Immunity and protection induced by outer membrane vesicles against Invasive non-Typhoidal *Salmonella*

Areej Ibrahim Alshayea

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Institute of Immunology and Immunotherapy
College of Medical and Dental Sciences
The University of Birmingham
Edgbaston
B15 2TT

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ABSTRACT

Salmonella enterica is responsible for causing both diarrheal and systemic illnesses, which remain significant global concerns. The development of vaccines that offer broad protection against multiple serovars holds potential in effectively managing diseases caused by *Salmonella*. Outer Membrane Vesicles (OMVs), which are naturally released by Gram-negative bacteria, show promising potential for developing subunit vaccines due to their high immunogenicity and protective effectiveness. The O-antigen of lipopolysaccharide (LPS) has been identified as a key target for inducing protective immunity. However, variation in O-antigen expression between closely related pathogens, such as *S.* Typhimurium and *S*. Enteritidis, limits cross-protection after natural infection. Modifications in the O-antigen chain length can alter outer membrane structure and composition. In this study, genetic mutations were introduced into *Salmonella* OMV-producing strains in order to reduce or delete LPS O-antigen expressions. The cellular, humoral, and functional immune responses to OMV from *S*. Typhimurium expressing wild-type length O-antigens (WT-OMV), ≤1 O-antigen repeat (wzy-OMV), or no O-antigen (wbaP-OMV) were assessed in mice immunisation studies. All OMV induced similar plasma cell and germinal centre responses, although the nature and outcomes of these responses differed. Surprisingly, wzy-OMV elicited comparable levels of anti-LPS IgG responses to WT-OMV. However, wzy-OMV and wbaP-OMV induced more robust responses against cell-surface-exposed porin proteins and cross-reactive antibodies targeting S. Enteritidis. Immunisation with wzy-OMV provided protection against *S*. Typhimurium comparable to that induced by WT-OMV. In contrast, after one immunisation, with any OMV type, there was minimal control of *S*. Enteritidis infections, except in the blood. However, after boosting with OMVs, there was an enhanced level of cross-protection.

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شكرا امي وابي من اعماق قلبي, لا توجد كلمات توفيكم حقكم, جزاكم الله عني خير الجزاء وحفظكم لي.

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Chapter 1. INTRODUCTION

1.1 *Salmonella* classification

Salmonella is a gram-negative non-spore forming facultative intracellular bacterium that belongs to the family Enterobacteriaceae. *Salmonella* is classified into two species: the first one is *Salmonella bongori* which often affects cold-blooded animals, and the other species is *Salmonella enterica* which includes the *Salmonella* serovars that most risk to humans (1, 2). There are six subspecies of *Salmonella enterica*, which are: *enterica, salamae, arizonae, diarizonae, houtenae, and indica*. *Salmonella enterica* includes more than 2500 serovars which differ in their surface antigens. These serovars are classified based on surface antigens, which are lipopolysaccharide (O-Ag) and flagellin (H-Ag) and capsule (K-Ag), according to the Kauffmann and White classification system (1). *S. enterica* subsp. *enterica* serovar Typhi (STy) expresses the capsular polysaccharide K antigen (Vi), while *S. enterica* subsp. *enterica* serovar Typhimurium (*S*. Typhimurium) and Enteritidis (SEn) do not express Vi. *Salmonella enterica* subsp. *enterica* includes many medically important human pathogens, but amongst the most prominent serovars are: Typhi, Paratyphi, Typhimurium, and Enteritidis, which are the main causes of Salmonellosis in humans (3). *Salmonella* enterica subsp. enterica can also be classified as causative or non-causative of Typhoidal Salmonellosis. *Salmonella* serovars, including *Salmonella* Typhi and *Salmonella* Paratyphi, are the main cause of enteric fever (4). However, *Salmonella* enterica serovar Typhimurium and *Salmonella* enterica serovar Enteritidis are causes of non-Typhoidal Salmonellosis (NTS) and are the most common serovars referred to throughout this project.

1.2 Cell wall structure of *Salmonella*

The cell wall composition of *Salmonella*, like all gram-negative bacteria, consists of two layers: the inner membrane (IM) and outer membrane (OM) (Figure 1.1). The inner membrane is called the cytoplasmic membrane and consists of a phospholipid bilayer. The IM and OM are separated by a viscous layer of peptidoglycan and proteins called the periplasm. The *Salmonella* outer membrane in turn consists of an inner layer composed of a phospholipid, while the outer layer is mostly made up of lipopolysaccharide (LPS) and other components, such as porins and other outer membrane proteins (OMPs). The outer membrane of *Salmonella* exposed to the environment contains some of the most accessible and immunogenic antigens which are key targets for recognition by the immune system and are able to trigger protective immune responses. Most of these components are shared between both typhoidal and nontyphoidal serovars. However, there is some heterogeneity, and a major one is the Vi capsular polysaccharide, a virulence factor in S. Typhi, which is not expressed in NTS strains *S*. Typhimurium and *S*. Enteritidis (5, 6)

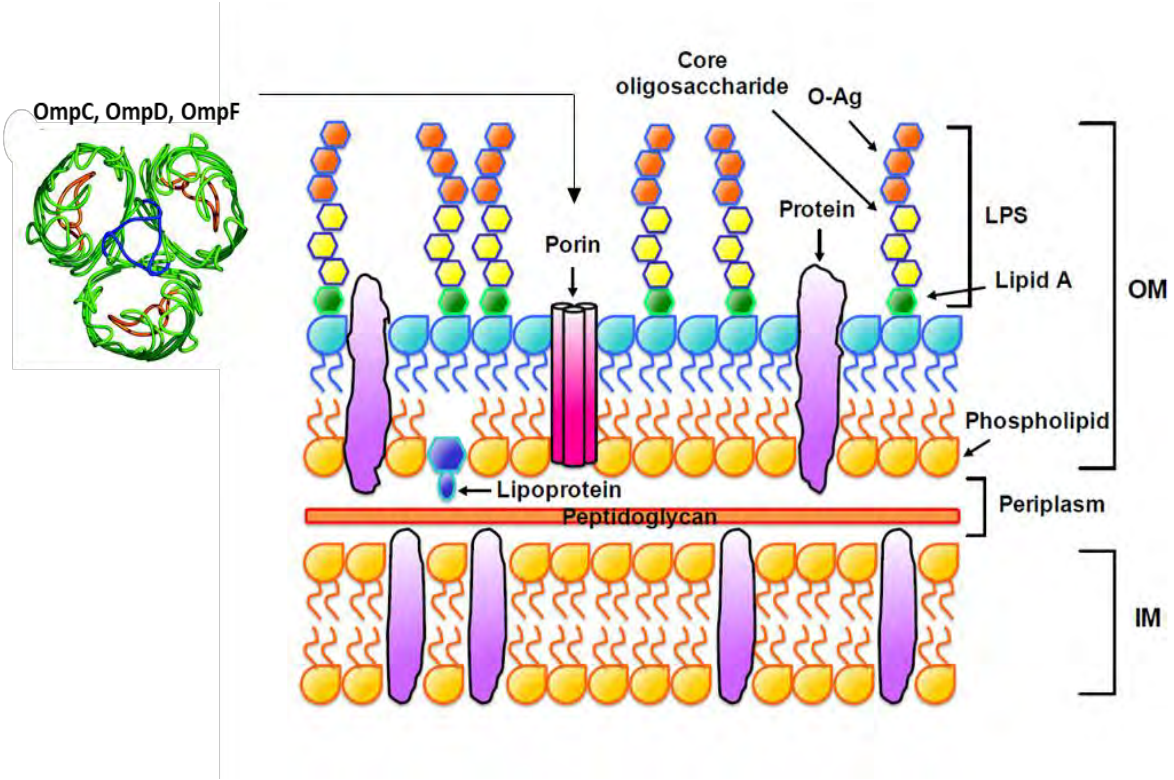


Figure 1.1 Gram-negative bacteria cell wall Structure.

The cell wall of *Salmonella* is composed of the outer membrane (OM) and the inner membrane (IM), separated by a layer of peptidoglycan which is called periplasm. Porins, the trimeric channel, found in the OM, it could be OmpC, OmpD, or OmpF. Lipopolysaccharide (LPS) is found in the outermost layer of the OM and is composed of three parts: lipid A, core oligosaccharide, and O-antigen (O-Ag). OAg and the core protrude from the OM surface. Figure taken and adapted from Brock Biology of Microorganisms, 11ed, 2006, Pearson Prentice Hall, inc.

1.2.1 Lipopolysaccharide (LPS)

LPS is found in the outer leaflet of all Gram-negative bacteria outer membranes and attached to the phospholipids. LPS potentially interacts with the immune system through each of its three main components: lipid A, a polysaccharide core, and the O-antigen (O-Ag) chain (7) (Figure 1.2). The hydrophobic lipid A consists of fatty acid chains and is connected to the core by an N-acetylglucosamine linkage (8). It is responsible for the endotoxic activity of LPS and can cause toxic shock through stimulating the Toll-like receptor 4 (TLR4) signal that causes the

release of pro-inflammatory cytokines by macrophages (9, 10). Modifications in lipid A can affect the recognition by Toll-like receptors (TLRs) and subsequent immune responses. Lipid A component can have different acyl chains, and the number and length of these chains can vary. These differences can affect the fluidity and stability of the outer membrane. The phosphorylation pattern of lipid A can differ, influencing the immunogenicity and interaction with host immune responses.

