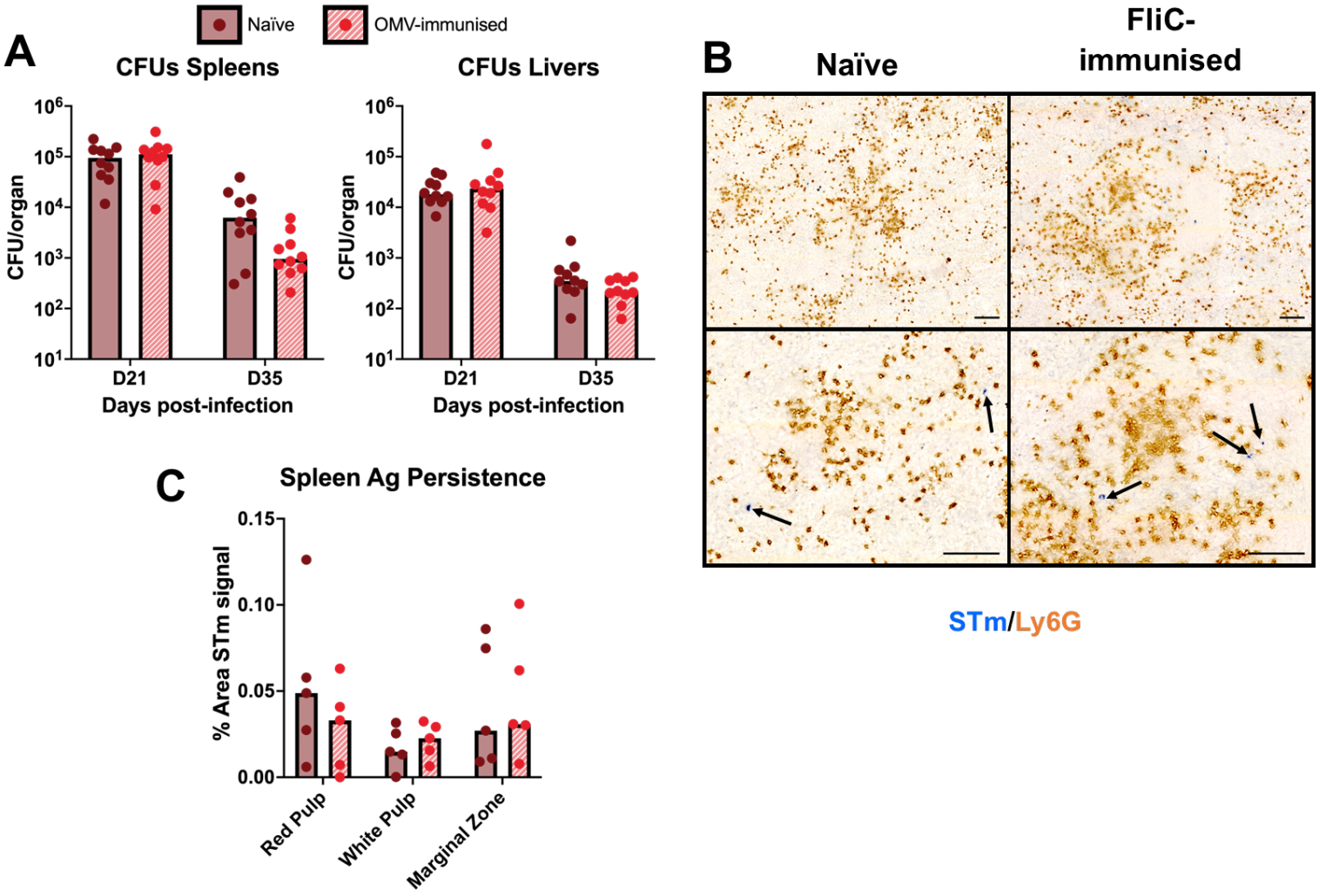

Figure 4.7: Mice immunised with sFliC do not show antigen persistence in spleens. C57BL/6 mice were immunised with 20 µg sFliC i.p. for 35 days prior to i.p. infection with 2 x 10^5 SL3261 STm. Spleens and livers were harvested and weighed 21- or 35-days post-infection. Organs were segmented and processed: (A) organs were mashed and bacterial burden enumerated; (B) spleens were snap-frozen and sectioned into 6 µM slices, stained using IHC, imaged with Zeiss AxioScan 7 and processed using Zeiss Zen Blue software v3.7; (C) frequency of STm^+ signal in IHC stained spleens was quantified using ImageJ v.1.54d with ColorDeconvolution2 plugin. In (B), STm is stained in blue and Ly6G in brown, RP = red pulp, MZ = marginal zone, WP = white pulp. Arrows highlight STm^+ stain and scale bars denote 100 µM. Data are representative of two independent experiments (*n* = 4). Each dot represents one mouse, bars represent the median of each group. D CFU data was statistically analysed using Kruskal-Wallis test with correction for multiple comparisons, persistence data was analysed using unpaired *t*-test.

4.2.6.2 Antigen persistence does not occur in spleens of Vi-immunised mice

I next examined vaccination with the antigen Vi. Vi is a capsular polysaccharide found on *S*. Typhi and *S.* Paratyphi serovars of *Salmonella enterica* (Wain et al., 2005). There are currently 3 vaccines against typhoid fever available for use in humans, including the whole cell Ty21a, non-conjugated Vi-polysaccharide, Vi-polysaccharide conjugated to a carrier protein (World Health Organisation, 2023). Vi-immunisation induces protective IgM and IgG responses to *S.* Typhi and boosting with conjugated Vi vaccines sustains immune response efficacy (Jossi et al., 2023). Investigation of this vaccine antigen would identify if antigen persistence is vaccine specific. Additionally, examination of antigen persistence in this experimental model would provide insight into whether this is specific to non-typhoidal infections, or if it also occurs in typhoidal infections. Mice were immunised with Vi-polysaccharide (Vi-PS) or Vi-tetanus toxin conjugate (Vi-TT) vaccines. After 14 days, mice were then infected with Vi-producing STm strain TH177 (Haneda et al., 2009). Spleens were investigated for bacterial burden and STm^+ stain as previously described. Tissues were kindly provided by Dr Siân Jossi.

As seen in **Fig. 4.8A**, immunisation with either Vi-PS or Vi-TT results in a marked reduction of CFU in both spleens and livers at 1-day post-infection. However, investigation of STm^+ stain in the spleens of these mice showed a lack of antigen persistence in these mice (**Fig. 4.8B and C**). Therefore, whilst Vi-PS and Vi-TT constitute protective vaccines, they do not induce antigen persistence.

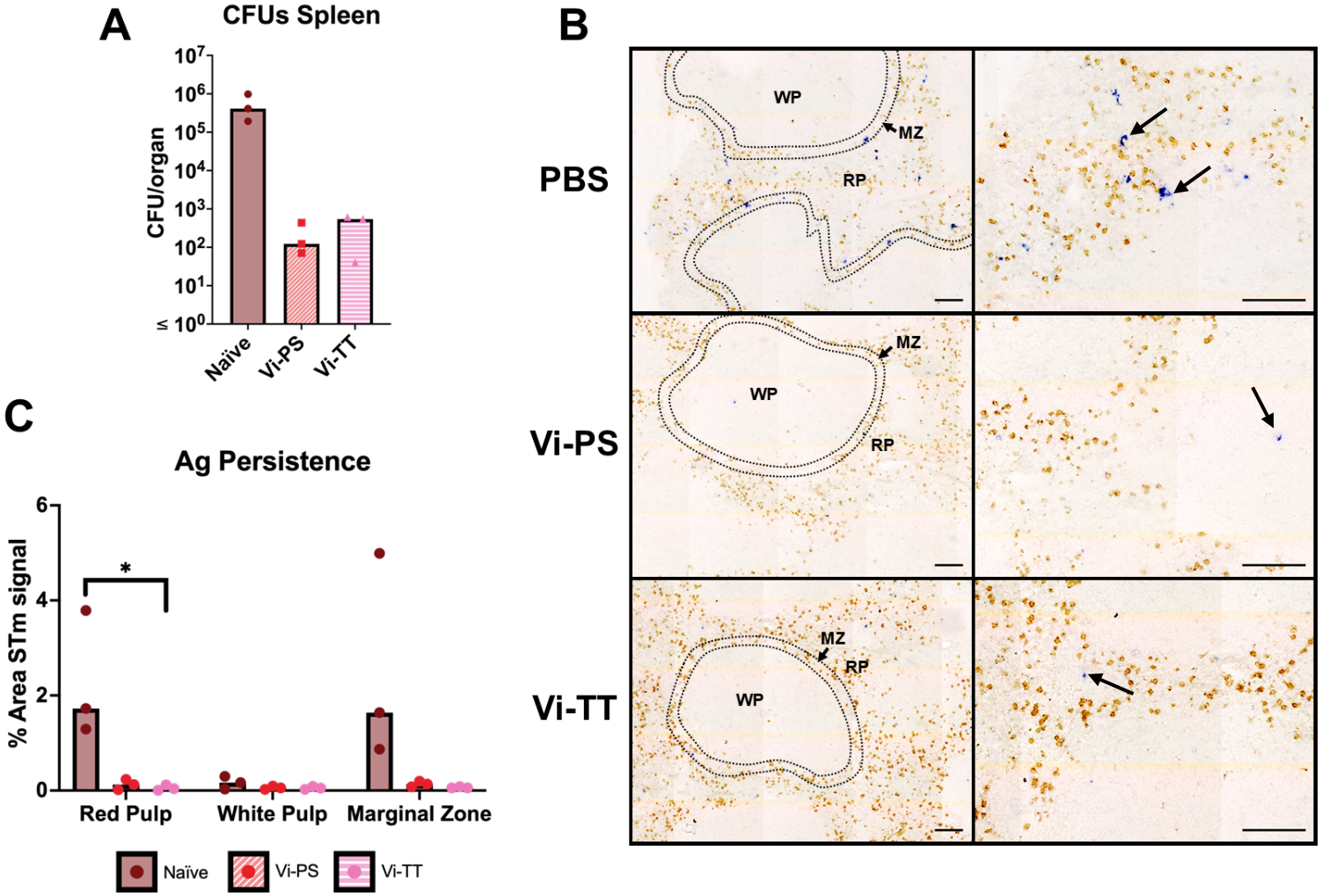

Figure 4.8: Mice immunised with Vi-PS or Vi-TT do not show antigen persistence in spleens. C57BL/6 mice were immunised i.p. with 2 µg of Vi-PS or Vi-TT for 14 days prior to i.p. infection with 1 x 10^5 CFU TH177 STm. (A) Spleens were snap-frozen, sectioned, and stained using IHC, imaged with Zeiss AxioScan 7 and processed using Zeiss Zen Blue software v3.7; (B) frequency of STm^+ signal in IHC stained spleens was quantified using ImageJ v1.54d with ColorDeconvolution2 plugin. In (B), STm is stained blue and Ly6G in brown, RP = red pulp, MZ = marginal zone, WP = white pulp. Arrows highlight STm^+ stain and scale bars denote 100 µM. Data are representative of one experiment (n = 3). Each dot represents one mouse, bars represent the median of each group. Data were statistically analysed using One-way ANOVA test with correction for multiple comparisons; * = $p \leq 0.05$.

4.2.7 Lymphocyte-deficient mice display persistent antigen when immunised and infected with *Salmonella* Typhimurium

To identify if antigen persistence depends on a specific immune cell population, both depletion and knockout mouse models were used in immunisation and infection studies. To examine whether lymphocytes were involved with the development of antigen persistence, *Rag2*$^{-/-}$ animals were used. *Rag2*$^{-/-}$ mice fail to produce mature B or T lymphocytes due to the *Rag2* gene being essential for V(D)J recombination in lymphocyte maturation (Shinkai et al., 1992). Therefore, if lymphocytes are responsible for the presence of persistent antigen in the spleen, we would expect to see a loss or change of the phenotype in immunised and challenged *Rag2*$^{-/-}$ mice. Mice were immunised with WT OMVs 7 days prior to i.p. infection with 5 x 10^5 CFU SL3261 for 1 day.

Immunisation of *Rag2*$^{-/-}$ mice does decrease the bacterial burden of spleens or livers (**Fig. 4.9A**), however this decrease is not statistically significant. This suggests that the lymphocytic cell compartment is important for the protective immune response induced by WT OMVs. Histological analysis of both spleens and livers indicates that a similar level of STm$^+$ staining occurs in the organs of naïve and immunised mice (**Fig 4.9B**). Further analysis and quantification of these images (**Fig. 4.9C**) shows that the percentage area with STm$^+$ stain is lower in the spleens of OMV-immunised mice compared to naïve controls, however this is not statistically significant. Percentage area with STm$^+$ stain in livers of OMV-immunised mice was slightly increased compared to the naïve group, however there was significant variation between mice. When we examined the histology slides more closely, a difference in the quality of STm$^+$ staining is observed. In naïve mice, STm$^+$ stain is generally larger with a deeper intensity compared to the more punctate and less intense staining seen in OMV-

immunised mice. This may indicate that the STm^+ stain seen in immunised tissues is similar to the persistent antigen seen in previous sections. However, it is also possible that this staining reflects the high bacterial burden observed in the OMV-immunised mice (**Fig 4.9A**). It is therefore unclear as to whether persistent antigen occurs in lymphocyte-deficient mice, as this may be a result of high bacterial burden in the organs due to lack of protection following OMV immunisation. Therefore, the role of lymphocytes in antigen persistence remains undetermined.