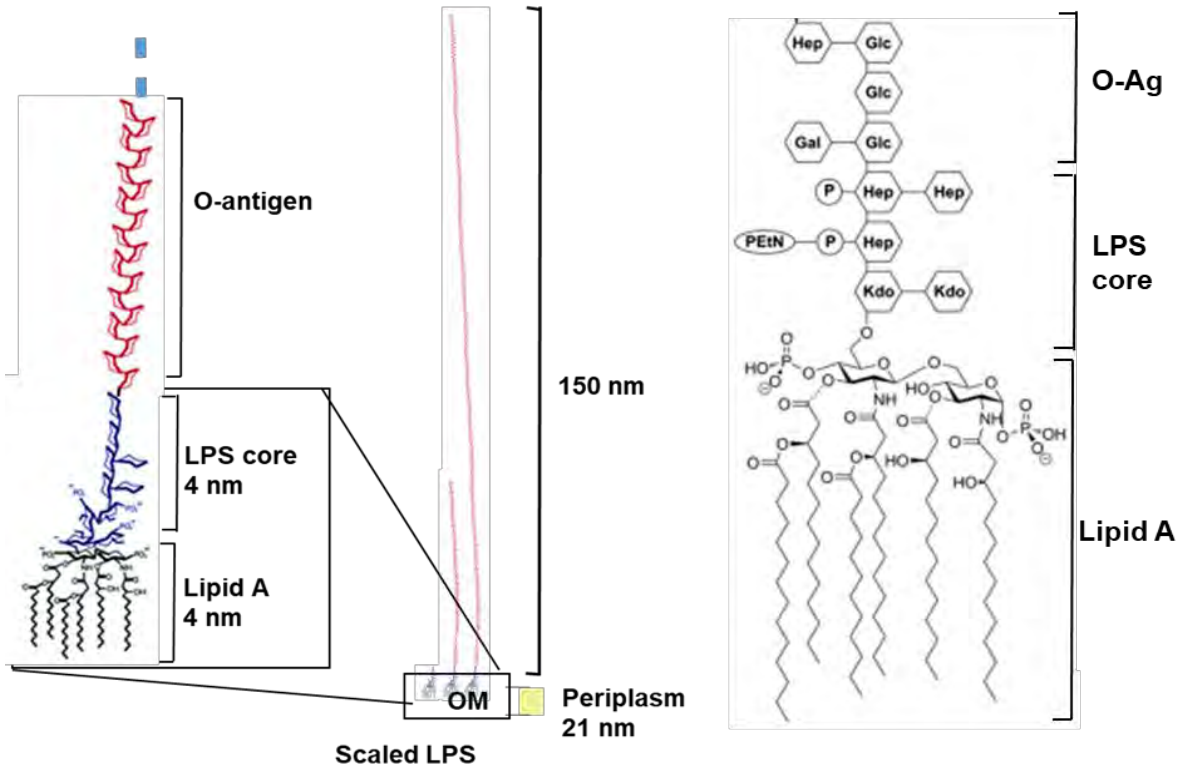


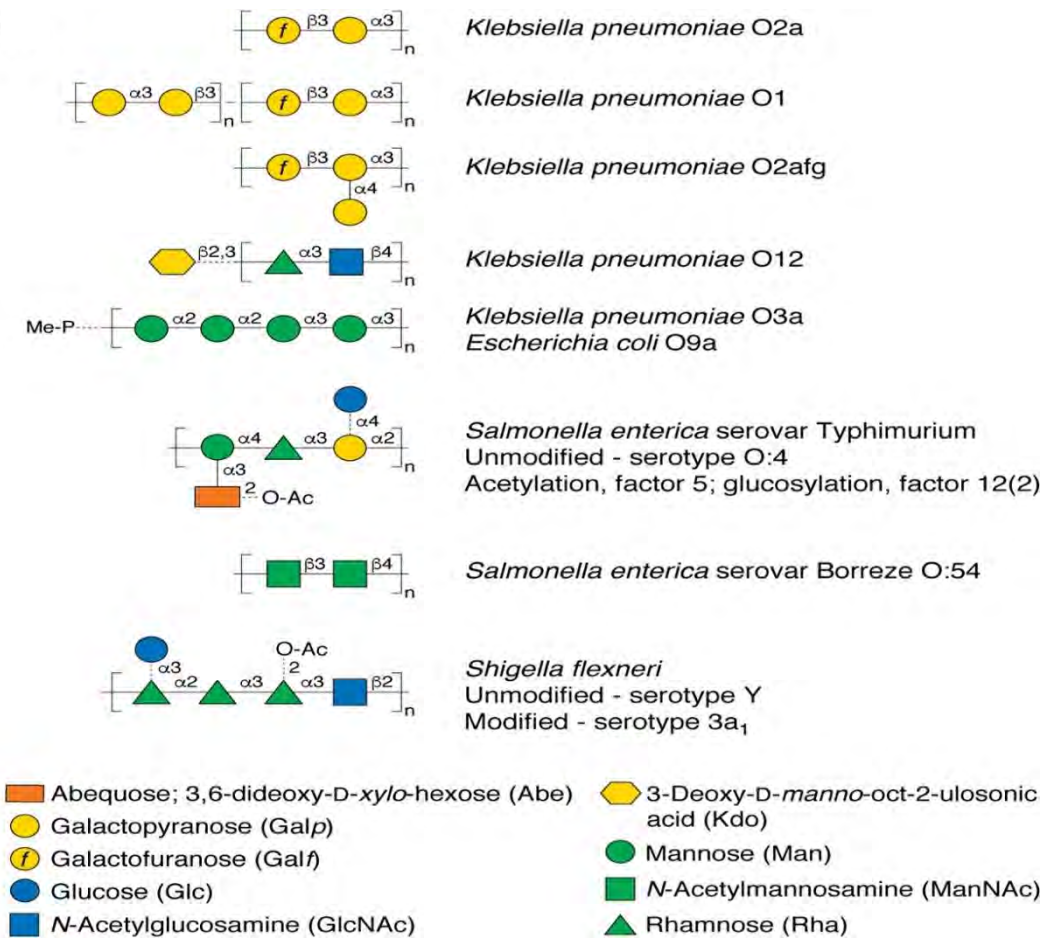
Figure 1.2 Lipopolysaccharide Structure in the outer membrane of non-typhoidal *Salmonella*.

Diagram shows the LPS structure: the Lipid A, core oligosaccharide, and O-Antigen. A proportionally schematic scaled LPS shows the repeating units of the O-Ag. some LPS molecules have short O-Ag attached, and some have a relatively long O-Ag. Figure adapted from Dr. Marcin Grabowicz, University of Princeton.

The core polysaccharide connects lipid A with O-antigen and can vary in composition and length. Changes in the core structure may affect the permeability of the outer membrane and its resistance to environmental stresses. Core antigen provides stability and is more conserved between different species and serovars. The variations in LPS structure can impact the barrier properties of the outer membrane. Changes in lipid A or core polysaccharide may affect the membrane's ability to prevent the entry of harmful substances.

O-antigen is often but not always made of repeating oligosaccharide units of variable length depending on the number of repeats. Each unit is about 1-1.6 nm long and shows a high degree of variation between different strains (11) (Figure 1.3 A). Variations in O antigen structure contribute to the antigenic diversity of bacteria, influencing their ability to evade immune recognition. In addition, even among closely related bacteria, LPS O-antigen can exhibit notable structural diversity. *Salmonella* Typhimurium and *Salmonella* Enteritidis express different O-antigen, although they share a common O polysaccharide backbone (Figure 1.3 B). The difference is just one sugar that is an acetylabequose in *S*. Typhimurium (O-antigen 4) and a tyvelose in *S*. Enteritidis (O-antigen 9) (12, 13). Despite the fact that the difference is relatively small, this could affect the molecular dynamics (MD) of the outer membrane making the latter more flexible than the former.

A


Klebsiella pneumoniae O2a
Klebsiella pneumoniae O1
Klebsiella pneumoniae O2afg
Klebsiella pneumoniae O12
Klebsiella pneumoniae O3a
Escherichia coli O9a
Salmonella enterica serovar Typhimurium
Unmodified - serotype O:4
Acetylation, factor 5; glucosylation, factor 12(2)
Salmonella enterica serovar Borreze O:54
Shigella flexneri
Unmodified - serotype Y
Modified - serotype 3a1
Abequose; 3,6-dideoxy-D-xylo-hexose (Abe)
Galactopyranose (Galp)
Galactofuranose (Galf)
Glucose (Glc)
N-Acetylglucosamine (GlcNAc)
3-Deoxy-D-manno-oct-2-ulosonic acid (Kdo)
Mannose (Man)
N-Acetylmannosamine (ManNAc)
Rhamnose (Rha)

B

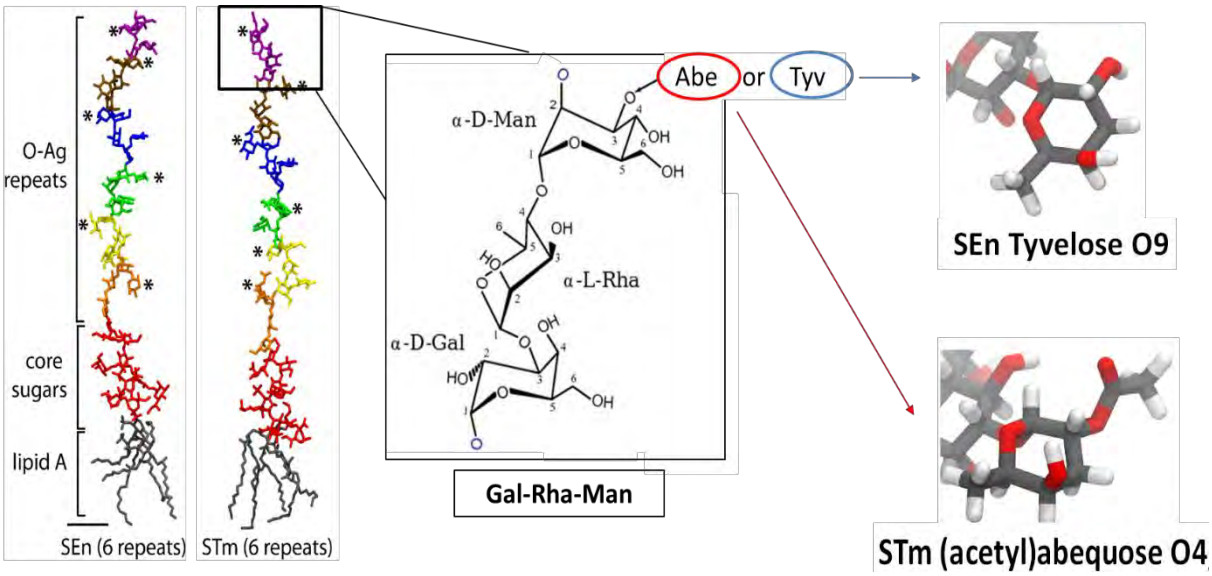

O-Ag repeats
core sugars
lipid A
SEn (6 repeats)
STm (6 repeats)
Abe
or
Tyv
α-D-Man
α-L-Rha
α-D-Gal
Gal-Rha-Man
SEn Tyvelose O9
STm (acetyl)abequose O4

Figure 1.3 Structural diversity in the O-antigen repeat units.

(A) The repeating unit polysaccharides comprising the O-antigen exhibit variability across different strains, characterized by differences in glycose and non-glycose components, linkages, and the overall topology of the repeat unit. Figure taken from Whitfield C et al. 2020. J Biol Chem. (B) *S*. Typhimurium and *S*. Enteritidis express different O-antigens although they are highly similar and share a common O Trisaccharide backbone. The difference is one sugar which attached to Mannose, an acetyl abequose in *S*. Typhimurium and a tyvelose in *S*. Enteritidis.