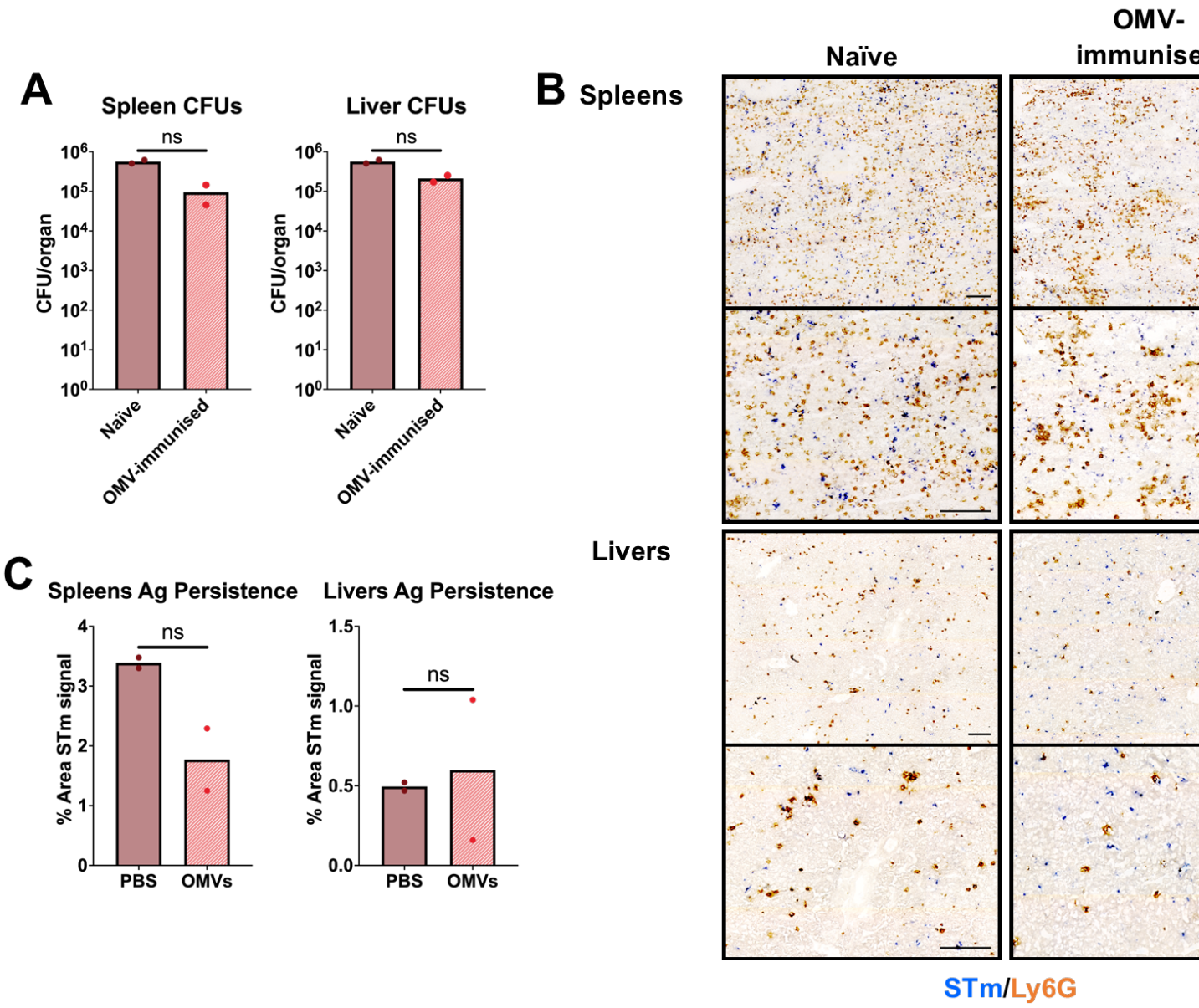

Figure 4.9: Lymphocyte-deficient mice do not lose antigen-persistence phenotype following immunisation and infection. *Rag2*$^{-/-}$ mice were immunised i.p. with 1 µg *ΔtolR* OMVs for 7 days prior to i.p. infection with 5 x 10^5 CFU SL3261 STm. Spleens and livers were harvested 24 hours post-infection. Organs were segmented and processed: (A) organs were mashed and bacterial burden enumerated; (B) spleens were snap-frozen and sectioned into 6 µM slices, stained using IHC, imaged with Zeiss AxioScan 7 and processed using Zeiss Zen Blue software v3.7; (C) frequency of STm$^+$ signal in IHC stained organs was quantified using ImageJ v.1.54d with ColorDeconvolution2 plugin. In (B), STm is stained in blue and Ly6G in brown, arrows highlight STm$^+$ stain, and scale bars denote 100 µM. Data represent a single experiment (n = 2), each dot represents one mouse and bars represent the median of each group. Data were analysed using unpaired t test.

4.2.8 Macrophage depletion alters the localisation of antigen persistence in the spleen

Since the role of lymphocytes in antigen persistence was unable to be determined due to high levels of STm^+ staining in both naïve and OMV-immunised groups, I then studied the effects of macrophages. Macrophages capture and phagocytose bacteria during innate immune responses, and it is well-documented that they provide an intracellular survival niche during *Salmonella* infections (Salcedo et al., 2001; Goldberg et al., 2018; Buchmeier and Heffron, 1989). Macrophages are not often associated with vaccine-mediated immune responses, though studies have shown that macrophage populations expand in response to BCG vaccination, with depletion of macrophages reducing protection (Yang et al., 2020). Others have shown that macrophages can act as APCs (Muntjewerff et al., 2020), or can accept antigen from activated B cells to bolster adaptive responses (Harvey et al., 2007).

Macrophages can be depleted from mice using liposome-encapsulated clodronate. The liposomes are phagocytosed by macrophages leading to an intracellular accumulation of clodronate and triggering apoptosis (Nguyen et al., 2021). Therefore, clodronate treatment of immunised and infected mice would indicate if macrophages were required for the development of antigen persistence. C57BL/6 WT mice were immunised i.p. with WT OMVs. After 13 days, mice were injected intravenously (i.v.) with liposome-encapsulated clodronate, or control liposomes and then infected 24 hours later with 5 x 10^5 CFU SL3261 STm i.p. Untreated mice received injections of PBS both at the immunisation and clodronate-treatment stage prior to i.p. infection and were used as controls. Spleens were then harvested at 1-day post-infection.

Bacterial burden of spleens was reduced by ~100-fold in OMV-immunised mice compared to naïve controls (**Fig 4.10A**), as observed previously. Clodronate treatment did not significantly affect immunisation protection afforded by OMVs. Mice that received liposomes + OMVs showed greater STm^+ staining than the PBS-treated controls (**Fig. 4.10B**). Interestingly, whilst antigen persistence was still observed in the clodronate-treated immunised mice, the localisation of the persistent antigen was no longer exclusive to the RP and was observed in both the RP and WP (**Fig 4.10B and C**). Indeed, the fraction of STm^+ signal in distinct splenic regions was higher in the WP of clodronate-treated mice compared to both the untreated and OMV-immunised groups, while STm^+ signal in the RP of clodronate-treated mice was lower than that of mice that were OMV-immunised, yet higher than those that were untreated (**Fig 4.10C**). These results suggest that macrophages may play a role in the capture and/or distribution of the antigen within the spleen following immunisation and demonstrate that depletion of these cells may promote free movement of antigen.

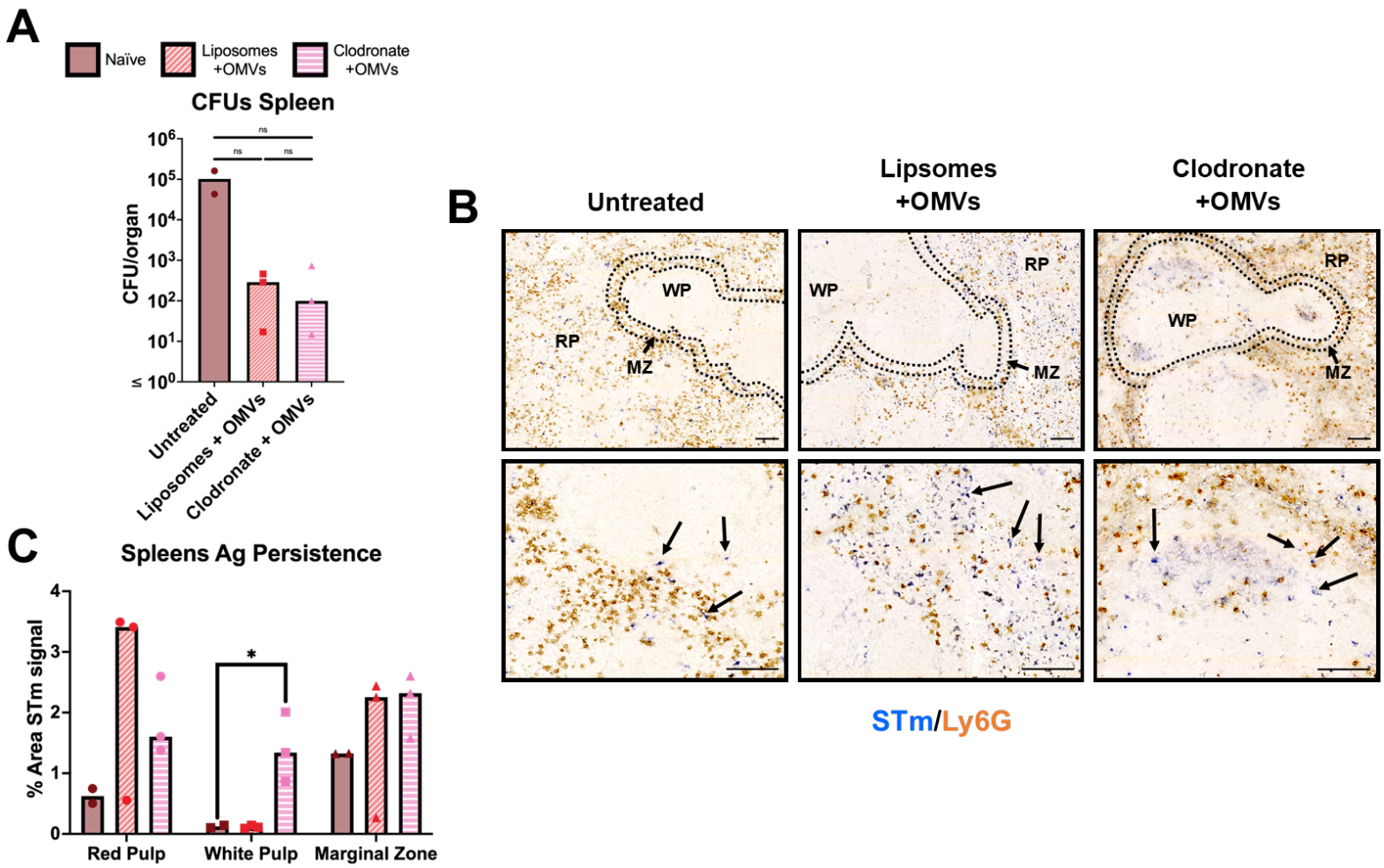

Figure 4.10: Macrophage-depleted OMV-immunised mice show antigen persistence in spleens, with different localisation. C57BL/6 mice were immunised with *ΔtolR* OMVs for 13 days. Mice were administered i.p. of liposome-encapsulated clodronate (or control liposomes) for 24 hours. Mice were then infected i.p. with 5 x 10^5 CFU SL3261 STm. Spleens were harvested and processed: (A) spleens were mashed and bacterial burden enumerated; (B) spleens were snap-frozen and sectioned into 6 µM slices, stained using IHC, imaged with Zeiss AxioScan 7 and processed using Zeiss Zen Blue software v3.7; (C) frequency of STm^+ signal in IHC stained spleens was quantified using ImageJ v.1.54d with ColorDeconvolution2 plugin. In (B), STm is stained in blue and Ly6G in brown, arrows highlight STm^+ stain, and scale bars denote 100 µM. Data represent a single experiment (untreated group *n* = 2, treatment groups *n* = 3), each dot represents one mouse and bars represent the median of each group. Data were analysed using One-way ANOVA test with correction for multiple comparisons; * = $p \leq 0.05$.

4.2.9 Liver macrophages do not play a definitive role in liver antigen persistence

As the clodronate experiments indicated that macrophages played a role in the capture and localisation of persistent antigen in the spleen, I next explored how macrophage depletion affected antigen persistence in the liver. Mice were immunised and treated as described in section 1.1.9, and livers harvested for analysis after 1 day of infection.

An approximate 100-fold reduction of bacterial burden was observed in livers in OMV-immunised mice compared to naïve controls, and reduced ~1000-fold upon treatment with clodronate prior to infection (**Fig. 4.11A**). Histological analysis of liver sections from these mice showed that persistent antigen can be observed in both the OMV-immunised and OMV-immunised clodronate-treated groups, but not in the untreated controls (**Fig. 4.11B**). The amount of STm^+ signal observed appeared lower in immunised mice that received the clodronate treatment compared to those that did not, which may reflect the lower bacterial burden of this group (**Fig. 4.11B and C**). These results suggest that depletion of macrophages lowers the recoverable CFU of livers from immunised and infected mice, which may have a knock-on effect on antigen persistence. This could relate to a limited availability of intracellular survival niches in the absence of macrophages, resulting in exposure to extracellular immune mechanisms and reduced bacterial survival.

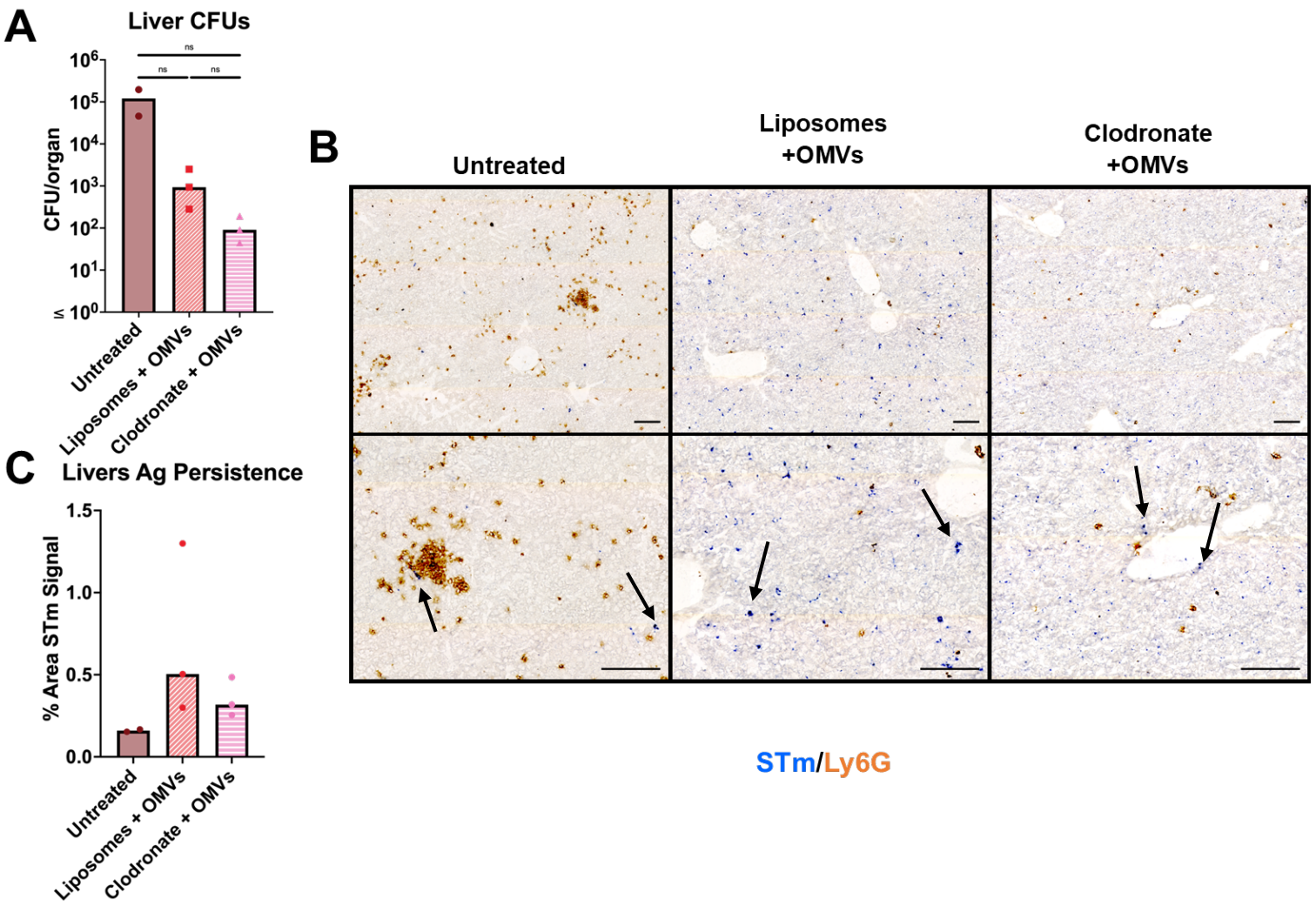

Figure 4.11: Macrophage-depleted OMV-immunised mice show antigen persistence in livers. C57BL/6 mice were immunised with *ΔtolR* OMVs for 13 days. Mice were administered i.p. of liposome-encapsulated clodronate (or control liposomes) for 24 hours. Mice were then infected i.p. with 5 x 10^5 CFU SL3261 STm. Livers were harvested and processed: (A) livers were mashed and bacterial burden enumerated; (B) livers were snap-frozen and sectioned into 6 μM slices, stained using IHC, imaged with Zeiss AxioScan 7 and processed using Zeiss Zen Blue software v3.7; (C) frequency of STm^+ signal in IHC stained livers was quantified using ImageJ v.1.54d with ColorDeconvolution2 plugin. In (B), STm is stained in blue and Ly6G in brown, arrows highlight STm^+ stain, and scale bars denote 100 μM. Data represent a single experiment (untreated group *n* = 2, treatment groups *n* = 3), each dot represents one mouse and bars represent the median of each group. Data were analysed using One-way ANOVA test with correction for multiple comparisons; * = $p \leq 0.05$.

4.2.10 Macrophage-depletion causes co-localisation of Ly6G$^+$ cells and persistent antigen in the spleen WP

Macrophages interact with multiple cell types in the spleen during infection, participating in coordinated immune responses (Saahene et al., 2022). To examine how other cell types were affected by macrophage depletion, and if these disturbances were co-localised with persistent antigen, I stained spleens from clodronate-treated tissues and stained them using immunofluorescent antibodies. A myeloid panel was used to stain for F480 (macrophages), Ly6G (neutrophils), and STm (**Fig. 4.12**). In both untreated and OMV-immunised groups, F480$^+$ stain was found to co-localise with STm$^+$ stain, however this was more frequent in the OMV-immunised group. Ly6G$^+$ cells were distributed evenly in the RP of untreated mice, apart from Ly6G$^+$ foci that are assumed to be neutrophil-containing thrombosis (Beristain-Covarrubias et al., 2019). Ly6G$^+$ cells in the OMV-immunised mice followed a similar pattern with the absence of thrombi, and a few were observed in the spleen WP.

As expected, the amount of F480$^+$ staining was notably decreased in the clodronate-treated mice, confirming depletion (**Fig. 4.12**). The remaining F480$^+$ cells in these mice do not appear to colocalise with STm antigen. Notably, Ly6G$^+$ cells appear to be more frequently present in the WP of spleens from clodronate-treated OMV-immunised mice, and these cells co-localised with the differentially distributed persistent STm$^+$ antigen.

These results suggest that F480$^+$ cells, most likely macrophages, have captured the persistent STm$^+$ antigen during infection of OMV-immunised mice. Depletion of this cell population results in the release of this antigen to other structures of the spleen, possibly to be captured by other immune cells, such as Ly6G$^+$ neutrophils.

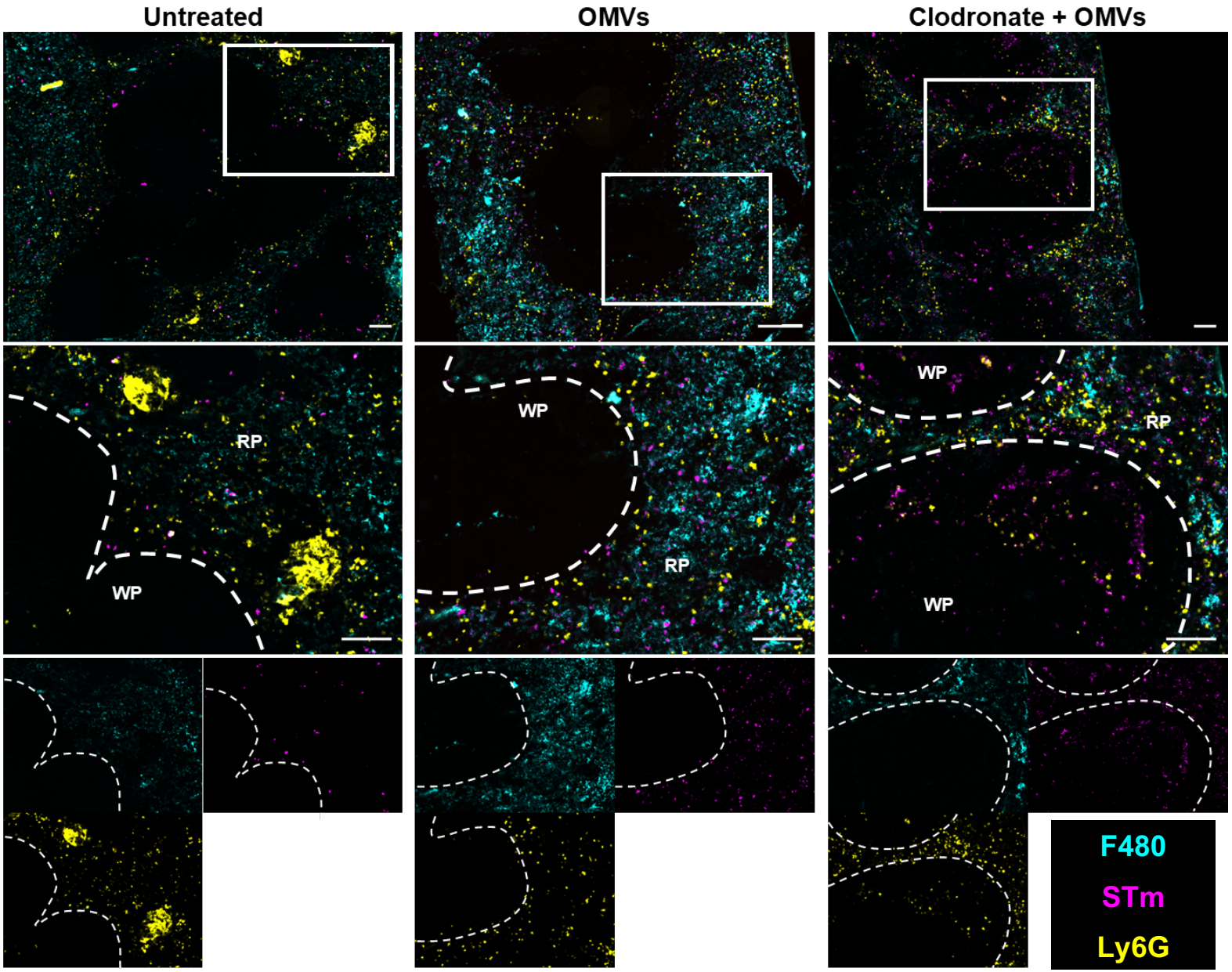

Figure 4.12: Macrophage-depleted mice show alternate distribution of STm$^+$ antigen that does not associate with myeloid cell types. C57BL/6 mice were immunised with *ΔtolR* OMVs for 12 days. Mice were administered i.p. with liposome-encapsulated clodronate (or control liposomes) for 24 hours. Mice were then infected i.p. with 5 x 10^5 CFU SL3261 STm. Spleens were harvested 24 hours post-infection, snap-frozen and sectioned into 6 µM. Sections were stained by IF, imaged using Zeiss AxioScan 7 and processed using Zeiss Zen Blue software v3.7. F480$^+$ cells are shown in cyan, STm$^+$ in magenta, and Ly6G$^+$ in yellow, WP = white pulp, RP = red pulp. Scale bars denote 100 µM.

4.2.11 Persistent antigen in the WP following macrophage depletion does not associate with lymphocytes

Macrophage-lymphocyte interactions are well studied. Interaction of T cells and mononuclear phagocytes were documented in study of anti-mycobacterial immune responses (Kaufmann and Flesch, 1988). T cells were shown to continuously migrate on macrophages during productive antigen presentation (Underhill et al., 1999), and B cells have been shown to transfer antigen to macrophages to improve antigen presentation to T cells (Harvey et al., 2007). I therefore tested how macrophage depletion affected lymphoid cell populations in vaccinated and challenged animals.

Perturbations on lymphoid cell subsets was investigated by staining for B220 (B cell stain), CD3 (T cell stain), along with STm (**Fig. 4.13**). Infected, untreated mice showed an expected distribution of $B220^+$ cells in the B cell follicles (BCF), and $CD3^+$ cells in the T cell zones (TCZ). These represent mature B and T lymphocytes in their standard microarchitectural zones. Few STm^+ cells appear in the WP amongst these cell populations, with most remaining in the RP. $B220^+$ and $CD3^+$ cells can also be seen distributed throughout the RP; however, these cells rarely associated with STm^+ cells.

The intensity of $B220^+$ and $CD3^+$ staining in the BCF and TCZ of OMV-immunised mice was greater than that seen in the untreated mice, suggesting an expansion of these cells in the WP due to OMV-immunisation. The persistent antigen that can be seen in the RP did not associate with either $B220^+$ or $CD3^+$ cells, suggesting that this antigen did not interact with B or T cells directly.

$B220^+$ and $CD3^+$ cells observed in OMV-immunised mice that received clodronate treatment shared a similar level of intensity as those who were immunised, suggesting that these cell populations had also expanded in response to immunisation and was

not affected by macrophage depletion. This is expected, as macrophage depletion was not induced until after OMV-immunisation. Persistent antigen that appears in the WP appeared to colocalise with some $B220^+$ cells (**Fig. 4.13**). However, the distinction between the BCF and TCZ of the WP is largely lost in immunised mice that received clodronate treatment, making specific analysis difficult. Taken together, these data indicate that a loss of macrophages influences the microarchitecture in the WP of the spleens and that persistent antigen that is usually RP-restricted is found in the BCF of clodronate-treated mice.

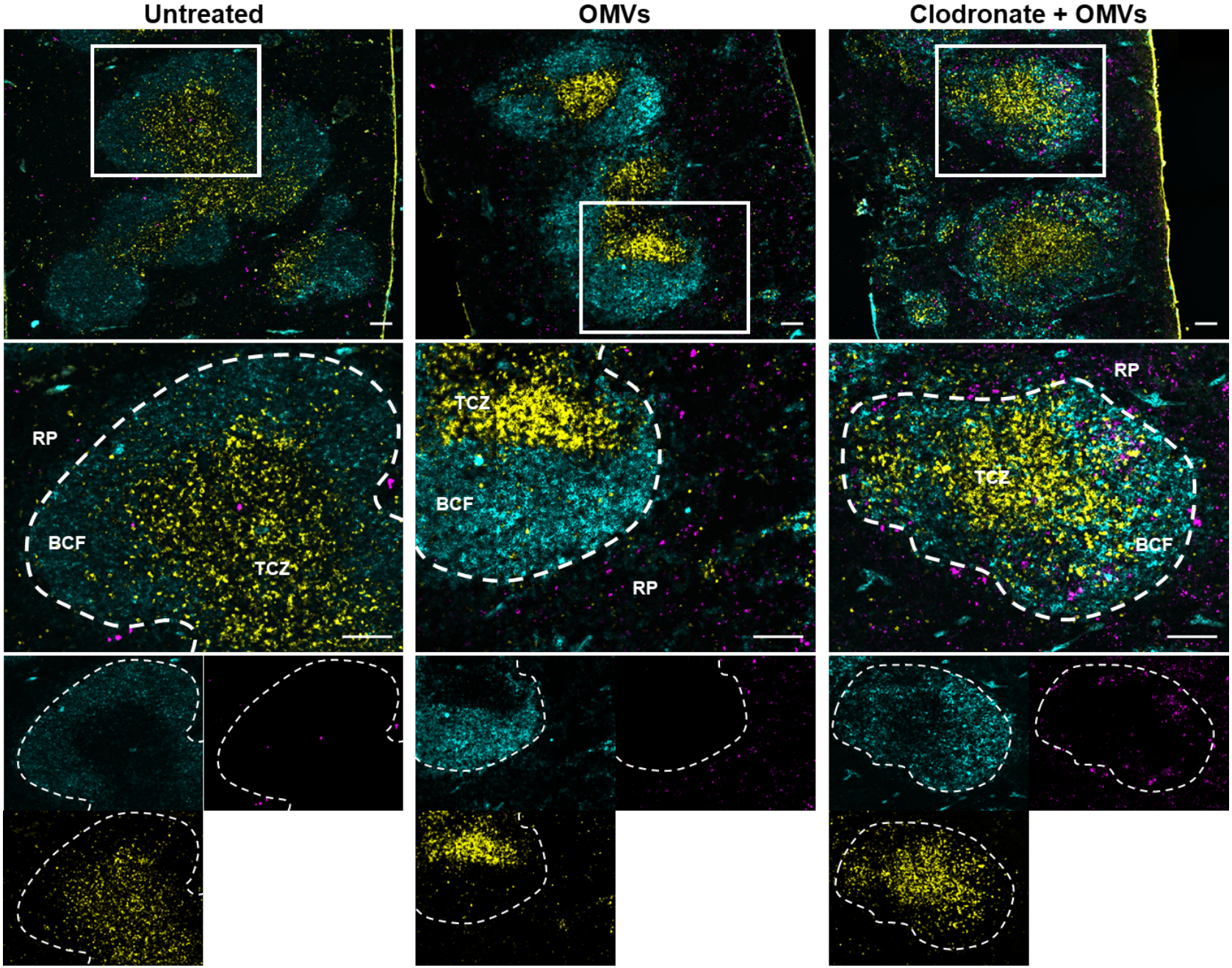

Figure 4.13: Persistent antigen in macrophage-depleted spleens does not associate with B220$^+$ or CD3$^+$ lymphocytes. C57BL/6 mice were immunised with *ΔtolR* OMVs for 12 days. Mice were administered i.p. with liposome-encapsulated clodronate (or control liposomes) for 24 hours. Mice were then infected i.p. with 5 x 10^5 CFU SL3261 STm. Spleens were harvested 24 hours post-infection, snap-frozen and sectioned into 6 µM. Sections were stained by IF, imaged using Zeiss AxioScan 7 and processed using Zeiss Zen Blue software v3.7. B220$^+$ cells are shown in cyan, STm$^+$ in magenta, and CD3$^+$ in yellow, WP = white pulp, RP = red pulp. Scale bars denote 100 µM.

4.2.12 Persistent antigen in macrophage-depleted mice associates with IgM$^+$ cells

Mature, resting naïve B cells express transmembrane IgM as part of the B cell receptor (Sathe and Cusick, 2022), and therefore identifies the BCF. Immune complexes (ICs) are formed with IgM, antigen, and other opsonising agents such as complement (Kranich and Krautler, 2016; Liu et al., 2019). Additionally, previous work has shown that IgG1 is essential for optimal protection against STm following immunisation with purified protein subunit vaccines (Zhang et al., 2017). As both soluble and cell-bound antibody is capable of capturing antigen and forming ICs, I wanted to test if specific antibody isotypes co-localise with persistent antigen. Spleens were stained for IgM, IgG1, and STm.

Regions of IgM^{hi} expression in the BCF is characteristic of early germinal centre (GC) responses (Sundling et al., 2021). Untreated mice infected with STm displayed regions of IgM^{lo}, which correspond to BCF, encompassing smaller foci of IgM^{hi} expression (**Fig. 4.14**). STm$^+$ staining was rarely localised to either of these regions, and IgG1 expression was minimal.

Similar IgM^{lo} and IgM^{hi} regions can be observed in the OMV-immunised mice, with the exception that the IgM^{hi} regions are much larger and more frequent, typical of immunisation responses. IgG1 expression was largely increased in this group also, and localised proximal to Cords of Billroth (cords of connective tissue between venous sinuses), separate from the WP (Lindberg and Lamps, 2018; Bellinger et al., 2004). STm$^+$ antigen rarely associated with either IgM or IgG1 in the OMV-immunised groups.

The IgM expression in clodronate-treated immunised mice was unlike that seen in either the untreated or OMV-immunised groups. The IgM^{lo} regions were sparse, with

IgM expression concentrating in the outer regions of what appear to be the BCF. As seen with **Fig. 4.13**, this may indicate a breakdown in the microarchitecture of the spleen in the absence of macrophages. IgG1 expression was still present in clodronate-treated mice, assumingly due to clodronate-depletion occurring post-OMV-immunisation, however $IgG1^+$ stain rarely co-localised with STm^+ antigen.