In *S. enterica* serovar Typhimurium, synthesis of LPS is regulated by the wzx/wzy pathway in which O-antigen units are synthesised in the cytoplasm and transported across the inner membrane to be assembled in the periplasm (14, 15) (Figure 1.4). The genes *wba* family encode the enzymes required for synthesising O-antigen polysaccharides, and mutants that lose any of these genes result in LPS lacking full-length O-antigen (16, 17). For instance, deletion of the *wbaP* gene, which is an enzyme involved in the transfer of sugar residues during the assembly of the O-antigen, results in a mutant unable to form an O-antigen chain (18, 19). The length of the O-antigen chain is determined by *wzz* proteins. *S*. Typhimurium can synthesise a modal length chain (16-35 units) and long O-antigen chain (> 100 repeating units) depending on which *wzz* genes it bears (20). If LPS has full-length O-chains, LPS is classified as smooth LPS. In contrast, the lack or shortening of O-chains would make the LPS rough. Since a rough LPS is more hydrophobic, bacteria with rough LPS have more permeable cell membranes to hydrophobic drugs (21, 22). The polymerisation of O-antigen multi-units requires O-antigen ligase, which is encoded by the *wzy* (also called *rfc*) gene. Deletion of *wzy* affects O-antigen polymerase resulting in the ligation of only a single O-antigen subunit with a complete core, and this phenotype is known as semi-rough LPS (23).

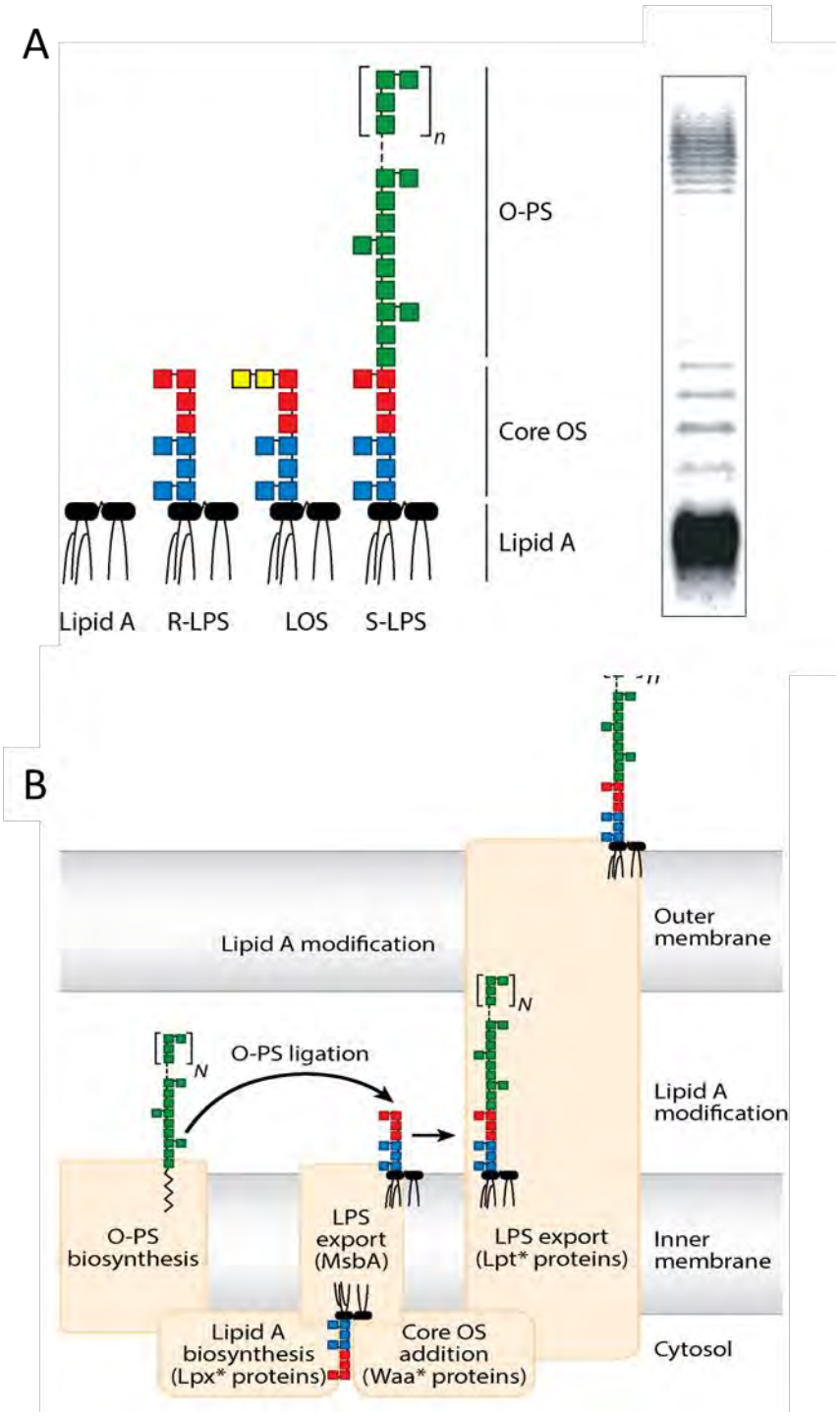


Figure 1.4 An overview of the structure and biosynthesis of LPS.

(A) an SDS-PAGE separation of LPS from *E. coli* demonstrates the diverse molecular species present in a growing culture. The heavily stained band corresponds to molecules containing lipid A and varying quantities of the core oligosaccharide. Each subsequent band represents lipid A-core molecules that have been progressively substituted with additional repeat units of the O-antigen. (B) outlines the primary steps involved in the biosynthesis and export of LPS. The asterisks indicate instances where multiple proteins are involved in a specific process. The abbreviations used are R-LPS (rough LPS lacking O-PS), LOS (lipooligosaccharide), and S-LPS (smooth LPS containing O-PS). Figure taken from Whitfield C, Trent MS. 2014. Annu Rev. Biochem.

The length of the O-antigen has an impact on how gram-negative bacteria interact with the complement proteins of the innate immune system, with longer side chains preventing complement from reaching the bacterial surface to cause lysis, as long chains can afford complement resistance, while shorter chains might facilitate host cell uptake (19). Previous studies in *P. aeruginosa* have demonstrated that mutants deficient in O-antigen are much more susceptible to complement-mediated killing than wild-type strains (24). In addition, alterations in O-antigen chain length can affect the recognition of bacteria by macrophages and neutrophils, either enhancing or impairing phagocytosis. Changes in O-antigen chain length may also either mask or expose certain immunogenic sites on the bacterial surface (25). This can influence the ability of the immune system to recognize and mount an effective response against the bacteria. Some bacteria can resist the bactericidal activity of serum by modifying their LPS, including the O-antigen. Changes in O-antigen chain length may contribute to the ability of bacteria to resist killing by serum components (26). Changing the O-antigen chain length not only affects recognition and evasion by immune cells but also influences the modulation of inflammatory responses, including cytokine production and TLR activation. Changes in chain length may lead to alterations in the host immune response, affecting the levels and types of cytokines produced during infection and the activation of TLRs, modulating the inflammatory signalling pathways (27).

1.2.2 Porins

Porins are proteins found in the OM and one of the most common outer membrane proteins (OMPs). They typically form a trimeric structure. Each monomer contributes to the formation of a pore, allowing the passage of molecules through the outer membrane (28) (Figure 1.1). Porins are essential for the bacteria to adapt to different environments, including surviving

within the host organism. *Salmonella* porins contribute to the transport of nutrients, such as sugars and amino acids, as well as ions like potassium and magnesium. They also play a role in resisting environmental stresses, such as antimicrobial peptides and other immune defences. OmpC, OmpD, and OmpF are the most common porins found in *Salmonella,* and they vary among different *Salmonella* strains and under different growth conditions (29). S. Typhi-porins are immunogenic and can induce protective antibodies and lifelong Ab responses in typhoid fever patients (30, 31). In addition, purified porins have been used as vaccines to protect mice from S. Typhi infection (32, 33). OmpD is found abundantly in the outer membrane of most *Salmonella*. It has been reported that Immunisation with *S*. Typhimurium -OmpD can confer protection in mice from *S*. Typhimurium infection (34). The sequence of OmpD-forming amino acids is highly conserved by up to 99% between *S*. Typhimurium and *S*. Enteritidis (35). *S*. Typhimurium-OmpD differs from *S*. Enteritidis-OmpD by only single amino acid, which is alanine in *S*. Typhimurium and serine in *S*. Enteritidis. Antibodies against OmpD from one serovar may cross-react with the other. Similar levels of binding were observed between anti- *S*. Typhimurium OmpD antibodies and purified porins from both *S*. Typhimurium and *S*. Enteritidis (36). However, OmpD is not found in *S.* Typhi (37).

1.2.3 Flagella

Flagella enable *Salmonella* to move and exhibit a chemotactic behaviour in response to chemical information from the environment (38). Flagellin is a target for both the innate and adaptive immune systems during *S*. Typhimurium infection (39, 40). Flagella are composed of three distinct elements, a basal body or transmembrane motor, the hook, and a filament. The filament consists of 20,000 flagellin protein monomers, and the expression of flagellin and flagella varies between serovars allowing antigenic diversity. Moreover, flagellin is encoded

by two genes which are fliC and fljB, allowing for phase variation, although not all serovars express both flagellin proteins (38, 41).

1.2.4 Other *Salmonella* protein antigens

Salmonella has other virulence proteins which have protective functions against host immunity and facilitate invasion and multiplication within host cells such as epithelial cells and macrophages. In order to actively engage with the host and alter biological processes, *Salmonella* has evolved a number of sophisticated virulence functions. These virulence factors include two clusters of distinct Type-3 secretion system (T3SS), which translocate bacterial effector proteins directly into host cells. T3SS is encoded on *Salmonella* Pathogenicity Islands 1 (SPI-1) and *Salmonella* Pathogenicity Islands 2 (SPI-2), which mediate cell invasion and intracellular survival, respectively (42, 43). This will be discussed further in section 1.4.

1.3 *Salmonella* infections in humans.

1.3.1 Typhoid fever

Typhoid fever is caused by S. Typhi and is also known as enteric fever. It is usually transmitted by ingestion of contaminated water or food and is a hygiene-related disease (44). There are around 21 million typhoid fever cases annually in South and Southeast Asia, with a 20% mortality rate in untreated individuals and 1% in antibiotics-treated individuals. Children under the age of five are most susceptible to the infection (45). One of the main features that distinguishes *S*. Typhi from most non-typhoidal *Salmonella* is the presence of typhoid toxin, Vi antigen (polysaccharide capsule) (Figure 1.5 A), which can act as a barrier to minimise both complement deposition and recognition of subcapsular antigens like LPS O-Antigen (46). The expression of Vi capsules has the ability to inhibit the recruitment of neutrophils to the

infection site. Various cell types, including epithelial cells, release lower levels of IL-6, IL-8, and TNFα when exposed to Vi. These cytokines typically serve as chemoattractants for neutrophils. Additionally, Vi actively suppresses T cell activation by interacting with the prohibitin molecule on the host cell surface. This interaction hinders intracellular signalling essential for processes like actin polymerization and the release of inflammatory mediators, thereby affecting proliferation.