Further examination of the isotype panel can be seen in **Fig. 4.15**, showing images from each mouse within the clodronate-treated group. **Fig. 4.15** indicates co-localisation of the persistent STm^+ staining within the IgM^{hi} regions of the BCFs in this group. Areas of colocalization are indicated by red arrows, indicating capture of the persistent STm^+ antigen, released in the absence of macrophages, either by ICs or IgM^+ cell within the BCF of the spleens. Therefore, WP-localised STm^+ antigen in immunised macrophage-depleted mice co-localises with IgM^+ cells and/or ICs, indicating capture of free antigen.

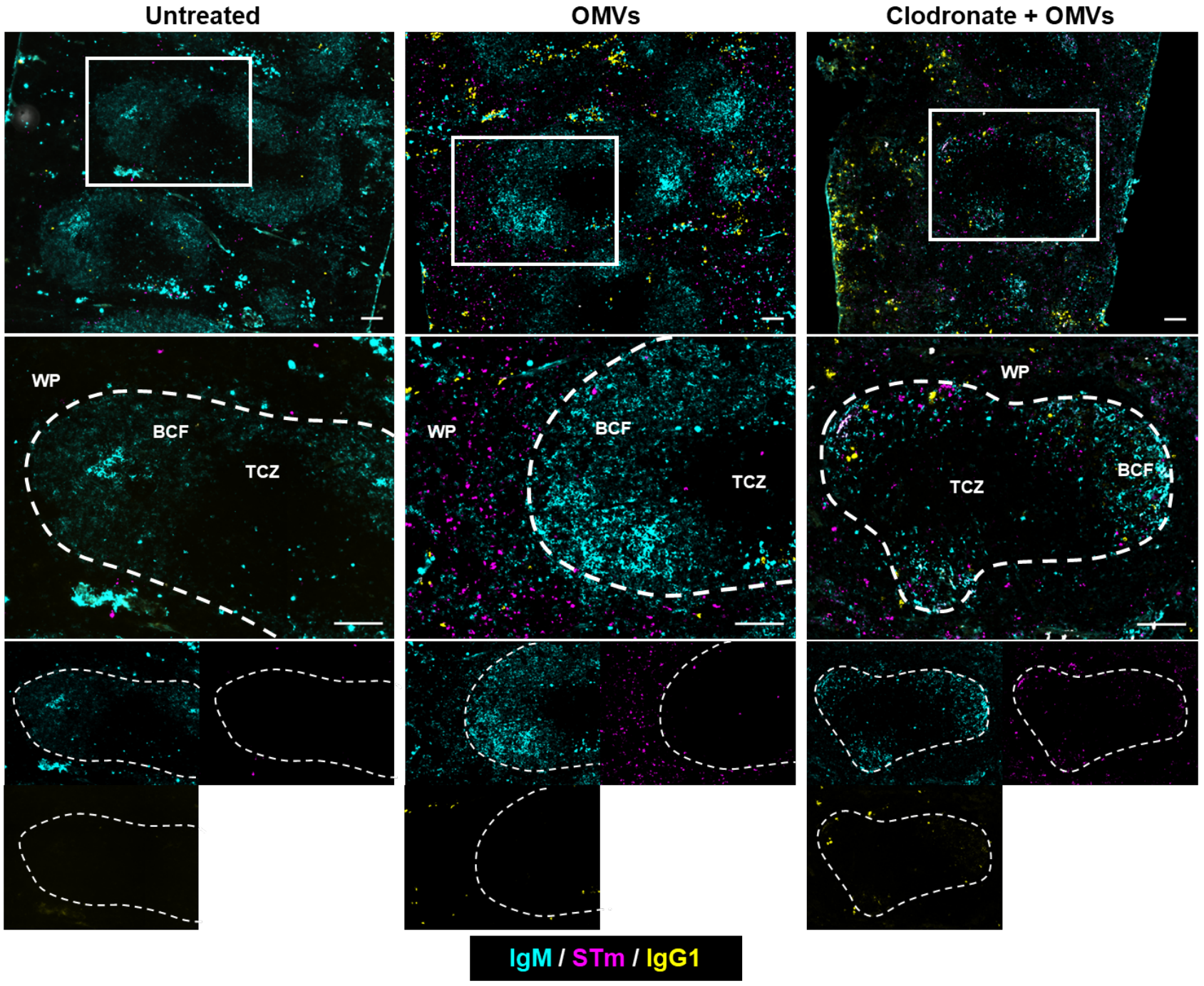

Figure 4.14: Persistent antigen in macrophage-depleted mice localise to IgM⁺ regions of B cell follicles. C57BL/6 mice were immunised with *ΔtolR* OMVs for 12 days. Mice were administered i.p. with liposome-encapsulated clodronate (or control liposomes) for 24 hours. Mice were then infected i.p. with 5 x 10^5 CFU SL3261 STm. Spleens were harvested 24 hours post-infection, snap-frozen and sectioned into 6 µM. Sections were stained by IF, imaged using Zeiss AxioScan 7 and processed using Zeiss Zen Blue software v3.7. IgM⁺ cells are shown in cyan, STm⁺ in magenta, and IgG1⁺ in yellow, WP = white pulp, RP = red pulp. Scale bars denote 100 µM.

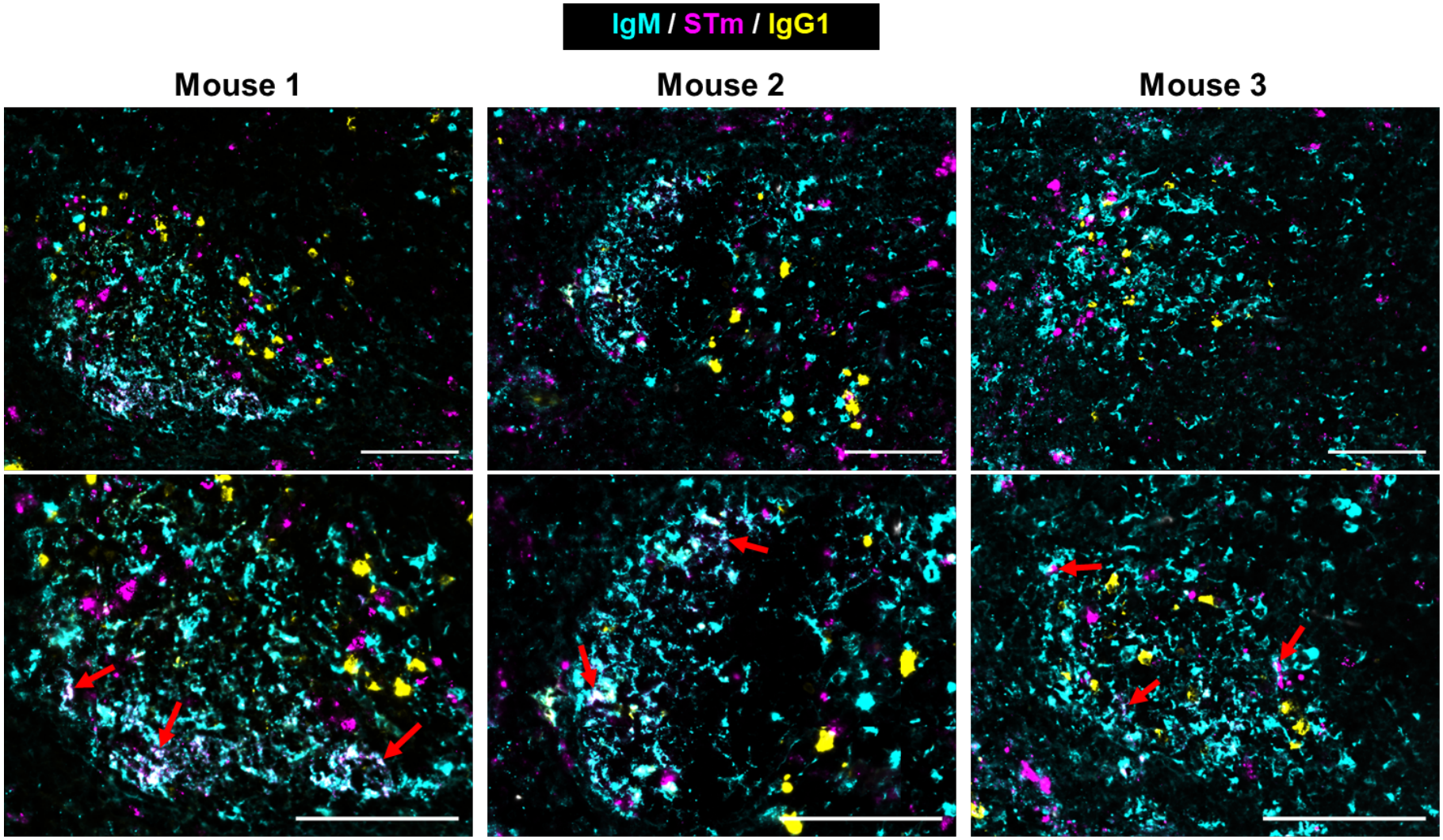

Figure 4.15: Antigen in macrophage-depleted mice associates with IgM⁺ cells. C57BL/6 mice were immunised with *ΔtolR* OMVs for 12 days. Mice were administered i.p. with liposome-encapsulated clodronate for 24 hours. Mice were then infected i.p. with 5 x 10^5 CFU SL3261 STm. Spleens were harvested 24 hours post-infection, snap-frozen and sectioned into 6 µM. Sections were stained by IF, imaged using Zeiss AxioScan 7 and processed using Zeiss Zen Blue software v3.7. IgM⁺ cells are shown in cyan, STm⁺ in magenta, and IgG1⁺ in yellow, arrows highlight areas of co-localisation. Scale bars denote 100 µM.

4.2.13 Loss of LPS O-antigen from OMVs alters protective ability and reduces antigen persistence

So far, I have identified that the mechanism of persistent antigen observed in OMV-immunised STm-infected mice is most likely macrophage-dependent, but lymphoid-, strain-, and site delivery-independent. However, as mentioned in **section 1.1.2**, the STm (and therefore OMV) is a complex bacterial cell surface, comprising of multiple proteins, polysaccharides, and bacterial antigen that aid in *Salmonella* pathogenesis and immune evasion mechanisms. STm-LPS has potent immunomodulatory properties and aggravates the host immune system through TLR4-dependent mechanisms (Lu et al., 2008). *Salmonella* strains that are mutated to truncate LPS O-antigen ($LPS_{O\text{-}Ag}$) production can also be used in OMV production to produce OMVs with varying lengths of O-antigen (**figure 2.2**). *Δwzy* mutants produce OMVs containing LPS with only 1 O-antigen monomer attached to the core oligosaccharide, and *ΔwbaP* mutants produce OMVs with no O-antigen monomers. For the sake of distinction, WT OMVs refers to purely *ΔtolR* OMVs in these experiments, though it should be noted that both *Δwzy and ΔwbaP* strains also possess the *ΔtolR* deletion as this induces the hyperblebbing phenotype required for OMV production and purification. C57BL/6 WT mice were immunised for 14 days with either WT, *Δwzy*, or *ΔwbaP* OMVs, prior to infection with 5 x 10^5 CFU SL3261. Mice were culled and tissues harvested after 1 day.

First the bacterial burden and therefore induced protection afforded by each type of OMVs was examined. Immunisation with *Δwzy* OMVs reduces bacterial burden by approximately 1000-fold compared to naïve controls. *ΔwbaP* OMVs, however, did not induce a similar level of protection in the spleen, resulting in a moderate 10-fold reduction of viable CFU (**Fig. 4.16A**). The equivalent protection afforded by *Δwzy*

OMVs in comparison to WT OMVs is likely due to enhanced access to the bacterial surface for antibody and complement proteins (due to loss of the long, inhibitory chains of $LPS_{O\text{-}Ag}$) whilst still able to induce production of protective neutralising antibodies. Further, the diminished protection exhibited by *ΔwbaP* OMV immunisation may be due to the lack of any $LPS_{O\text{-}Ag}$ epitope resulting in poor antibody production.

Groups immunised with either *Δwzy* or *ΔwbaP* OMVs did not display persistent STm^+ antigen (**Fig. 4.16B & C**). Some STm^+ staining could be observed in the *ΔwbaP* OMV-immunised mice; however, this may be attributed to the higher bacterial burden in the spleens within this group.

These results indicate that specific anti-$LPS_{O\text{-}Ag}$ immunity contributes to the protective properties against STm of OMVs when used as a vaccine in mice. Additionally, they infer an $LPS_{O\text{-}Ag}$-dependent mechanism in the antigen persistent phenotype observed throughout this chapter. *Δwzy* OMV-immunised mice displayed the equivalent protective abilities as WT OMVs as seen by the reduced bacterial burden, in addition to the lack of persisting antigen, which indicates that antigen persistence does not correlate with protection, as previously discussed.

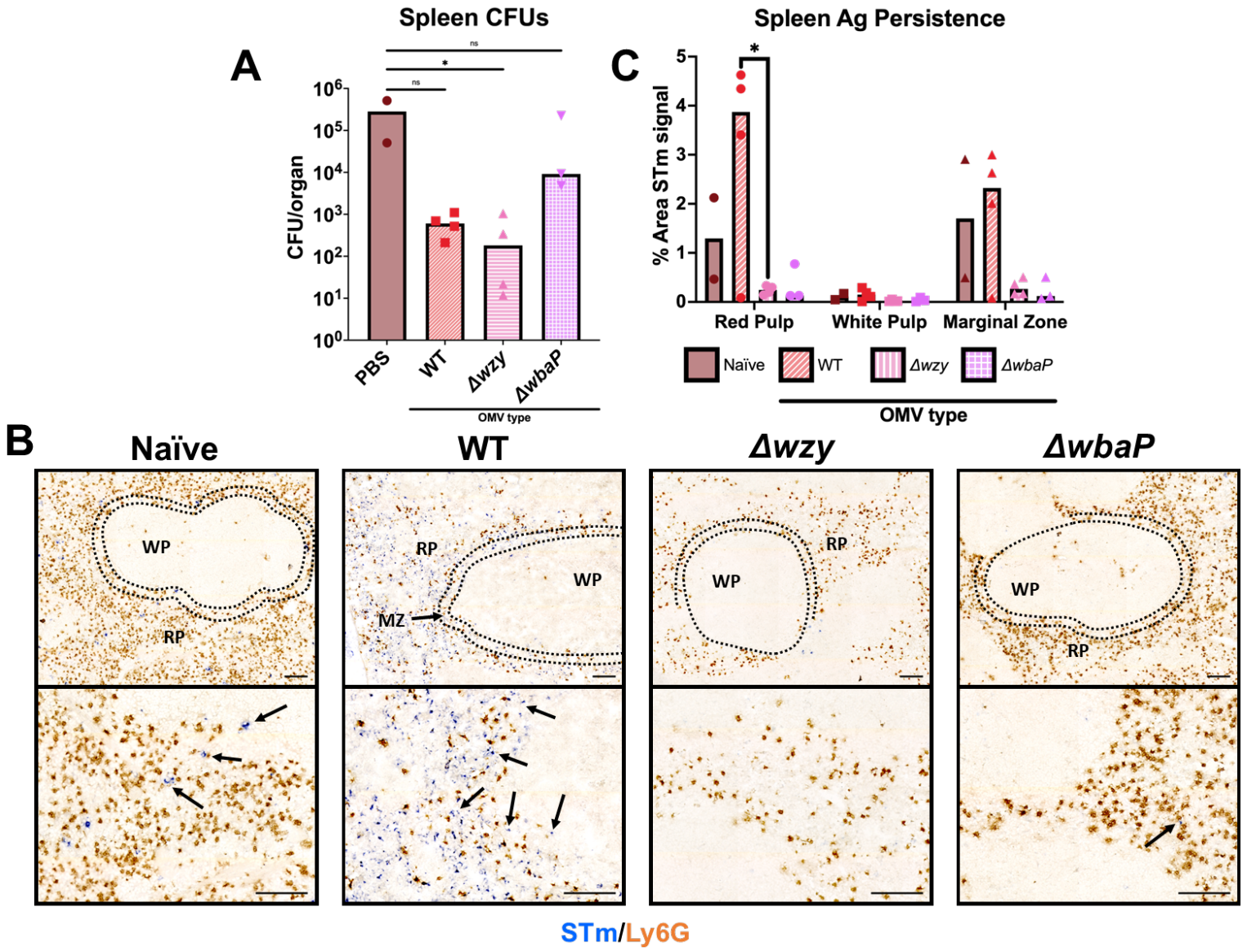

Figure 4.16: LPS status of OMVs determines persistence of antigen in spleens. C57BL/6 mice were immunised with WT, *Δwzy*, or *ΔwbaP* OMVs for 14 days prior to infection with i.p. with 5 x 10^5 CFU SL3261 STm. Spleens were harvested 24 hours post-infection. (A) spleens were mashed, and bacterial burden enumerated; (B) spleens were snap-frozen, sectioned, and stained using IHC, imaged with Zeiss AxioScan 7 and processed using Zeiss Zen Blue software v3.7; (C) frequency of STm^+ signal in IHC stained spleens was quantified using ImageJ v.1.54d with ColorDeconvolution2 plugin. In (B), STm is stained in blue and Ly6G in brown, arrows highlight STm^+ stain, RP = red pulp, MZ = marginal zone, WP = white pulp, and scale bars denote 100 μM. Data represent a single experiment (untreated group *n* = 2, vaccine groups *n* = 3-4), each dot represents one mouse and bars represent the median of each group. Data were analysed using One-way ANOVA test with correction for multiple comparisons; * = $p \leq 0.05$.

4.2.14 Loss of LPS O-antigen from OMVs alters protective ability and the persistence of antigen in the liver

To confirm the phenotypes observed in **section 4.3.14**, livers of mice immunised with WT, *Δwzy*, and *ΔwbaP* OMVs were also analysed for bacterial burden and antigen persistence. Like the spleen, the WT and *Δwzy* OMVs provided the most protection against STm burden in the liver, yet the enhanced protection observed in the spleens of *Δwzy* OMV-immunised mice was not observed in the liver. *ΔwbaP* OMV-immunised mice did not show a reduced bacterial burden compared to the naïve controls, and therefore also showed a limited protective ability as observed in the spleen (**Fig. 4.16A**).

Histological analysis of naïve mice livers (**Fig. 4.17B**) showed occasional STm^+ cells, frequently co-localised with $Ly6G^+$ cells. WT OMV-immunised liver displayed higher levels of persistent STm^+ antigen as observed in the spleen. Mice immunised with *Δwzy* OMVs showed virtually no STm^+ antigen staining, indicating robust protection and a lack of the antigen persistence phenotype. Mice immunised with the *ΔwbaP* OMVs, on the other hand, displayed a greater frequency of both STm^+ cells and surrounding $Ly6G^+$ cells, indicating an immune response similar to naïve mice including an absence of antigen persistence. In conclusion, these results show that antigen persistence observed in both lymphoid and non-lymphoid organs that are targeted by invasive STm infection is dependent on the $LPS_{O\text{-}Ag}$ status of the OMVs used for immunisation. Further, this antigen persistence phenotype is not strictly correlated with protection, as indicated by the greater protective ability of the *Δwzy* OMVs in the absence of persistent STm antigen.

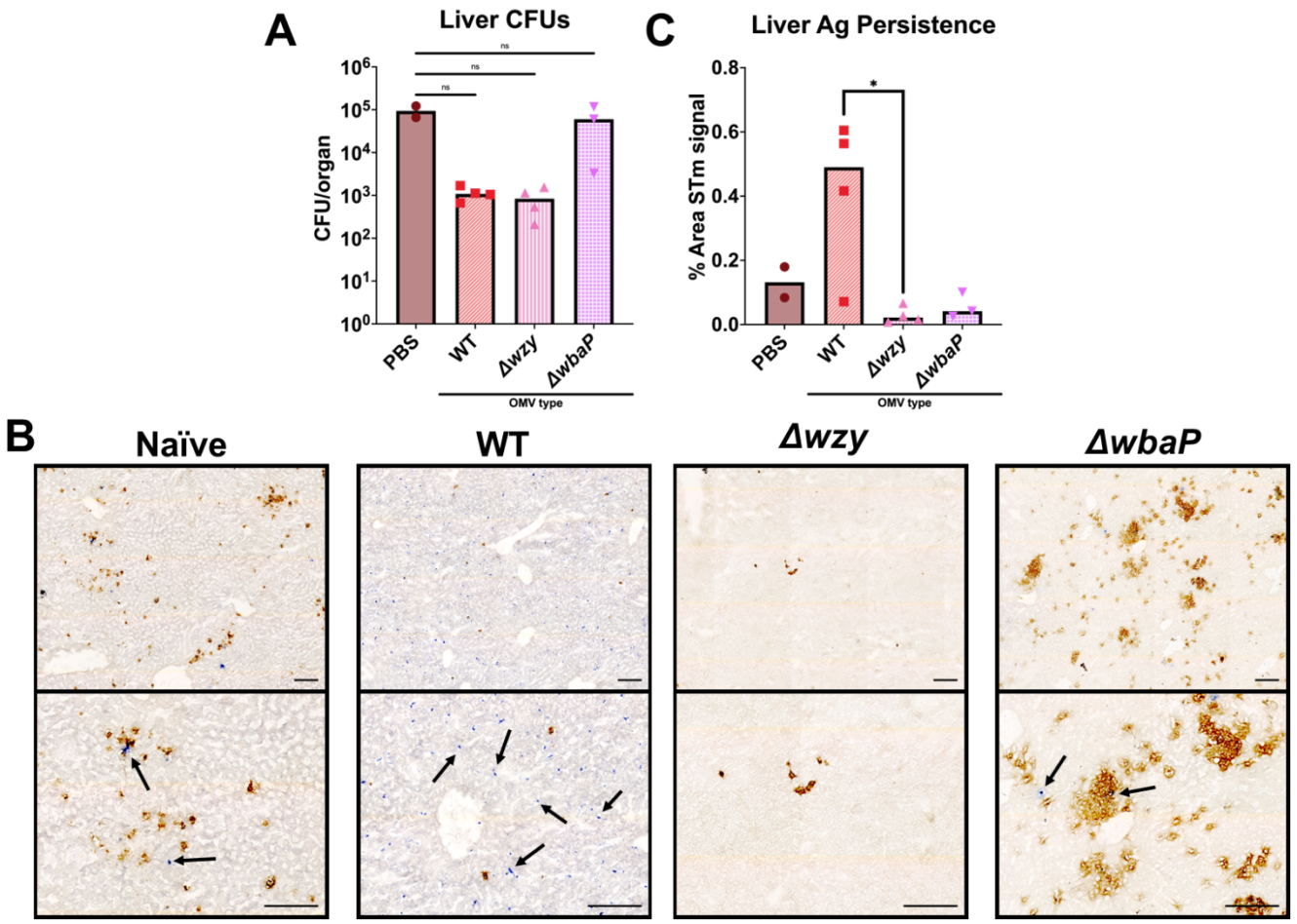

Figure 4.17: LPS status of OMVs determines persistence of antigen in livers. C57BL/6 mice were immunised with WT, *Δwzy*, or *ΔwbaP* OMVs for 14 days prior to infection with i.p. with 5 x 10^5 CFU SL3261 STm. Livers were harvested 24 hours post-infection. Livers were harvested and processed: (A) livers were mashed and bacterial burden enumerated; (B) livers were snap-frozen and sectioned into 6 µM slices, stained using IHC, imaged with Zeiss AxioScan 7 and processed using Zeiss Zen Blue software v3.7; (C) frequency of STm^+ signal in IHC stained spleens was quantified using ImageJ v.1.54d with ColorDeconvolution2 plugin. In (B), STm is stained in blue and Ly6G in brown, arrows highlight STm^+ stain, and scale bars denote 100 µM. Data represent a single experiment (untreated group $n = 2$, treatment groups n = 3-4), each dot represents one mouse and bars represent the median of each group. Data were analysed using One-way ANOVA test with correction for multiple comparisons; * = $p \leq 0.05$.

4.3 Discussion

The findings in this chapter illustrate a novel phenotype of antigen-persistence that occurs in both the spleens and livers of OMV-vaccinated and STm-challenged animals that persists for up to 5 weeks post-infection. This phenotype is not induced in sFliC or Vi polysaccharide/conjugate vaccines and is dependent on the presence of whole $LPS_{O\text{-}Ag}$ chains on the OMV surface at the point of immunisation. Subcutaneous immunisation affects the intensity of this phenotype observed in the spleen, however, is not dependent on i.p. administration of the vaccine. Finally, presence of the phenotype is not dependent on lymphoid cell populations, but localisation of persistent antigen in the spleen is influenced by depletion of macrophage populations.

First, it is important to address the caveats of experimental design and analysis, as these will be key factors to consider when drawing conclusions in this chapter. Examination of the localisation of antigen also gave insights into what cell types may be involved with the development of persistence. In most cases where STm+ antigen was noted and quantified, the highest proportion of antigen was found to localise to the red pulp (RP) and marginal zones (MZ) of the spleen, and rarely found in the white pulp. This indicated a potential myeloid-dependent mechanism behind this phenotype, as the red pulp consists mainly of myeloid-derived cells such as monocytes, macrophages, and neutrophils. Whilst examination of the MZ of immunised and infected spleens did allude to higher proportions of persistent STm+ antigen in these areas, the antigen did not significantly congregate to the MZ when examined against MZ macrophage marker CD169 (Grabowska et al., 2018). It could be suggested that the higher quantities of STm+ antigen in the MZ could be a result of biased analysis, where a smaller measured area (compared to the RP) with a similar density of antigen would present a higher proportion of antigen in these areas. However, it could

alternatively be argued that a similar density of antigen in a smaller area would indicate a higher concentration of antigen in this region, suggesting an enhanced biological effect on the present cells. One way to analyse this could be to use laser microdissection techniques to isolate different splenic structures, and measure LPS concentrations in these regions relative to the size of the area extracted. Alternatively, use of fluorescently labelled STm and fluorescence-activated cell sorting with specific gating for RP or MZ macrophages might give more accurate quantitative measures of association between STm+ antigen and specific cell subsets within the spleen.

Another limitation of this study is to consider how high bacterial burden within tissues may obscure visualisation of the antigen persistence phenotype. This is most notable in the use of *Rag2*$^{-/-}$ mice, where differentiation between live bacteria and bacterial antigen was difficult due to high recoverable CFU from these tissues. This raises a further question of whether the antigen persistence is representative of live-but-non-culturable persisting bacterial cells, or whether it is isolated bacterial antigen. Exploration of this question would inform the safety/efficacy of OMVs as vaccines, as if the former were true, it may indicate a population of persisting bacteria and induction of a 'carrier state', as seen in asymptomatic carriers of typhoid fever. This may be experimentally investigated by use of fluorescently labelled bacteria as shown by Helaine et al., where differential expression of two fluorescent reporters shows distinction between actively dividing or persisting non-mitotic cells (Helaine et al., 2014). Additionally, tissues from mice of this experiment could be stained by a fluorescently labelled antibody specific for STm $LPS_{O\text{-}Ag}$, identifying regions that are strictly antigen or those that are live bacteria by dual channel fluorescence.

There are a few caveats to consider with the clodronate-depletion experiment. Most importantly is the schedule in which macrophages were depleted from OMV-

immunised mice. Mice were first immunised with OMVs for 12 days before being administered liposome-encapsulated liposomes. Mice were then infected 24-hours post-depletion. As the antigen persistence phenotype was only observed in immunised animals that suffered subsequent infection, this schedule was chosen to examine the effects of macrophage depletion in the infection stage. However, the cell type under study using knockout models were absent from the beginning of the immunisation schedule, and so capture of the OMV antigen and subsequent stimulation were perturbed from day 0. Clodronate-treated mouse macrophages were not depleted until 12 days post-immunisation i.e., mice still had a fully functioning immune system from onset. This is not a true measure of macrophage-deficiency in terms of this phenotype, and thus future experiments investigating this phenotype should include a model of macrophage depletion that occurs prior to OMV-immunisation that is sustained until the endpoint of infection.

Additionally, use of clodronate as a method of macrophage depletion is not a strictly macrophage-exclusive intervention. Previous work has demonstrated the ingestion of clodronate-liposomes by polymorphonuclear neutrophils, resulting in functionally arrested neutrophils and an anti-inflammatory phenotype (Culemann et al., 2023). Others have shown that intradermal administration of clodronate liposomes results in elimination of $CD11c^+$, $F480^+$, and $CD11b^+$ cells in the skin, in addition to reduced $CD8^+$ T cell numbers (Ward et al., 2011). Therefore, clodronate treatment of mice prior to immunisation and infection studies may not be descriptive of an exclusively macrophage-depleted landscape, and rather a cumulative phenotype of multiple depleted myeloid-derived cell types including macrophages, dendritic cells, and neutrophils. Use of genetically modified mice that can be conditionally depleted of macrophages through drug or toxin administration (Hua et al., 2018), Cre/loxP

technology to induce specific tissue- or cell- gene knockouts (Shi et al., 2018) would allow investigation of specific macrophage population roles in antigen persistence.

Another caveat to clodronate-depletion as an exploration of the macrophage role in this context is the removal of intracellular survival niches for STm. Clodronate depletion of macrophages results has macro effects on other branches of immunity, thus results might be skewed to an overall aberrant immune landscape rather than displaying macrophage-specific influences on this phenotype. This could be framed as an argument for use of $MD2^{-/-}$ mice in these studies, as it removes the ability of macrophages to bind/capture and be stimulated by LPS whilst still being susceptible to STm infection (Nagai et al., 2002). As mentioned previously, use of Cre/loxP technology might be a method of achieving this specifically in macrophages (Shi et al., 2018).

The results in this chapter implicate a role for macrophages in the antigen persistence phenotype. Invasive Salmonella infections are consistently shown to infect multiple organs, with the main sites of colonisation being the spleen and liver (Beristain-Covarrubias et al., 2019). A question that arose during the initial identification of this phenotype was its dissemination to non-lymphoid organs. Indeed, the persistence phenotype was observed in the livers of OMV-immunised and infected animals. By default, the immune status of the liver is generally anti-inflammatory and immune tolerant (Kubes & Jenne, 2018). Though not considered a lymphoid tissue, the liver contains one of the largest pool of phagocytes in the body, and is ideally positioned to detect, capture, and destroy gut-derived pathogens (Dong et al., 2019; Kubes & Jenne, 2018; Robinson et al., 2016). Kupffer cells (KCs) are specialised tissue resident macrophages derived from foetal yolk-sac progenitors. Replenishment of KCs can occur either through homeostatic mechanisms of other tissue resident KCs, or by bone

marrow-derived monocytes during episodes of severe depletion (Roth et al., 2019). When taken into consideration with the splenic localisation results presented earlier in the chapter, these data pointed to a role for myeloid cells due to similar spatial localisation between tissue macrophages and persistent antigen. Macrophage cells are a common cell type that are resident to both hepatic and splenic RP/MZ. Splenic macrophages are poised to detect and capture pathogens and antigen from the circulation (Borges Da Silva et al., 2015).