S. Typhi can invade the gut wall to reach the intestinal epithelium by infecting the intestinal microfold M cells, which are specialised epithelial cells found in the gut mucosa or lymphoid tissue and act as antigen-presenting cells (47, 48). The severity of typhoid fever is normally associated with systemic spread. These bacteria can travel from the gut to the spleen, liver, bone marrow, and gallbladder, leading to widespread and systemic disease. Interestingly, this dissemination occurs without causing inflammation in the intestines (49) (Figure 1.5 B). Symptoms begin to appear after about one week of incubation and include cough, abdominal pain, nausea, anorexia, persisting fever, and constipation or diarrhoea (50). If typhoid fever is diagnosed in its early stages, it can be then treated with antibiotics, such as fluoroquinolones. However, some strains of *S*. Typhi bacteria have developed a resistance to some antibiotics, and this has become a problem with typhoid infection in some areas like southeast Asia and Africa.

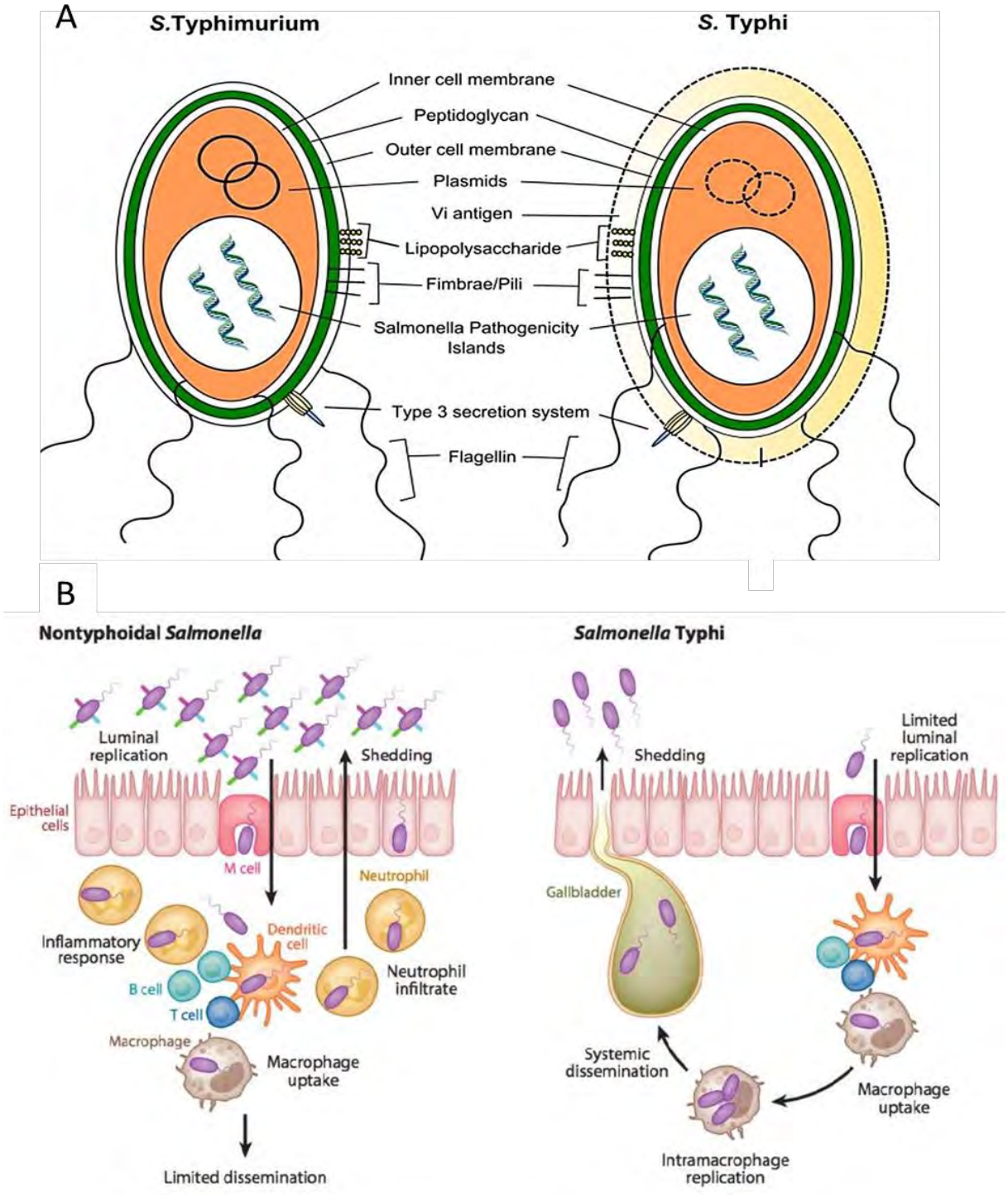


Figure 1.5 Differences in structure and pathogenesis of *S.* Typhimurium and *S.* Typhi

(A) A graphical representation of *S.* Typhimurium and *S.* Typhi bacterial cells, with key shared structural components highlighted. Diagram taken from (de Jong et al., 2012), CC. (B) NTS often enters the gut and invades the epithelial or M cells, causing a local rapid inflammatory response with massive neutrophil invasion and self-limiting gastrointestinal symptoms (left). In some cases, NST spreads through blood to other tissues such as the spleen, liver, and mesenteric lymph nodes (MLN), resulting in invasive NTS (iNTS) infection. In contrast, S. Typhi has virulence factors enabling them to infect the M cells and spread to MLN causing a systemic infection with the absence of local inflammation in the intestine (right). Figure taken from (40).

1.3.2 Nontyphoidal salmonellosis (NTS)

Severity of NTS infection is also associated with systemic spread, with invasive infection having a much higher mortality rate (51). Symptoms of NTS may occur within hours or days of ingestion of contaminated food, and these symptoms include nausea, diarrhoea, abdominal pain, and sometimes fever. Infection may rarely spread and cause bacteraemia, especially in young children, the elderly, and other immunocompromised people. In such cases, treatment requires the use of antibiotics (52). However, an invasive form of non-typhoidal salmonellosis is commonly present in developing countries, and this frequently occurs with no symptoms. This usually occurs in immunocompromised with a higher risk of bacteremia and life-threatening septicaemia (53, 54).

1.3.3 Invasive Non-Typhoidal Salmonellosis (iNTS)

Invasive Non-typhoidal Salmonellosis (iNTS) is an endemic disease in Sub-Saharan Africa and often affects HIV-infected patients and children under the age of 2 and is one of the major causes of bacteraemia among young children there (55, 56). Invasive NTS is commonly caused by *S*. Typhimurium and Enteritidis (SEn) (57). The severity of the disease is usually associated with the systemic spread of bacteria through the host. Approximately 535,000 cases of invasive nontyphoidal *Salmonella* disease and 77,500 fatalities reported in 2017, the majority, accounting for 78.9% of illnesses and 85.9% of deaths, were concentrated in countries within sub-Saharan Africa. The highest incidence of the disease is observed in individuals under the age of 5 (56). Normally, most iNTS cases in adults are associated with HIV infection (approximately 90% of all cases) (58, 59). Also, HIV-positive individuals have increased rates of iNTS recurrence because iNTS that have been poorly controlled might persist in the bone marrow and reappear years later (60). Depletion of CD4+ T cells, particularly T-helper 17 (Th-

17), in the gastrointestinal tract has been associated with the risk of iNTS infections in HIV-infected adults. High levels of anti-LPS IgG antibodies have also been linked to poor immunity in HIV patients due to their failure to clear bacteria in serum, indicating the potential significance of humoral responses in iNTS (61). Young children between 1 to 3 years are also at a high risk of getting iNTS, with the majority of bacteraemia cases being observed in newborns between the ages of 6 and 24 months (62). However, maternal IgG specific to *Salmonella* may have a significant role in providing protection against iNTS in infants under the age of four months (54). Data from a previous study showed that these maternal antibodies may be lost after weaning (63). Prevalence may also be influenced by additional risk factors, including malnutrition, severe malaria infection, and anaemia (64). Patients with iNTS may develop severe fever and bacteraemia but without gastroenteritis. Moreover, hepatosplenomegaly and pneumonia, which are frequently associated with co-infection like Mycobacterium TB and Streptococcus pneumoniae, can occur in iNTS patients (52, 58). Invasive NTS can be treated with antibiotics such as ampicillin, chloramphenicol, fluoroquinolones and cephalosporins, but antibiotic resistance has become common worldwide, making treatment difficult for such cases. In Sub-Saharan Africa, multidrug resistance was found in 75% of iNTS cases (65). Higher mortality rates are seen in NTS patients infected with multi-drug resistance (MRD) strains (66). Efforts are being made to make successful vaccines to protect against iNTS in developing countries because of the increase in MDR strains.

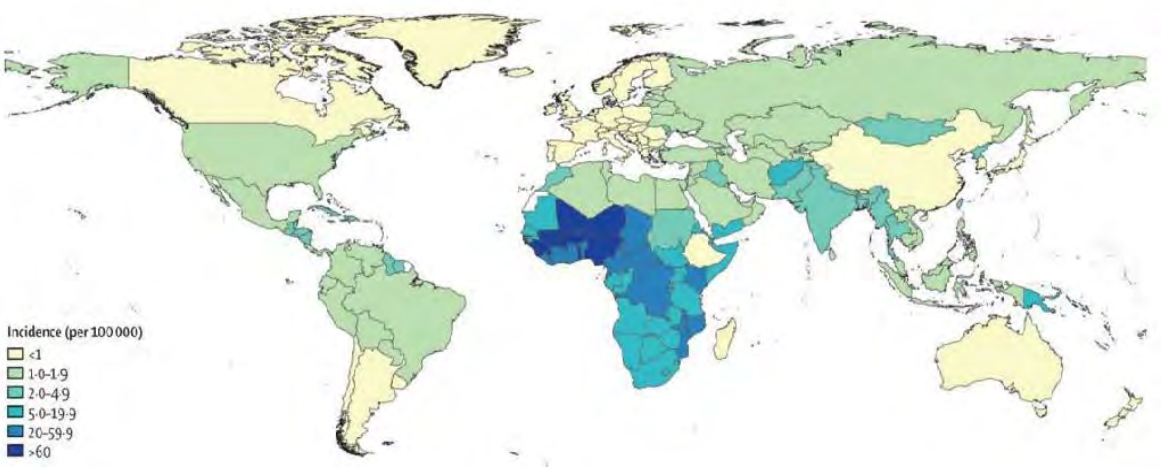


Figure 1.6 Global Epidemiology of invasive non-typhoidal *Salmonella*.

This figure demonstrates the geographical distribution of iNTS worldwide. Incidence iNTS per 1000,000 by country in 2017. The dark blue colour indicates iNTS disease's high burden. Figure adapted from (Stanaway, Parisi, et al., 2019; Stanaway, Reiner, et al., 2019).

1.4 Pathogenesis of NTS

NTS is typically acquired through the consumption of contaminated food. *Salmonella* survive exposure to the acidic environment of the stomach, allowing them to reach the small intestine. *Salmonella* adheres to the intestinal epithelial cells using fimbriae and other adhesion factors. It then invades the epithelial cells through a process involving type 3 secretion systems (T3SS) and effector proteins (67, 68). Once inside the host cells, *Salmonella* evades host defences and avoids degradation by manipulating the host cell machinery. The bacteria induce the rearrangement of the host cell's actin cytoskeleton, leading to the formation of membrane ruffles and a *Salmonella*-containing vacuole (SCV) around the bacteria (43). *Salmonella* has evolved strategies to avoid fusion with lysosomes, which contain enzymes capable of degrading pathogens. By inhibiting the maturation of the phagosome into

a phagolysosome, *Salmonella* prevents exposure to lysosomal enzymes. *Salmonella* actively maintains the integrity of the SCV by modifying the membrane of the vacuole. The bacteria use effector proteins to modulate host cell processes and prevent the fusion of the SCV with lysosomes. Once the SCV is established, *Salmonella* begins to replicate within this protected environment. The bacteria exploit host cell nutrients and resources for their own growth and survival. *Salmonella* employs various strategies to modulate the host immune response. This includes inhibiting the activation of certain immune signalling pathways and evading detection by host immune cells (69).

The invasion of intestinal epithelial cells triggers an inflammatory response characterised by the release of pro-inflammatory cytokines such as IL-1, TNF-α, IL-6, and IL-8. This inflammation leads to the clinical symptoms of gastroenteritis, including diarrhoea, abdominal pain, and fever, as well as recruiting immune cells to the site of infection. In severe cases, *Salmonella* may breach the intestinal barrier, enter the bloodstream, and disseminate to other organs, leading to invasive non typhoidal *salmonella* infection. Immunocompromised individuals are particularly at risk for severe manifestations. The host immune response involves the activation of immune cells, such as macrophages, which attempt to eliminate the intracellular bacteria. However, *Salmonella* has evolved mechanisms to evade immune detection and manipulate host cell processes, contributing to its persistence (70). In immunocompetent individuals, the infection is often self-limiting, and the immune system successfully clears the bacteria. However, in certain cases or individuals with compromised immune systems, chronic infections may occur. Figure 1.5 B illustrates a summary of NTS pathogenesis. A more detailed review of immune responses has been presented in Section 1.6.3.

1.5 Vaccines against *Salmonella*

Immunisation represents a remarkable achievement in global health and development, saving millions of lives annually. Currently, immunisation saves 3.5-5 million lives each year by preventing diseases like diphtheria, tetanus, pertussis, influenza, and measles (71). Vaccines can be administrated as a pathogen or one of its components in a way that can trigger an immune response without actually causing an infection. Today, there are licenced vaccines based on different strategies. One of these is whole-cell or live attenuated vaccines which are based on altering pa-thogens so that it becomes less virulent or harmless but still able to induce an immune response, such as whole cell Pertussis or Tuberculosis BCG, respectively. Killed or inactivated vaccines can also be used in which pathogens are grown under controlled conditions and then killed to reduce infectivity, such as the cholera vaccine and most influenza vaccines. It is also possible to use pathogen components like toxin subunits (such as Diphtheria) and antigens subunits (such as Hepatitis B). Nucleic acid vaccines, which encapsulate genetic material in artificial carriers like liposomes and trigger transcription of the antigenic material once they enter a host cell, have only lately been given licenses such as SARS COV-2 BNT162b2. Conjugate vaccine is a strategy commonly employed for licensed vaccines, involving the covalent attachment of a less potent antigen to a more potent one. This process results in an enhanced immune response to the weaker antigen. Typically, the weaker antigen is a polysaccharide linked to a robust protein antigen. One of the most widely used conjugate vaccines is the *Haemophilus influenzae* type B vaccine, also known as the Hib vaccine.

Human vaccines against *Salmonella* infections that are now available only target *S*. Typhi. However, there are currently no available licensed vaccines for *S*. Typhimurium. The initial

form of vaccination against *Salmonella*, known as an inactivated whole cell vaccine, had a history spanning over a century. Similar to Ty21a and Vi CPS, this vaccine targeted *S*. Typhi and was never implemented at a country-wide level. Its introduction dates back to 1896 (72), and it was used by the British (73) and US military (74), leading to a significant decrease in cases of typhoid fever and related fatalities. Among the three types of *Salmonella* vaccines, the inactivated whole cell vaccine demonstrated the highest effectiveness, with a three-year cumulative efficacy of 73% (75). However, its discontinuation stemmed from its notable drawback: a high level of reactogenicity. Although tolerated by military personnel in the past, this adverse reaction rendered the vaccine unsuitable for general use.

Currently, five *S*. Typhi vaccines are available commercially. These vaccines are based on three strategies: Attenuated live vaccine (Ty21a-based), Vi capsular polysaccharide (Vi-PS), or Vi-conjugate vaccines. The Ty21a-based vaccine is derived from a live attenuated mutant strain which lacks Vi-antigen but is still able to stimulate T-cells and produce antibodies against other antigens (76). in addition, evidence suggests that it may provide cross protection against other serovars such as *Salmonella* Paratyphi A and B (77). The effectiveness of three doses over a period of three years was found to be 51% (75).

Purified Vi-PS elicits a T-independent B cell immune response. It exhibits a substantial seroconversion rate, and its efficacy over a span of three years is comparable to that of Ty21a (75). Nonetheless, after a period of 2-3 years, the level of protection diminishes (78). However, most studies showed that both vaccines are not effective in children under two years old, and none are licensed for this group (79). In younger children, a protein-conjugated Vi-PS Vaccine seems to be more effective and has the potential to induce T-dependant responses (80, 81). Currently, there are three licensed typhoid conjugate vaccines which are conjugate Vi-Ag to

recombinant *Pseudomonas aeruginosa* exotoxin (Vi-rEPA), and two Vi polysaccharide-tetanus toxoid conjugate vaccines, Typbar-TCV and Peda Typh (82). When compared to Vi-PS, anti-Vi IgG titres in school-aged children were maintained at higher levels after immunisation with Vi-rEPA (83). Additionally, Typbar-TCV demonstrated promising seroconversion rates in both adults and children and antibody titres persist at a high level for five years (84). The Protective efficacy of Typbar-TCV in children was 95% and 100% for Peda Typh (85, 86). In a human challenge study that directly assessed the protective efficacy, both Vi-PS and Typbar-TCV vaccines demonstrated a comparable level of protection (87). Nevertheless, applying alternative experimental endpoints or including participants who closely resemble endemic populations may reveal more pronounced distinctions between Vi-PS and Typbar-TCV (88).

1.5.1 Non-typhoidal *Salmonella* vaccines

No NTS vaccines are currently licensed for humans, but there are some *S*. Typhimurium and *S*. Enteritidis vaccines under development and preclinical research is being conducted on some vaccine candidates. Live attenuated vaccines can be given orally in multiple doses and induce T-dependent responses and mucosal immunity. However, the reduced level of immunogenicity is a concern with live attenuated vaccines (72, 89). Clinical human studies utilising live attenuated *S*. Typhimurium vaccine, generated by mutating SPI-2 in combination with either purine synthesis or aromatic amino acid synthesis genes, demonstrated the induction of anti-LPS IgG following immunisation (90, 91). Subunit vaccines such as *S*. Typhimurium porins and flagellin showed promising results as potential vaccines by inducing B and T cell responses (72) and offering a protection response against *S*. Typhimurium in mice. Porin-based vaccines could offer some cross protection as porins are highly conserved

between serovars (34). Moreover, OmpD from *S*. Typhimurium has also been shown to promote protective immunity in mice (92).

Although O-antigen is one of the crucial targets of protective immunity, it is unable to elicit T cell-dependent response alone (93). In contrast, conjugating O-antigen to a carrier protein can confer protective immunity and protection in mice challenged with invasive *S*. Typhimurium (94, 95). Additionally, SEn-OAg has been conjugated to the flagellin monomer (FliC), providing protection against *S*. Enteritidis challenge in adults and infants mice (96). Recently, the Outer membrane vesicles (OMV)-based vaccine technology, also called Generalized Modules for Membrane Antigens (GMMA), has emerged as a promising alternative for delivering O-antigen to traditional glycoconjugate vaccines (97) (discussed further in section 1.5.2).

1.5.2 Outer Membrane Vesicles (OMV)

OMVs are naturally released by Gram negative bacteria and are outer membrane blebs. These OMVs are 20-250 nm in diameter, and production can be accelerated from bacteria genetically engineered to increase blebbing (Figure 1.7 A) (98, 99). This can be accomplished by disturbing the membrane integrity through knocking out the *tolR* gene, which is responsible for the tol-pal system (100) (Figure 1.7 B). OMV contain all the outer membrane components and imitate the bacterial exterior, presenting a pathogen-like appearance. However, since they are non-living entities, they lack the capacity to induce disease. OMVs predominantly consist of components from the bacterial outer membrane, including crucial antigenic elements necessary for triggering a protective immune response. Consequently, shortly after their identification, OMVs were suggested as a potential vaccine platform and an alternative method for delivering O-antigen compared to conventional glycoconjugate vaccines (97, 101, 102).

1.5.2.1 Types of OMV

Chatterjee and Das discovered OMV in 1967 while studying the cell-wall structure of *Vibrio cholera in vitro* (103). The spontaneous release of vesicles from bacteria gives rise to the formation of native outer membrane vesicles (nOMV). However, in many species, this occurs at insufficient levels for practical use in vaccine manufacturing. To address low yields, vesicle-like aggregates of insoluble outer membrane proteins are chemically extracted from whole bacteria using detergents, such as deoxycholate. This process yields detergent-extracted outer membrane vesicles (dOMV). In addition to enhancing production, the use of detergents also diminishes lipopolysaccharide (LPS) content, thereby reducing the endotoxicity of OMV. Nevertheless, this approach results in the loss of crucial protective lipoprotein antigens, compromises vesicle integrity, and introduces contamination with cytoplasmic proteins in the final preparation (104, 105).

More recently, there has been a genetic manipulation of bacteria to enhance blebbing, resulting in the creation of mutant-derived outer membrane vesicles (mdOMV), also known as GMMA. The augmentation of extracellular vesicle production is achieved by disrupting genes responsible for crosslinking the outer membrane and the peptidoglycan layer in the periplasm. The primary genetic target for disruption, leading to the high-yield production of mdOMV, is tolR of the Tol-Pal system, a component found in the majority of Gram-negative bacteria. However, it is essential to note that the Tol-Pal system is not universally present. Moreover, the removal of the Tol-Pal system in certain E. coli strains results in a deficiency in the polymerization of LPS O-antigen (106) .