It is well established that tissue resident macrophages are capable of cross-presentation of antigen, similar to dendritic cells (DCs) (Muntjewerff et al., 2020). Mice depleted of both splenic DCs and macrophages via $CD11c^+$- and $CD11b^+$-dependent depletion were shown to have poor antigen-specific T cell activation than those depleted of only $CD11c^+$ DCs (Schliehe et al., 2011). However, this study presents caveats in that CD11b expression alone is not sufficient to distinguish macrophages from DCs, and the $CD11c^+$ populations may be contaminated with $CD11c^{int}F480^+$ RP macrophages (Muntjewerff et al., 2020). In other studies, cross-presentation of fluorescently labelled ovalbumin by $CD11c^{int}F480^+$ RP macrophages resulted in faster OT-I cell activation and proliferation (Enders et al., 2020). Side-by-side comparison of liver-resident Kupffer cells demonstrated an equal ability of antigen cross-presentation and stimulation of OT-I $CD8^+$ lymphocytes when compared to $CD11c^+$ splenic DCs (Ebrahimkhani et al., 2011). When the ability of tissue resident macrophages to capture and retain antigen is taken into consideration with the localisation of the antigen persistence phenotype presented earlier in the chapter, these data pointed to a role for myeloid cells due to similar spatial localisation between tissue macrophages and persistent antigen.

The role of macrophages in antigen persistence was further confirmed by clodronate-dependent macrophage depletion studies. Clodronate treatment is a validated and commonly used method of macrophage depletion in animal models (Yu et al., 2021). Clodronate-mediated macrophage depletion did not prevent antigen persistence in OMV-immunised and infected mice, however it did alter the distribution of this antigen in the spleens of these animals, where a greater amount of antigen can be observed in the WP of the spleens as opposed to the RP-restricted antigen in macrophage-sufficient OMV-immunised tissues. The distribution of persistent antigen in the livers was not affected, however this is most likely due to the uniform distribution antigen persistence in macrophage-sufficient immunised and infected animals. It is therefore difficult to conclude whether depletion of macrophages influences liver antigen persistence in OMV-immunised tissues. Macrophages have been shown to enter an inflammatory state upon LPS stimulation of surface-bound CD14 resulting in secretion of pro-inflammatory cytokines TNFα, IL-1, and IL-6 (Meng & Lowell, 1997). Therefore, the perceived persistent antigen seen in OMV-immunised mice may be $LPS_{O\text{-}Ag}$ captured on macrophages during active inflammatory mechanisms. This however would not explain the stark increase in antigen in OMV-immunised mice compared to naïve controls, as if this were a simple matter of macrophage binding of LPS or endotoxin we would expect to see this phenotype regardless of immunisation status. Previous studies have demonstrated a mechanism in which B cell acquired antigen on the BCR is specifically transferred to APCs, including macrophages, leading to activation of $CD4^+$ T cells (Harvey et al., 2007). Thus, it may be possible that prior immunisation with OMVs that have been confirmed to induce ag-specific humoral and therefore B cell responses, could lead to the “priming” of macrophages by B cells in the event of active infection.

Further examination of macrophage depleted tissues indicated a role for macrophages in the structure of the splenic microarchitecture. Cell-cell interactions between macrophages and other immune cell types (both myeloid and lymphoid) are well documented. Therefore, knock-on effects of macrophage depletion on other cell types could have implications on the differential antigen localisation observed in the clodronate-treated tissues. Examination of these tissues by immunofluorescence revealed that WP localisation of antigen persistence was accompanied by an increased frequency of neutrophils, in addition to structural breakdown between the BCF and TCZ within the WP. This suggests an influence of macrophages on organisation of persistent antigen to the RP, in addition to organisation of $Ly6G^{+}$ neutrophils, and B and T lymphocyte zones within the WP. In a review by Scapini et al., it is stated that RP and MZ macrophages are proposed to orchestrate neutrophil recruitment and education, but these mechanisms are largely unexplored (Scapini & Cassatella, 2017). RP $Ly6G^{high}$ neutrophils have been previously shown to clear Streptococcus pneumoniae bacteria from the surface of RP macrophages and effect bacterial killing through poorly defined mechanisms (Deniset et al., 2017). If this mechanism was common across other bacterial infections, it may be possible that in the absence of macrophages, neutrophils migrate towards other cell types that capture or co-localise with extracellular bacterial antigen, leading to increased frequency of neutrophils within the BCF of the spleen where persistent STm^{+} antigen is accumulating. It is worth noting that *S. pneumoniae* is an extracellular bacterial infection, and so mechanisms of antigen capture may not be entirely comparable to that of intracellular *Salmonella* infections. However, in the context of immunised animals where enhanced antibody responses may neutralise bacteria and prevent

their intracellular entry, this mechanism may have some merit in describing antigen persistence and attraction of neutrophils to STm antigen-rich areas.

Moreover, differentially localised persistent STm^+ antigen was found to co-stain with IgM+ regions or cells within the BCF of the spleen. This was not shown in mice that received OMVs in the macrophage-sufficient context, and thus is a phenotype that results from removal of macrophages in OMV-immunised and infected mice. IgM^+ cells are characteristic of early GC responses; additionally, IgM^+-antigen immune complexes can accumulate during infectious immune responses (Baumann et al., 2001; Ferguson et al., 2004). A cell type of interest in this context may be follicular dendritic cells (FDCs); FDCs are stromal cells of mesenchymal origin that function to coordinate splenic microarchitecture via cytokine secretion (specifically BCFs) and retain antigen in its native conformation on their cell surface, aiding affinity maturation of GC B cells (Allen and Cyster, 2008; Heesters et al., 2013; Marcial-Juárez et al., 2023). FDCs are also still detectable in clodronate treated mice (Lisk et al., 2021), and so can be assumed to be present under these experimental conditions. In the absence of macrophages, which appear to capture persistent STm^+ antigen in OMV-immunised mice, it is possible that free-floating antigen is captured by the increased level of OMV-induced antibody, APCs and/or FDC networks. This STm^+ antigen could then accumulate within the BCFs on FDC networks, aiding in initiation and maintenance of GC reactions (Wu et al., 2009). Potentially, in the context of pre-immunised mice, macrophages might hinder the action of protective humoral responses by providing an intracellular survival niche for Salmonella bacteria. In their absence, persistent STm^+ antigen is free to be shuttled towards the FDC networks and GC reaction, improving humoral and cell-mediated adaptive responses that prove important in protective ability of the OMV vaccine. However, as previously mentioned, clodronate does not

exclusively deplete macrophage cell types, and therefore these perturbations in splenic structure and antigen shuttling might be reflective of other cells that are affected by clodronate treatment, for example stromal cell types such as $CD11b^{+}$ DCs.

The dependency on macrophages for antigen persistence would raise suggestions as to why subcutaneous (s.c.) immunisation resulted in persistence with a less intense phenotype. The s.c. experiments were initially conducted to address the less clinically relevant intraperitoneal (i.p.) method of immunisation used in all other experiment schedules throughout this chapter. Clinically available vaccines are traditionally administered through s.c., intramuscular (i.m.), or interdermal (i.d.) routes. With these methods, antigen may be transported to the draining lymph nodes (DLNs) by cell-mediated transport by APCs or may drain passively into the lymphatic vessels to reach the DLNs. Alternatively, antigen may diffuse into the blood vasculature and enter systemic circulation, reaching distal organs such as the liver, spleen, and kidneys (Ding et al., 2021; Irvine et al., 2020). In contrast, the peritoneal cavity is regarded as an excellent gateway to systemic circulation for i.p. administered substances, due to its large surface area and vast blood supply (Al Shoyaib et al., 2019; Kuzlan et al., 1997). Unlike the LNs, the spleen lacks afferent lymphatic vessels meaning all cells and antigen enter the spleen through the blood (Lewis et al., 2019). Therefore, antigen administered via i.p. injection would rapidly diffuse into the blood circulation and from there disseminate to distal organs such as the spleen and liver. This contrasts the more convoluted route antigen would take to disseminate to the spleen or liver when administered by other routes, having to first traverse the lymphatic system before entering systemic circulation. In this case, persistent antigen may be an artefact of the proximity of injection site to the spleen and liver, and due to its limited use in human vaccination, not a clinically relevant phenotype.

The results in this chapter indicated antigen persistence in s.c. immunised tissues, albeit with a lower intensity as seen with i.p. immunised tissues. S.c. immunisation has previously shown that antigen can be captured by skin infiltrating monocytes, and that these monocytes can develop into monocyte derived DCs and migrate to the draining lymph nodes (DLNs) in surrounding tissues (Schetters et al., 2020). Another study demonstrated that uptake of fluorescently labelled HIV-1 envelope protein is found in a diverse range of innate cell types, likely due to the high abundance of resident APCs in the skin (Ols et al., 2020). Classical dendritic cells have been shown to traffic bacteria between spleen structures (Doan et al., 2022), which presents the possibility that antigen or bacteria could be captured and trafficked to sites distal from the injection site. The prolonged trafficking, in addition to the accompanied processing that antigen will undergo between a subcutaneous injection site and the spleen, could explain the relatively lower level of antigen persistence in s.c. immunised tissues. However, these studies mainly address trafficking of antigen locally and to DLNs, rarely discussing the spleen. Alternatively, reduced antigen in the spleen of s.c. immunised mice may simply be a matter of a reduced amount of OMV antigen reaching the spleen. Uptake by various tissue resident monocytes, macrophages, and DCs during s.c. immunisation would result in less antigen entering the circulatory system compared to i.p. immunisation. This would result in lower levels of antigen reaching the spleen, and therefore less antigen to be captured by splenic macrophages. The questions presented in this section could be addressed by used of fluorescently labelled OMVs as a vaccine, and examination of proximal and distal tissues from the site of immunisation. Identification of fluorescent OMV^{+} cells in a similar fashion to Ols et al. may indicate the route that antigen takes to reach the spleen in this context. Further, use of an alternatively fluorescent STm strain in these

experiments would reveal whether persistent antigen captured in these tissues are vaccine or infection derived.

An important question that arises with this discussion is whether antigen persistence is relevant for protection against STm infections. This was first tackled with identification of this phenotype in OMV-immunised mice that were infected with clinical multidrug resistant isolate D23580. Often, observations made in mouse models have been shown to have little significance in the clinic due to a myriad of factors, including use of experimental lab strains of bacteria or use of irrelevant/non-clinical methods of intervention against infections and disease. Antigen persistence was found in mice immunised with OMVs and infected with the D23580 isolate at day 1 post-infection. The indication that this phenotype is apparent in mice infected with a more aggressively distinct strain from the attenuated SL3261 indicated a correlation between OMV-induced protection and persistence.

This association was further compounded after examination of sFLiC-immunised tissues. Immunisation with sFliC (protein subunit vaccine) did not induce protective immunity in mice infected for 21 or 35 days, nor was persistent STm+ antigen identified in the spleens of infected animals. However, sFliC has been previously shown to be protective at later time points of infection (day 18 – 35) (Bobat et al., 2011; Flores-Langarica et al., 2015), and so the lack of protection seen in my own experiments was unexpected. One explanation for this might be the differences in infection doses used, since previous studies used a dose that was ~20-fold larger than the dose used in my experiments. In any case, the level of protection (~10-fold reduction in splenic bacterial burden) seen in the previous study was not of the same magnitude as what has been seen with the OMVs. Therefore, the infection dose used in my experiments may not have been high enough to see subtle reductions in bacterial CFU.

Investigation of Vi-immunised mice revealed that protection against Vi^+ STm strain TH177 was achieved without antigen persistence. The Vi studies were initially used to identify whether antigen persistence holds relevance against typhoidal vaccines and infections, in addition to NTS. This could suggest that antigen persistence is specific to OMV-immunisation, and the second is that antigen persistence may not be indicative of protective vaccine responses. However, only a limited range of vaccine doses and time points have been examined, and thus these conclusions are not yet definitive until further studies are completed.

Localisation of persistent STm^+ antigen in spleens indicated an involvement of a myeloid-derived cells (macrophages, monocytes, neutrophils) due to localisation of these cell types in the splenic RP. *Rag2*$^{-/-}$ mice were immunised with OMVs and challenged to rule out lymphocytic cell involvement. *Rag2*$^{-/-}$ mice showed a small level of protection following immunisation and challenge, however this was significantly blunted compared to wild-type mice due to the lack of lymphocytes. Presence of antigen persistence was difficult to identify, due to high bacterial loads of both naïve and immunised groups. However, the quality of STm^+ staining (that is the more punctate staining as observed previously with WT mouse strains) in these mice could indicate an ongoing antigen persistence mechanism in the absence of all lymphocytes and antibody. This experiment first confirms previous findings that OMV-induced immunity against iNTS infections in mice relies heavily on lymphocyte-driven humoral and cellular immunity (Schager et al., 2018; Zhang et al., 2017). These results may also suggest a lymphocyte-independent mechanism of antigen capture, due to the potential of ongoing antigen persistence in *Rag2*$^{-/-}$ mice, however more studies with larger *n* numbers and alternative methods of detecting persistent STm^+ antigen in the context of high bacterial burden are required to confirm this. Finally, the data also

argue the point that persistent antigen is not necessarily indicative of protection, or vice versa.

Exploration of vaccine-driven influences of this phenotype lead to the discovery that the persistence of STm^+ antigen in immunised and infected mice is dependent on the $LPS_{O\text{-}Ag}$ status of the OMVs used for immunisation. Mice that received *Δwzy* OMVs did not show persistent antigen within the spleens or livers, despite offering a level of protection as previously seen with the WT (*ΔtolR*) OMVs. *Δwzy* OMVs possess only one $LPS_{O\text{-}Ag}$ subunit, and *ΔwbaP* OMVs are deficient of LPSO-Ag. This indicates first that the persistent STm^+ antigen observed could possibly be free or captured and presented $LPS_{O\text{-}Ag}$. LPS is known to interact with and activate immune cells by receptors such as Toll-like receptor (TLR)-4 (Fang et al., 2004; Rosadini & Kagan, 2017). Second, it suggests that the persistent antigen phenotype observed in infected OMV-immunised mice is not a protective mechanism, due to its absence in mice immunised with *Δwzy*-OMVs who showed equal protection as those immunised with WT OMVs. Previous work on OMVs and GMMA have described that OMVs with truncated LPS can confer cross-protection against multiple serotypes of Salmonella infection (Liu et al., 2016), confirming that $LPS_{O\text{-}Ag}$ length is not a critical parameter for immunogenicity of GMMA or OMVs (Gasperini et al., 2021). Therefore, the presence of persistent STm^+ antigen is unlikely to have any protective benefit, as both this data and previous work have shown OMVs with shorter $LPS_{O\text{-}Ag}$ chains are shown to be equally protective as WT OMVs.

In conclusion, the results in this chapter suggest that antigen persistence is indicated to be macrophage-dependent, potentially lymphocyte-independent, and $LPS_{O\text{-}Ag}$-dependent. It is not necessarily a protective mechanism of immunisation with OMVs, due to its absence in mice immunised with *Δwzy* OMVs. Further investigations into

this phenotype are required to draw definitive conclusions of the nature of the persistence, which are discussed in the chapter 5.

CHAPTER 5: FINAL DISCUSSION

The necessity for vaccines against *Salmonella* infections, particularly those causing iNTS disease, is becoming increasingly crucial with the rise of AMR strains and diminishing pool of treatment options. In aid of successful vaccine design, the initial aims of this thesis were to explore how immunisation and protective immunity to OMVs is regulated using mouse models of iNTS infection. This was achieved by investigation of two objectives: identification of immune components required for successful immunisation with OMVs against iNTS infections; and identification of any consequences of vaccination with OMVs against iNTS on either the host or pathogen.