To mitigate OMV endotoxicity in bacterial strains producing OMV, further mutations can be incorporated. In the context of detergent-extracted OMV, the removal of LPS and lipoproteins

is nearly complete, leading to diminished endotoxicity. In the case of mdOMV with elevated LPS content, a typical strategy for reducing endotoxicity involves altering the structure of lipid A, resulting in decreased stimulation of Toll-like receptor (TLR) 4 (107). LPS-lipid A is responsible for the toxicity of Gram-negative bacteria and reactogenicity in humans. Alteration to lipid A structure of *S*. Typhimurium-OMV by mutation of the *msbB* and *pagP* genes, which encode acetyltransferases, reduces the reactogenicity through lowering TLR4 activation (108). Although *S*. Typhimurium-OMVs with modified lipid A show immunogenicity and protectivity against wild-type *S*. Typhimurium, a direct comparison to non-altered OMVs has not yet been published.

1.5.2.2 OMV as vaccines against Gram-negative bacteria

Currently, OMV-basedu vaccines against *Neisseria meningitidis* (MeNZB and Bexsero) have been licensed for use in humans (109, 110). MenB was first licensed for use in Cuba against *Neisseria meningitidis* serogroup B in 1987 (111). Following that, OMVs have proven effective as vaccines in averting meningococcal group B outbreaks in Norway and New Zealand. In a recent retrospective case-controlled study, MeNZB demonstrated a 31% effectiveness (95% CI 21–39%) in protecting against gonorrhea (112), suggesting the potential of OMVs for cross-genus protection.

OMV-based candidate vaccines are currently in the preclinical stages of development for other Gram-negative pathogens. These vaccines have shown the capability to trigger both humoral and cellular immune responses (113). Previous studies on adults from both endemic and non-endemic areas have demonstrated that OMVs or GMMA derived from *Shigella sonnei* induces anti-S. *sonnei* LPS serum IgG antibodies (114, 115). OMV derived from Gram-negative bacteria, engineered to increase blebbing and decrease endotoxicity by de-acylating the lipid A moiety,

have emerged as promising vaccine candidates against non-typhoidal *Salmonella* (116). When compared to corresponding CRM197 glycoconjugates formulated with alum, OMV from *S*. Typhimurium and *S*. Enteritidis induced robust anti-O-antigen-specific IgG responses. Immunization with OMV resulted in a more diverse IgG subclass profile and heightened bactericidal activity, in contrast to glycoconjugates that predominantly induced IgG1 (117). Furthermore, *S*. Typhimurium-OMVs induced IgM and IgG antibodies specific for both LPS and porins (118). Another recent study on *S*. Typhimurium -OMV has also shown protection against *S*. Typhimurium challenge and induction of rapid and persistent *S*. Typhimurium O:4,5-specific serum IgM and IgG. In addition, long-lived plasma cells (PC) and antigen-specific memory cells were found in the spleens and livers of immunised mice (119). OMVs derived from *S.* Enteritidis also protect against oral challenge with wild-type *S*. Enteritidis and elicit strong humoral and mucosal immune responses (120).

OMVs might be the preferable option for vaccine development to control *Salmonella* infection due to their efficient, rapid and affordable purification from culture (98, 121). Moreover, one of the factors that may make OMVs an advantage over other vaccines is that there is no need for additional chemical or enzymatic modification or carrier proteins used with the classical glycoconjugate vaccines. Another feature of OMV is their ability to be decorated with particular proteins or polysaccharides derived from different pathogens. OMVs are also highly immunogenic at low doses in animals and able to elicit robust innate immune responses. Compared to other corresponding vaccines, such as polysaccharide conjugate and purified protein subunits, *S*. Typhimurium and SEn-OMVs induce serum antibodies with significantly better functional activity (117).

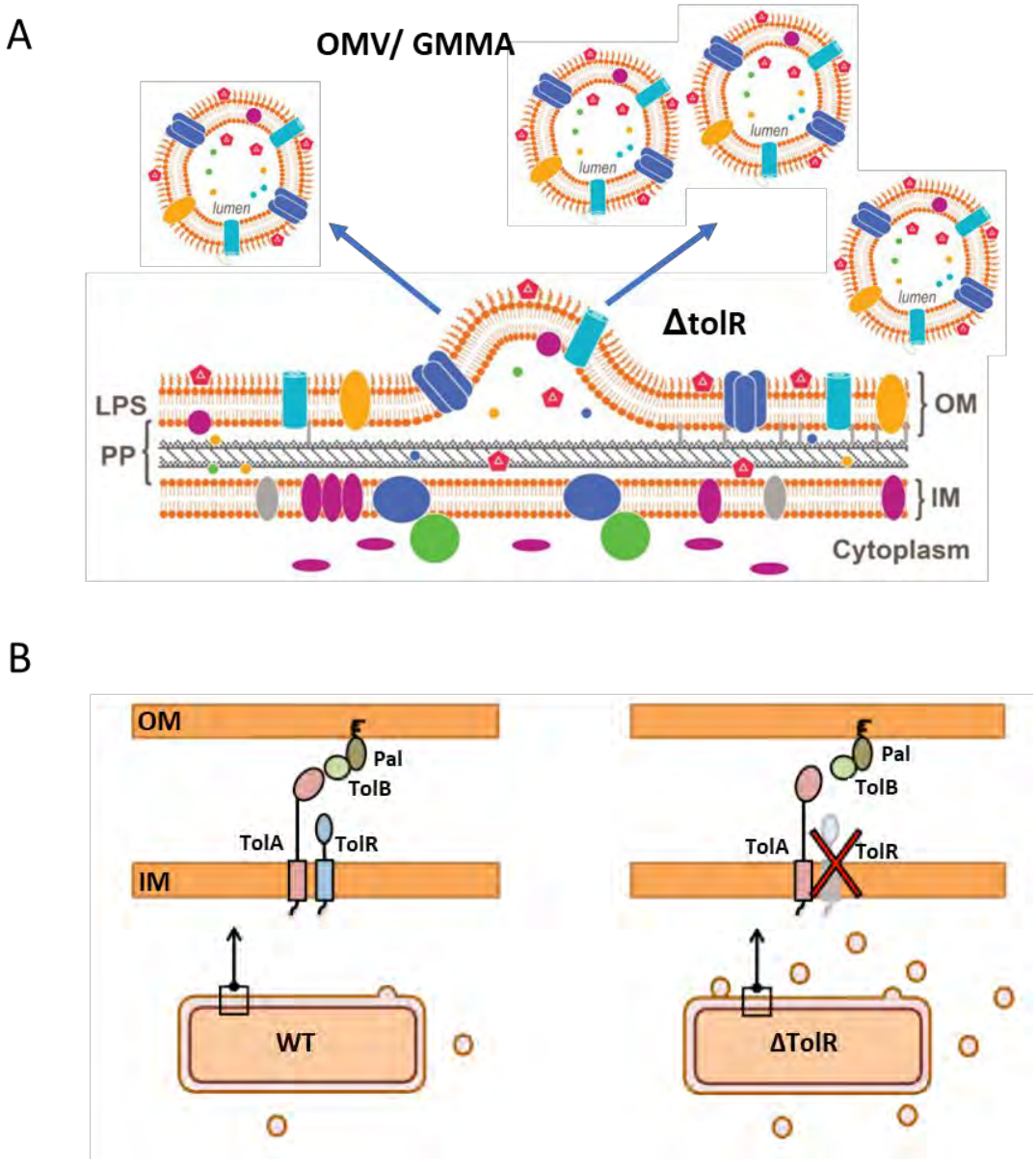


Figure 1.7 Production of Outer membrane vesicles (OMV).

(A) OMV are nanostructures released naturally by Gram-negative bacteria and present all surface antigens. (B) Bacteria is genetically mutated to increase blebbing. Deletion of *tolR* gene affects The Tol-Pal protein system, disturbing the membrane stability. Figure adapted from (95).

1.6 The immune system

The immune system is a group of barriers, cells and organs that work together to protect the human body from infection and consists of three functional parts: physical barriers, innate immune system, and adaptive immune system (122). They often work in conjunction, and

communication between these two responses is through soluble factors such as cytokines and cell-to-cell contact. The first lines of defence against invasive pathogens are physical barriers, with the skin being the most evident. However, there are other defences, including lung mucus and cilia, stomach acidity, lysozyme, saliva, and other secretions. When microorganisms do manage to get past these barriers, the innate immune system aids in limiting their spread (123). Innate immunity recognises pathogen-expressed molecular patterns, and these assist in the control of early infection phases and triggers the adaptive immune response. The innate immune response is non-specific and fast, while the adaptive immune response provides a specific and tailored response against specific pathogens or foreign substances. This response involves the activation of immune cells, such as T cells and B cells, which recognise and respond to specific antigens. Adaptive immunity is characterised by its ability to "remember" past encounters with pathogens, allowing for a more rapid and efficient response upon re-exposure. It works in coordination with other components of the immune system to maintain overall immune homeostasis (124, 125).

1.6.1 The innate immune response

The innate immune system reacts rapidly in a non-specific way to invading pathogens or limiting their growth when they breach anatomical barriers and enter the host. The innate immune system is made up of distinct haematopoietic stem cell-derived cells located in the bone marrow. Common myeloid cells are the progenitor cell for most innate cells, with dendritic (DCs) and natural killer (NK) cells being the notable exceptions (126, 127) (Figure 1.8). Cells of the innate immune system express receptors that recognise conserved patterns shared by large groups of pathogens known as Pathogen-Associated Molecular Patterns

(PAMPs). This recognition may lead to pro-inflammatory cytokines release, phagocytosis, cell migration, or cell death by apoptosis or necroptosis, depending on the cell type (128, 129).

The macrophage, neutrophil, monocyte, and NK cell types are key cells engaged in defending against bacterial invasion. When bacteria invade intestinal epithelium, epithelial cells and tissue-resident macrophages recognise them by Pattern recognition receptors (PRRs) and cause the release of cytokines, which in turn recruit neutrophils from the circulation. Neutrophils are the most common type of granulocyte circulating in the blood and help in capturing and ingesting pathogens by phagocytosis and the clearance of antibody-opsonised pathogens (130). However, they could need support from other immune cells to clear certain infections. In the blood, monocytes, which are mononuclear phagocytes, can also ingest and eliminate infections whether antigens are opsonised or directly via PRRs. Monocytes travel to tissues and differentiate into macrophages or dendritic cells. A pathogen is ingested by a macrophage or monocyte, and antigens are presented on a major histocompatibility class II (MHC-II) protein at the cell surface to boost immune responses (131).

DCs are important in the induction of protective immune responses as they are the main link between innate and adaptive immunity. DCs are competent antigen-presenting cells (APCs) which present antigens on MHC II; however, they can also do so on MHC I. DCs that have been activated migrate to the secondary lymphoid organs, where they can stimulate T cells to efficiently induce a cell-mediated adaptive immune response (132).