The work presented in this thesis has implications for vaccine design. In Chapter 3, I found that complement deficiency had little effect on the ability to control primary STm infection yet discovered that C3 was important for the development of productive OMV-induced immune responses that protect against subsequent STm infection. This demonstrates that the role of C3 is determined by the context in which antigen is presented to the immune system. It has previously been shown that antigen presented in the context of active infection induces distinct immune responses and pathways than those presented in a vaccine. Immunisation with soluble flagellin skews immune responses in mice towards a Th2 bias as characterised by production of IL-4 and class-switching to IgG1, whereas exposure to flagellated bacteria induces flagellin-specific Th1 responses and switching to IgG2a (IgG2c in mice) (Cunningham et al., 2004b; Bobat et al., 2011). In a similar way, infection may activate different immune pathways depending on exposure to live bacteria or OMVs, resulting in the discrepancies between primary and secondary infections in $C3^{-/-}$ mice.

Examination of B cells and antibody titres at separate timepoints of infection in naïve or immunised tissues would give insight into the temporal components responsible for this discrepancy. This could inform as to if the humoral response to infection is present but short-lived or non-existent in $C3^{-/-}$ mice. Additionally, antibody titres measured in this chapter were focused to total IgM and IgG titres. Considering previous findings of Th1 vs Th2-skewed responses to either soluble flagellin or live bacteria, it would be important to investigate if a similar pattern occurs when comparing live bacteria and OMV immunisation. This could be done by measuring antibody titres of specific IgG subclasses, cytokine ELISAs, and measuring of specific Th subset-specific mRNA transcripts as described previously (Cunningham et al., 2004b).

The interaction between C3 and B cell responses and regulation in humans is well established (Frade, 1990; Fearon, 1983; Ghannam et al., 2008; Kremlitzka et al., 2019). Receptors for C3 breakdown products are abundantly expressed on B cells (Erdei et al., 2021). Investigation of the interaction between serum derived C3 and DNA in human B cells have demonstrated C3 bound to histone H1 in a dose-dependent manner *in vitro*. Changes in histone structure or function induce chromatin remodelling, which alters DNA accessibility to transcription factors and regulates gene transcription (Kremlitzka et al., 2019). However, a limitation of this study lies in the differences between mouse and human complement systems. Early complement components are largely similar between mice and humans, however, complement receptors 1 and 2 (CR1 and CR2) are encoded by two separate genes in humans but only one gene in mice that is alternatively spliced resulting in two distinct gene products (Jackson et al., 2020; Jacobson and Weis, 2008). Murine CR2 shares only 65% protein sequence homology with human CR2 (Fingeroth, 1990). A review of C3-mediated processes and C3 receptors highlighted that mouse CR1 contains structural

elements of the CR2 receptor due to its encoding by the *Cr2* gene, indicating that most functions of mouse CR1 are almost identical to that of mouse CR2. CR1 and CR2 do not possess identical binding characteristics as their human counterparts, where mouse CR1 efficiently binds C3dg yet human CR1 does not (Michael Holers et al., 1992), possibly resulting in different biological activity. Functionally, studies have identified that mouse complement is functional and mimics a dependence of complement on bactericidal activity against *Salmonella* (Micoli et al., 2018), whereas others have shown that mouse serum is unable to kill *Salmonella* compared to human complement (Siggins et al., 2011), and that WT mouse sera has approximately ten-fold lower complement activity compared to human sera (Latuszek et al., 2014). Further, the review discusses how human CR2 exhibits inhibitory roles as a coreceptor to the BCR during B cell activation, which is opposite to the costimulatory properties of mouse CR2 (Erdei et al., 2021; Józsi et al., 2002). Therefore, whilst there is a clear link between C3 and B cell responses, conclusions made in the mouse complement system should be compared to humans with caution.

Both the immunisation and challenge experiments, and adoptive transfer experiments using $C3^{-/-}$ mice in this chapter highlight the importance of the innate immune components during induction and persistence of adaptive responses stimulated by vaccination. This is a feature of OMVs due to their inherent adjuvant features that make them an attractive vaccine candidate against various pathogens (Poltorak *et al*., 1998; Mancini *et al*., 2020; Micoli *et al*., 2018). The data emphasize the importance of complement stimulation during vaccination to induce robust B lymphocyte and humoral responses against STm antigen and confirm previous findings that the adjuvanticity afforded by OMVs is beneficial.

Failure of $C3^{-/-}$ mice to mount protective immune responses to STm with prior OMV-immunisation, in addition to the failure in rescuing this phenotype without prior supplementation with an exogenous complement source, highlight the importance of innate immune components (i.e., complement) during induction and persistence of protective vaccine responses. A major benefit of OMV's is their inherent adjuvant features that make them attractive vaccine candidates against a variety of pathogens (Poltorak et al., 1998; Micoli et al., 2018; Mancini et al., 2020, 2021). This is thought to be driven by abundant PAMPs and immunopotentiator activities of TLR agonists on the OMV surface as a result of its mimicry of the bacterial outer membrane (Mancini et al., 2020). Various studies have compared the activities of OMV-based and 'conventional' vaccines in mice. Exploration of meningococcal vaccines demonstrated that immunisation with both purified PorA and PorA-containing OMVs resulted in IgG induction, however only those raised against the OMVs were bactericidal (Arigita et al., 2003). Use of STm-OMVs to deliver *S. pneumoniae* protein PspA resulted in antibody induction to PspA, in addition to STm-LPS and outer membrane proteins, yet antibody was not detected in mice immunised with purified PspA. Additionally, mucosal IgA was raised against the OMV-PspA, but not for purified PspA. (Muralinath et al., 2011). OMV vaccine derived from *Bordetella pertussis* induced the highest antibody levels against all subclasses (IgG1/G2a/G2b/3) compared to whole cell vaccines, but only IgG1 was raised against the traditional acellular subunit vaccine (Raeven et al., 2015). Generalised modules for membrane antigen (GMMA) derived from STm induced the most diverse antibody profile with greater bactericidal activity compared to an STm O-Ag-CRM_{197} glycoconjugate, which almost exclusively induced IgG1. Further, the bacterial burden in tissues of GMMA-immunised mice was markedly lower than those that received the glyoconjugate (Micoli et al., 2018). Among these

studies, the inherent adjuvanticity of OMVs is the most common theme discussed for the superiority these vaccines. The most common immune potentiator discussed was LPS or LOS, a potent TLR4 activator, however it is also noted that OMVs may contain bacterial DNA and would additionally stimulate TLR9 responses (Raeven et al., 2015). Less complex vaccines such as the subunits or glycoconjugates, whilst immunogenic, are unable to drive the magnitude of immune responses as OMVs alone, relying on co-administration of synthetic adjuvants such as alhydrogel to achieve similar levels of antibody induction (Micoli et al., 2018). Additionally, studies where purified microbial products are used may not necessarily represent relevant forms. OMVs present bacterial antigens in their native context where TLRs and antibody recognise complexes of PAMPs and TLR-ligands (Alaniz et al., 2007; Domínguez-Medina et al., 2020), representing a more biologically relevant method of immune stimulation. With their relatively low cost and ease of production, in addition to their self-adjuvanticity and superior biological relevance, OMVs are therefore a simpler method of immune stimulation to pathogenic antigen, owing to their appeal as a vaccine method.

Exploration of the role of complement in vaccine-mediated protection uncovered an unexpected STm antigen persistence phenotype in OMV-immunised and challenged tissues, despite recovering low bacterial numbers. Pre-clinical research of vaccine efficacy primarily focuses on the success or failure to mount a protective immune response against infection. Consequential phenotypes that arise during infection are little studied unless the resulting phenotypes are immediately pathogenic or fatal. Successful protective immune responses were induced in mice immunised with *Δwzy* OMVs in the absence of the persistent antigen phenotype, indicating that antigen persistence does not strictly correlate with protection in the host. If not a protective mechanism, this could suggest that the persistence phenotype could lead to potentially

pathogenic outcomes such as vaccine-associated pathology reported in RSV vaccine studies (Kapikian et al., 1969; CHIN et al., 1969), or more recently highlighted in cases of vaccine-associated enhanced respiratory disease (VAERD) during the design and implementation of vaccines against SARS-CoV-2 (Ebenig et al., 2022). $LPS_{O\text{-}Ag}$ is highly immunogenic, and the vaccine-induced retention may lead to host-driven pathology within tissues due to prolonged $LPS_{O\text{-}Ag}$-directed inflammation. This could be assessed by measurement of pro-inflammatory cytokines such as IFNγ or TNFα by cytokine ELISA in mice immunised with WT OMVs and compared to those immunised with *Δwzy* OMVs. Discrepancies between pro-inflammatory markers in these two groups would indicate a pathogenic outcome to immunisation with OMVs, thereby informing vaccine design.

It is unclear whether the persisting STm antigen represented live bacterial cells or STm antigen only. Non-culturable viable cells, also termed 'persister cells' have been identified in models of intracellular *Salmonella* infections (Helaine et al., 2014). Identification of whether the persisting STm antigen is the result of captured STm antigen, or persisting cells that are not detectable by traditional microbiological methods, would raise important concerns that would influence OMV vaccine design. Detection of bacterial RNA within tissues by qPCR, or by use of RNAScope techniques that fluorescently stain bacterial mRNA, would indicate the presence of replicating bacterial cells in immunised mice due to its short half-life (Wang et al., 2012; Bonifacio and Schmolke, 2021). Alternatively, use of the fluorescent-reporter *Salmonella* strains would address this question. Helaine and colleagues describe a fluorescence-dilution reporter system that enables direct quantification of actively dividing *Salmonella* cells due to possession of a the pDiGc vector (Helaine et al., 2010). This vector constitutively expresses GFP and DsRed under an arabinose-inducible promoter.

Removal of the arabinose source during subculture (i.e., when injected into mice) leads to dilution of the red fluorescence if cells are actively dividing, however subsequent work with this reporter system indicated the presence of persisting, non-replicating *Salmonella* within macrophages (Helaine et al., 2014). Use of this reporter system could indicate whether antigen is indeed live, and if it is, whether it is actively dividing.

Examination of the antigen persistence phenotype identified a role for macrophages. This could indicate that persisting antigen was more likely to be live bacteria, as macrophages are a well-established intracellular survival niche for *Salmonella* (Buchmeier and Heffron, 1989; Salcedo et al., 2001), however experiments with LPS-truncated OMV mutants could alternatively suggest that persisting antigen may be retention of $LPS_{O\text{-}Ag}$ on macrophages. In these experiments, clodronate was used to deplete macrophages. As previously discussed in chapter 4, clodronate treatment does not exclusively affect macrophages, and other cell types may be depleted (Ward et al., 2011) or rendered functionally inert (Culemann et al., 2023), leading to a larger perturbation of immune cells than anticipated. Additionally, clodronate treatment was only conducted prior to infection, and not from the beginning of the immunisation schedule. Immunisation and infection of mice that are either genetically deficient or mutated to induce specific macrophage depletion with certain drug or toxin administration (Hua et al., 2018) would give more valid insights into the relationship between macrophages and persistent STm antigen in immunised and infected mice. For instance, macrophage Fas-induced apoptosis (MAFIA) transgenic mice possess a Fas-suicide system driven by the mouse *Csf1r* gene which, when dimerised by administration of AP20187 drug, leads to apoptosis of *Csf1r* transgene-expressing

cells (Burnett et al., 2004). This results in a reversible depletion of macrophages *in vivo* without widespread side effects of clodronate.

Furthermore, the signalling pathways and receptors responsible for maintaining antigen persistence are unclear. Receptor knockout mutants would provide insight into the specific ligand-receptor mechanisms that contribute towards the persistent STm^{+} phenotype. TLR4 is a noted receptor of LPS, however it is well established that the immunogenicity of LPS lies in the lipid A moiety of bacterial LPS, where the core and O-specific carbohydrates play little role in recognition by the host (Park and Lee, 2013). Nevertheless, immunisation and infection of $TLR4^{-/-}$ mice would confirm the influence of TLR4 in this phenotype. Alternatively, macrophages in $MD2^{-/-}$ mice (a TLR4 co-receptor that aids LPS recognition) are unresponsive to LPS whilst still being susceptible to *Salmonella* infection (Nagai et al., 2002), and would also provide insight into the effects of the TLR4-MD2-LPS binding pathway on the persistent antigen phenotype.

In conclusion, the data presented in this thesis both confirm essential components of vaccine-mediated immune responses using OMVs, and additionally inform of a novel consequential phenotype in the use of WT OMVs as a vaccine against STm infections in mice. The data not only highlight the importance of innate immune components in driving humoral responses that govern protective immunity against STm, but also a need for consideration of non-protective phenotypes that arise due to immunisation against specific pathogens, and the implications they may have on vaccine design in the context of reactogenicity.

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APPENDIX A: REAGENTS, BUFFERS, AND MEDIA

All buffers and media used throughout this study are listed below:

Luria-Bertani (LB) broth

20 g LB broth (Lennox) powder (Thermo Fisher Scientific)

1 L dH_2O

LB agar

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

1 L dH_2O

Broth & agar were autoclaved at 121ºC

1X Tris-buffered saline (TBS)

1.5 L NaCl 0.2M

1 L Tris 1M

1.5 L HCl 0.1M

ELISA wash buffer

500 µL Tween20

1L D-PBS

ELISA dilution buffer

125 µL Tween 20

2.5 g BSA

250 mL D-PBS

Table A1: Reagents

Reagent	Use	Manufacturer	Cat #
SIGMAFAST™ p-Nitrophenyl phosphate Tablets	ELISA substrate development	ThermoFisher	N2770-50SET
ELISA Wash Buffer, 1L packets	ELISA washes	Invitrogen	00-0400-46
Dulbecco's Phosphate buffered saline	ELISA buffers	Sigma-Aldrich	P4417-50TAB
N,N-Dimethylformamide	IHC substrate	Sigma-Aldrich	270547-2.5L
Fast Blue RR Salt	IHC substrate	Sigma-Aldrich	F0500-25G
3,3′-Diaminobenzidine tetrahydrochloride	IHC substrate	Sigma-Aldrich	D5905
VectaMount® Permanent Mounting Medium	IHC mounting	Vector Laboratories	H-5000-60
RPMI 1640 Medium	Cell culture/FACS	Gibco	11875093
Penicillin-Streptomycin (10,000 U/mL)	Cell culture/FACS	Gibco	15140122
Heat-inactivated fetal bovine serum	Cell culture/FACS	Gibco	10500064
Bovine Serum Albumin (BSA)	Buffers for ELISA, IF	Sigma-Aldrich	A3059
Bugbuster	Protein purification	Merck	70584
TAE Buffer 10X	Molecular Biology	Millipore	1.06023
Sodium Chloride (NaCl)	IHC Buffers	Sigma-Aldrich	S9888
Hydrochloric Acid, 37%	IHC Buffers	Fisher Scientific	10184763
Tris base	IHC Buffers	Sigma-Aldrich	10708976001
LPS	ELISAs/ELISpots	Enzo Life Sciences	ALX-581-011
Tissue-Tek OCT compound	Cryosectioning	Sakura Finetek USA	4583
Gentamicin solution	Gentamicin protection assay	Sigma-Aldrich	G1272
EDTA solution pH 8.0 (0.5M) for molecular biology	Cell culture/FACS	PanReac AppliChem	A4892.0100
ACK Lysis Buffer	Cell culture/FACS	Gibco	A1049201

Trypan Blue Solution, 0.4%	Cell culture/FACS	Gibco	15250061
LB Broth	Bacterial culture	ThermoFisher	12780052
LB Agar	Bacterial culture	Sigma-Aldrich	L2897
Tween20	ELISA	Sigma-Aldrich	P1379

Table A2: Kits

Kit	Use	Manufacturer	Cat #
Pierce™ BCA Protein Assay Kit	Protein concentration measurement for antigen prep	Thermo Scientific	23227
VECTASTAIN® ABC-AP Kit Alkaline Phosphatase (Standard)	IHC colormetric development substrate	Vector Laboratories	AK-5000
Foxp3/Transcription Factor Staining Buffer Set	FACS Intracellular staining	Invitrogen	00-5523-00

APPENDIX B: GATING STRATEGIES

Gating strategies used for flow cytometry analysis throughout Chapter 3 are outlined here:

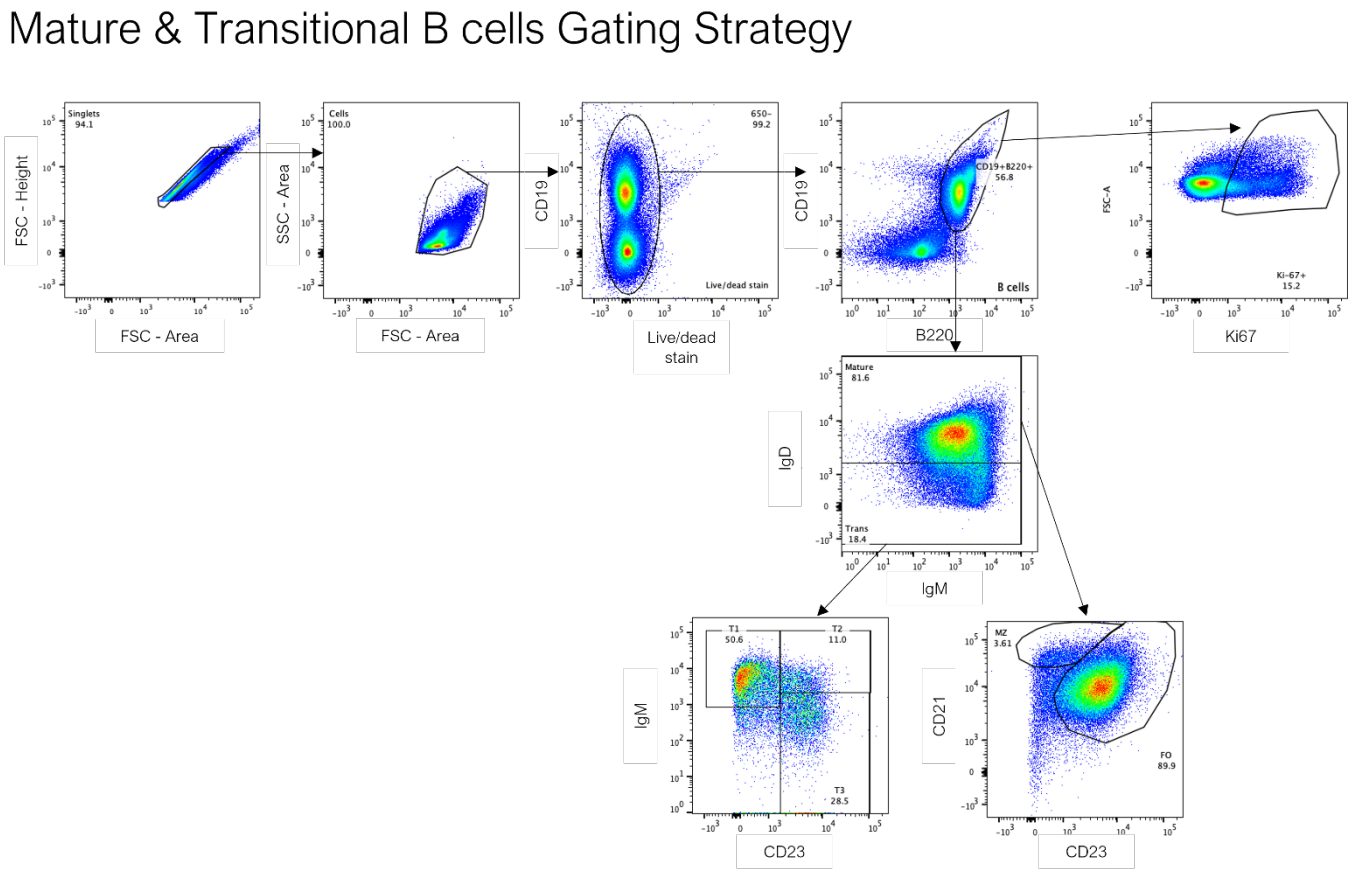

Fig. B1: Illustration of representative FACS plots used to gate mature and transitional B cells in chapter 3. WT or C3-deficient mice were vaccinated i.p. with 200 µL PBS or 1 µg ΔtolR OMVs for 14 days before challenge with 5×10^5 CFU SL3261 STm for 24 hours. Spleens were harvested, mashed, processed, and measured for cell populations by flow cytometry.

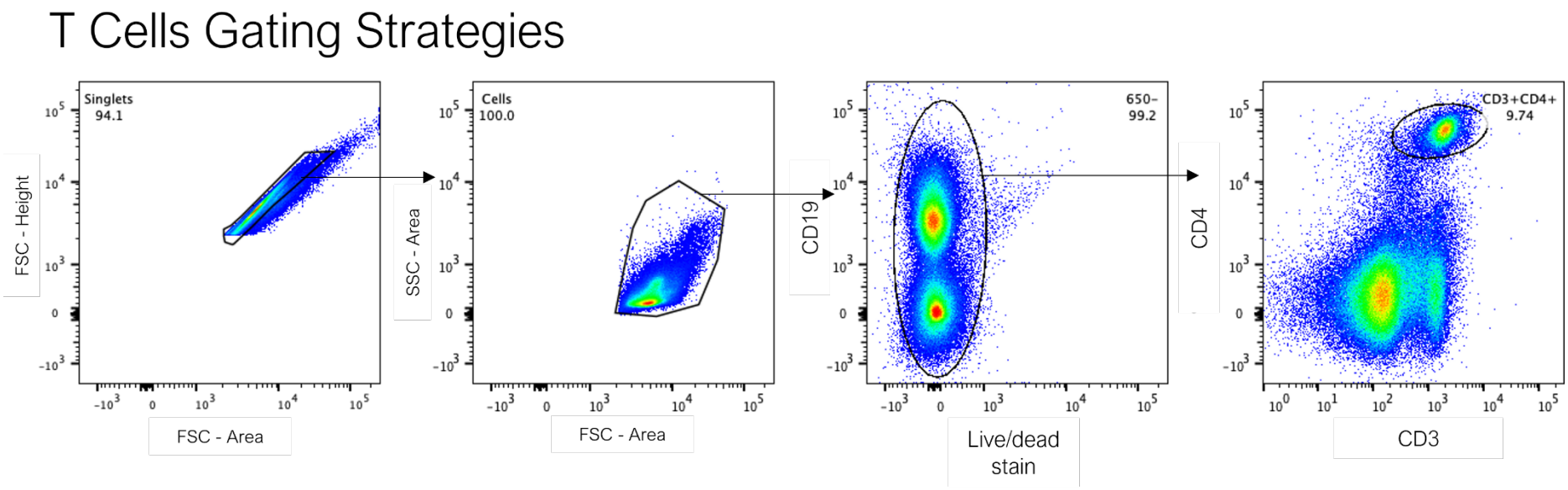

Fig. B2: Illustration of representative FACS plots used to gate T cells in chapter 3. WT or C3-deficient mice were vaccinated i.p. with 200 µL PBS or 1 µg ΔtolR OMVs for 14 days before challenge with 5×10^5 CFU SL3261 STm for 24 hours. Spleens were harvested, mashed, processed, and measured for cell populations by flow cytometry.

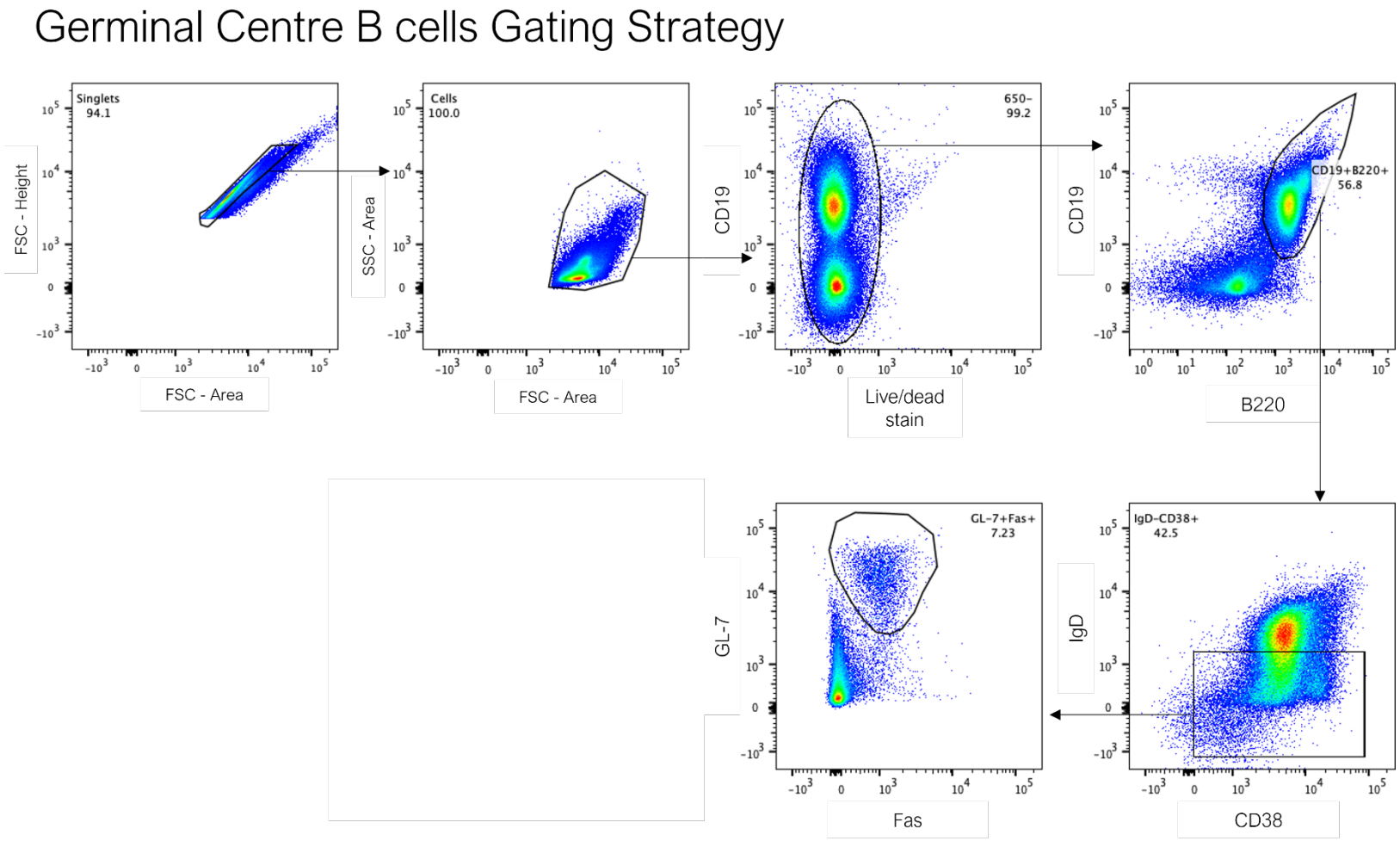

Fig. B3: Illustration of representative FACS plots used to gate germinal centre B cells in chapter 3. WT or C3-deficient mice were vaccinated i.p. with 200 µL PBS or 1 µg ΔtolR OMVs for 14 days before challenge with 5×10^5 CFU SL3261 STm for 24 hours. Spleens were harvested, mashed, processed, and measured for cell populations by flow cytometry.

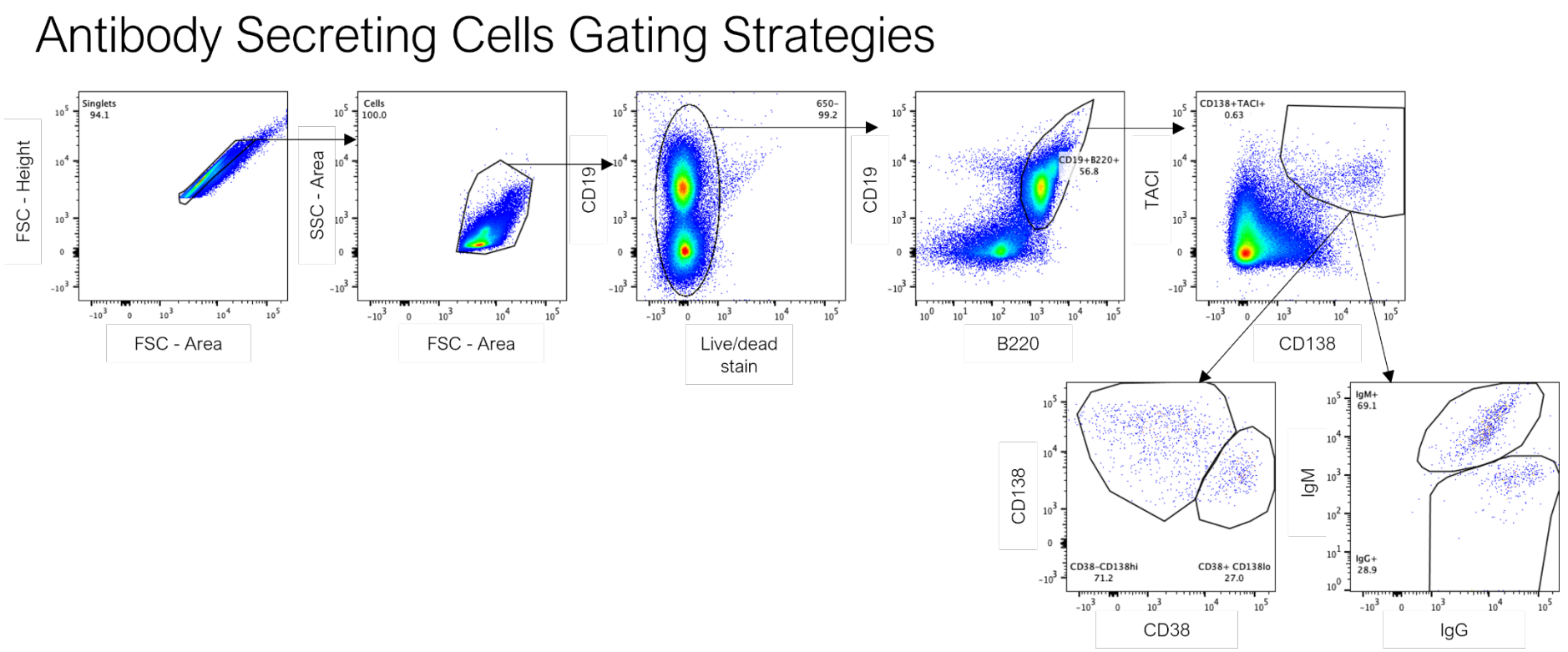

Fig. B4: Illustration of representative FACS plots used to gate antibody secreting B cells in chapter 3. WT or C3-deficient mice were vaccinated i.p. with 200 µL PBS or 1 µg ΔtolR OMVs for 14 days before challenge with 5×10^5 CFU SL3261 STm for 24 hours. Spleens were harvested, mashed, processed, and measured for cell populations by flow cytometry.