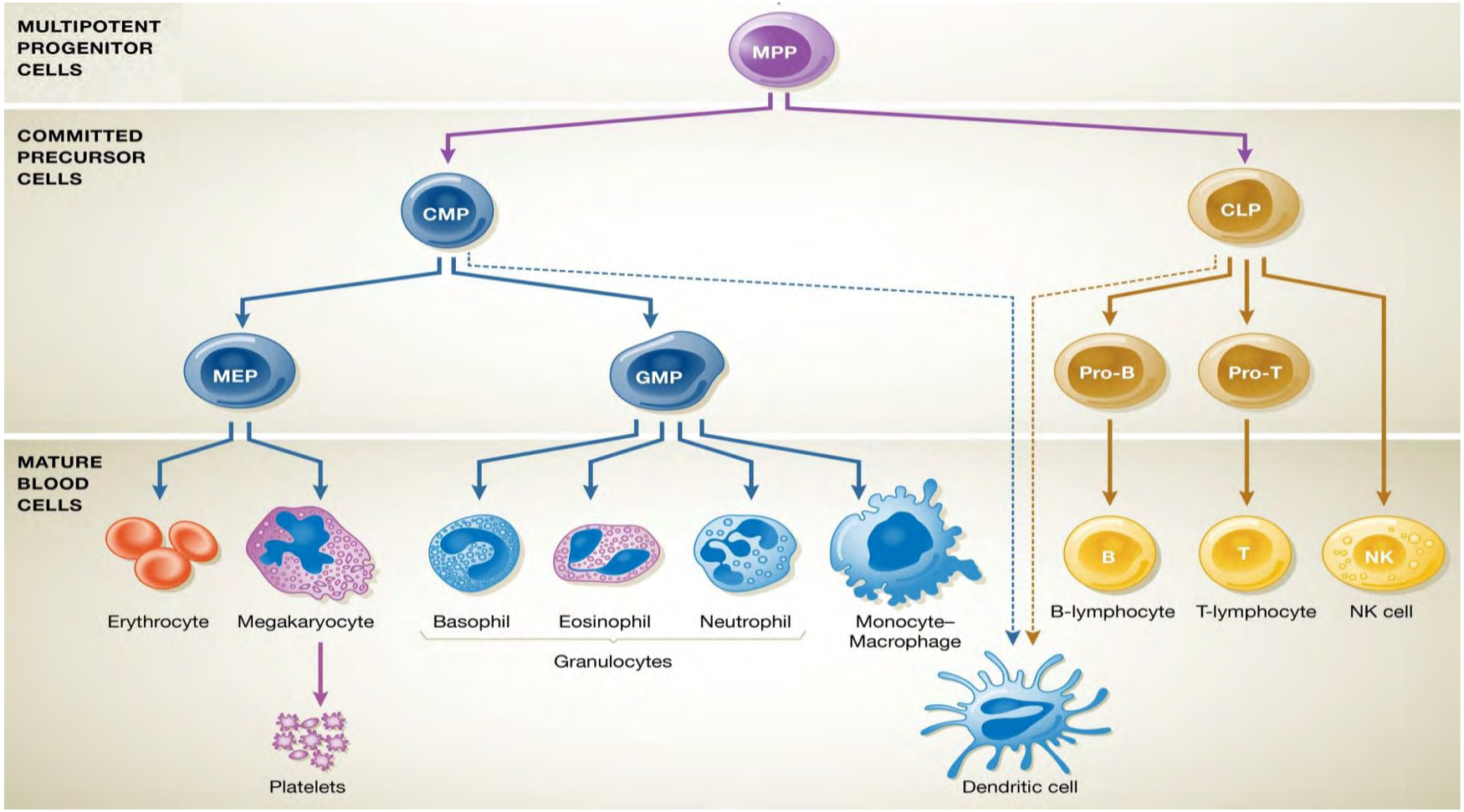


Figure 1.8 Immune cell haematopoiesis in the bone marrow.

Within the bone marrow, Haematopoietic Stem Cells generate multipotential progenitor cells (MPP) that have the capacity to differentiate into either common myeloid or lymphoid progenitor cells (CMP or CLP). Megakaryocyte erythroid progenitors (MEPs) and granulocyte-macrophage progenitors (GMPs) can give rise to all differentiated cells of the myeloid lineage within the bone marrow. CLPs further differentiate into T cells, B cells, and natural killer cells (NK). Dendritic cells (DCs) can be originated from CMPs or CLPs. Figure taken and adapted from Ackermann et al, EMBO Mol Med, 2015, 7:1388-1402.

1.6.1.1 Pattern recognition receptors

Upon breaching anatomical barriers and penetrating the epithelium, tissue-resident macrophages detect the presence of a pathogen via their pattern recognition receptors (PRRs). Subsequently, this causes the release of pro-inflammatory cytokines which attract additional immune cells, including neutrophils, to the infection site and facilitate the uptake of antigens by APCs (133). The main PRRs involved in the immunity against intracellular bacteria are TLRs and NLRs.

TLRs are expressed by immune and non-immune cells and recognise a wide range of distinct PAMPs present in many types of pathogens, such as LPS or RNA. Twelve functional TLRs have been identified in mice, compared to ten in humans. Both humans and mice have 9 conserved TLRs, and TLR10 is only functional in humans (134, 135). TLRs are expressed intracellularly or on the surface of the cell and are monomeric in their steady state. They are made up of an extracellular ligand-binding domain that is responsible for recognising PAMPs, a transmembrane region, and a Toll-interleukin-1 receptor (TIR) domain, that is a protein involved in signal regulation. TLRs form dimers upon ligand binding, sending signals intracellularly that can activate myeloid differentiation primary response gene 88 (MyD88) or TIR domain-containing adapter-inducing interferon β (TRIF) dependent signalling pathways (MyD88-independent pathway). Except for TLR3, all TLRs have a MyD88-dependent signalling pathway. TLR4 is expressed in the cell wall and is essential to control *Salmonella* infections as it recognises the lipid A portion of bacterial LPS, which can activate both MyD88-dependent and independent pathways. TLR4 is the only receptor that can mediate both pathways. Pro-inflammatory cytokines are often secreted in response to MyD88-dependent signalling, while

Type 1 interferons (IFNα and IFNβ) are produced in response to the MyD88-independent pathway (136, 137).

1.6.1.2 Complement

The complement system is crucial for inflammation and defence against certain bacterial infections. It comprises a series of more than 20 proteins circulating in blood and tissue fluids. While most of these proteins are inactive, they can be sequentially activated in response to microorganism recognition, forming an enzyme cascade. The complement system has three main functions: opsonise pathogens to help the immune cells recognise them and enhance phagocytosis, neutralise the toxic they release, or cause bacterial lysis by directly making pores in their cell walls (26, 138). There are three pathways through which complement can be activated, leading to the cleavage of C3 into C3a, which acts as an inflammatory mediator, and C3b, which acts as an opsonin. These pathways are classical, alternative, and mannose-binding lectin (MBL) (Figure 1.9). The classical pathway (CP) is activated when immune complexes are formed by antibodies binding to pathogens or foreign antigens. The C1 complex, composed of C1q, C1r, and C1s molecules, binds to the Fc portion of the immune complex, leading to the activation of C1s and C1r. C1s then cleaves C4 and C2 to generate the CP C3 convertase, C4bC2a (139). The alternative pathway (AP) is activated by foreign surfaces containing carbohydrates, lipids, and proteins (140). C3 undergoes hydrolysis to produce C3b, which binds to targets like bacteria. Factor B and Factor D are then recruited to C3b, resulting in the formation of the C3 convertase (C3bBb). The activation of the mannose-binding lectin pathway occurs when the mannose-binding lectin (MBL) attaches to mannose residues located on the surface of the pathogen. This attachment triggers the activation of MBL-

associated serine proteases (MASP-1 and MASP-2). As a result, these enzymes initiate the activation of C4 and C2, leading to the formation of the C3 convertase. (C4b2a) (141, 142).

The C3 convertases generated from the three pathways cleave C3, releasing C3a and C3b. C3a acts as a chemoattractant attracting other inflammatory mediator cells and promoting inflammation. C3b can attach covalently to the surface of microbial pathogens and works as an opsonin to promote phagocytosis. Furthermore, C3b complexes with the C3 convertases to form the C5 convertases, which cleave C5 into C5a and C5b. The membrane attack complex (MAC) is formed by C5b binding to C6 and C7, followed by the binding of C8 and multiple molecules of C9. The MAC complex inserts itself into cell membranes, forming a pore which disrupts the pressure and osmotic balance within the bacterial cell, leading to cell lysis (139).

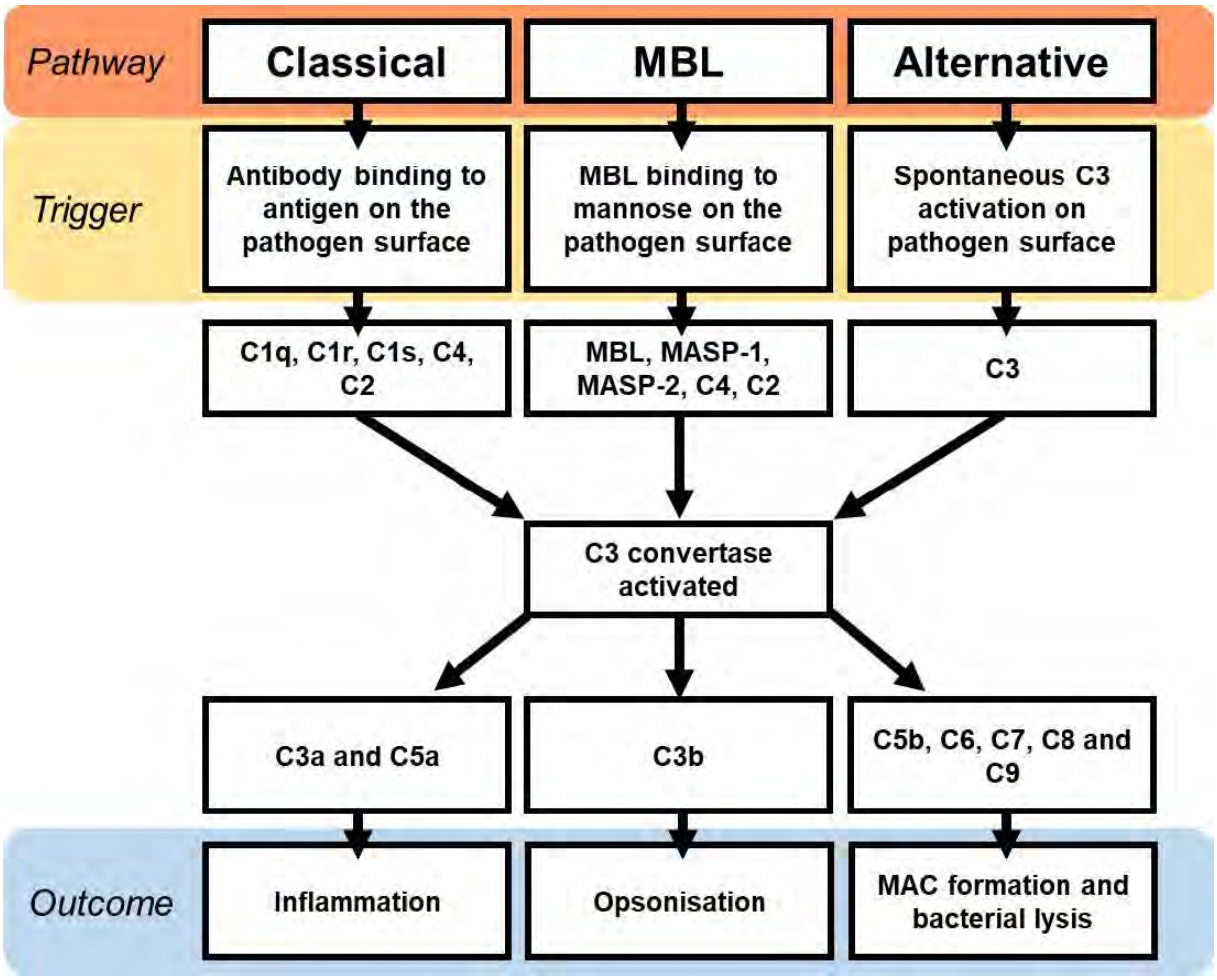


Figure 1.9 Simplified scheme of the complement cascade.

Three pathways can activate the complement system leading to the cleavage of C3 and the production of C3a, C3b, and C5a. The initiation of each pathway varies in their respective mechanisms. The classical pathway (CP) is triggered by the interaction between C1 and the surface of the pathogen or the binding of antibodies (Ab) to antigens (Ag) on the pathogen surface. On the other hand, the lectin pathway (LP) relies on the binding of mannose-binding lectin (MBL) to mannose on the cell wall. As for the alternative pathway (AP), it is spontaneously activated when C3 becomes activated on the surface of the pathogen.

1.6.2 The adaptive immune response

The adaptive response is highly specific and is required when innate responses are unable to contain infection. This response is also known as acquired immune responses and is crucial for offering protection against secondary infection. After a second exposure, the adaptive response develops rapidly and more efficiently after primary responses, promoting faster clearance (122). Adaptive immunity depends on T and B lymphocytes which are derived from the common lymphoid progenitor cells in the bone marrow (Figure 1.8). T cells then migrate to the thymus for maturation and selection, while B cells mature in the bone marrow and undergo positive and negative selection.

1.6.2.1 T cell-mediated responses

T cells can be categorised as CD4+ or CD8+ T cells which recognise Ag presented by MHC complexes on the surface of APCs. This recognition is achieved by T cell receptors (TCRs) which express αβ-chains. However, γδ-chains are expressed on a small subset of TCRs found in the epithelium and do not need to bind MHC to be activated (143, 144). CD4+ T cells recognise MHC Class II (expressed in B cells, DCs, and macrophages) and are important in isotype class switching in B cells and supporting the activity of other immune cells (Figure 1.10 A). While CD8+ T-cells recognise MHC class I which is expressed by all nucleated cells and are important for killing infected cells, releasing cytotoxic molecules, and defending against tumours by directly killing transformed cells (145, 146).

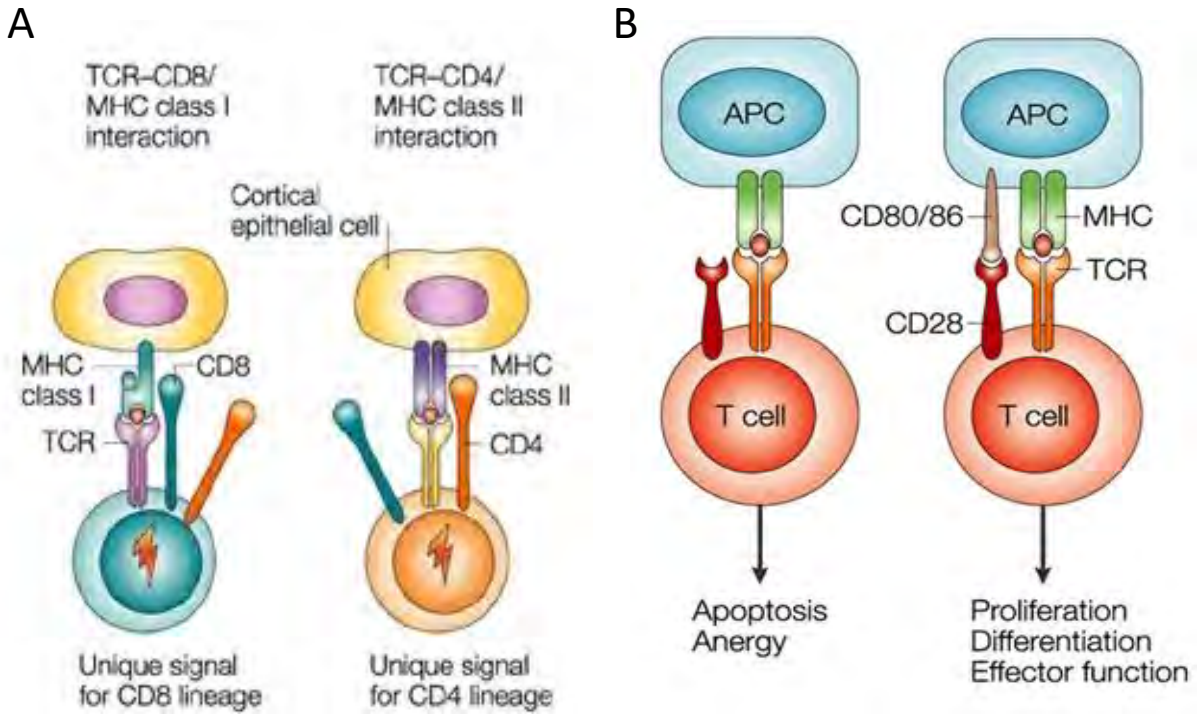


Figure 1.10 Activation of naïve T cells and TCR engagement.

(A) Activation of CD8+ and CD4+ T cells requires binding of antigen presented on antigen presenting cells (APCs) to MHC class I and MHC class II molecules, respectively. (B) T cell proliferation and differentiation is required concurrent recognition of antigen-MHC complex by the T cell receptor (TCR) and co-stimulatory receptor CD28 molecule by CD80/CD86 molecules on APCs. T cells commit apoptosis or develop anergic states if CD28 co-receptors are not activated. Figure A and B are taken and adapted from (132) and (136), respectively.

After leaving the thymus, naive T cells travel through the blood to reside in secondary lymphoid tissues until they are activated by the interaction of the TCR with the peptide-MHC complex. Moreover, activation of naive T cells requires co-stimulatory molecules, such as CD28 on T cells, to bind with CD80 or CD86 on APCs (Figure 1.10 B) (147, 148, 149). Activation of CD4+ T cells allows differentiation into different subsets based on their transcription factor (TF) and cytokine signals, including T helper 1 (Th1), T helper 2 (Th2), Th17, T follicular helper (Tfh), or regulatory T (T-reg) cells (Figure 1.11) (150, 151).

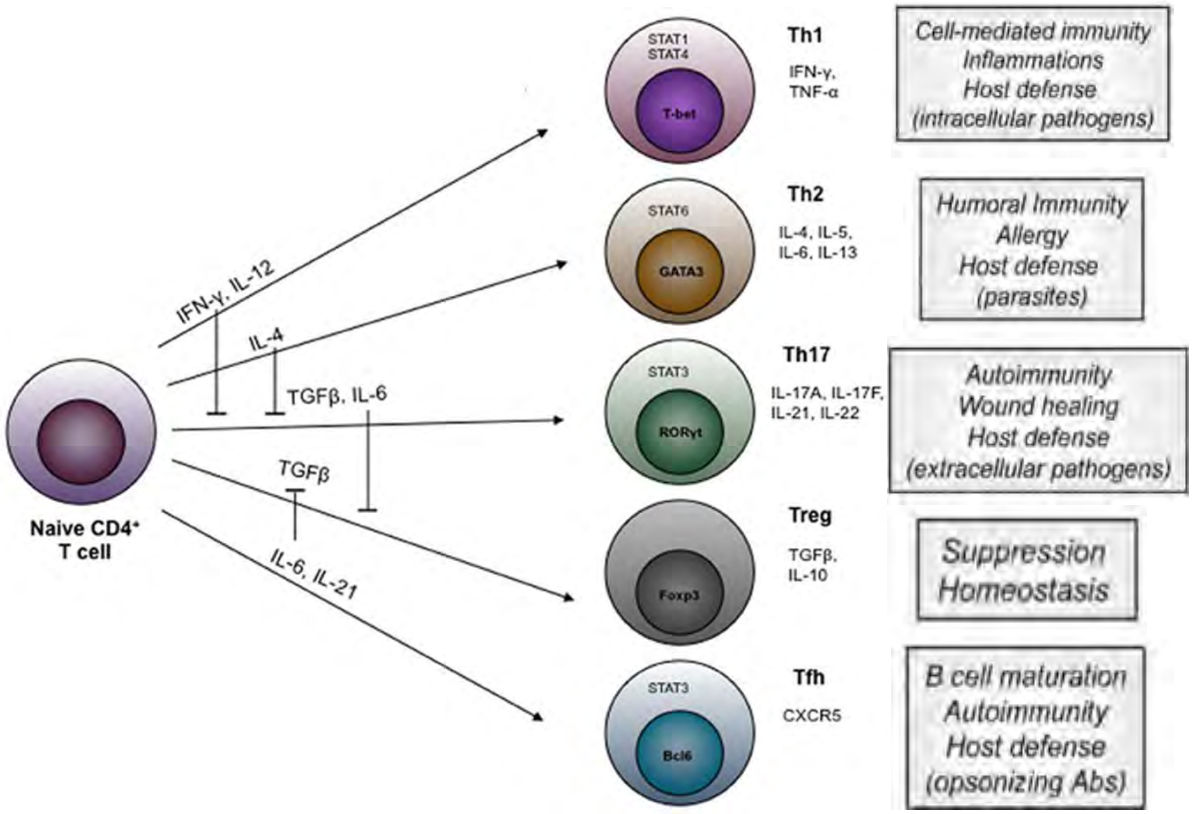


Figure 1.11 CD4+ T-cell subsets and their roles.

Following activation through TCR-MCH complex and in conjunction with the cytokines produced upon activation, Naïve CD4+ T cells differentiate into different effector subsets including, Th1, Th2, Th17, Treg and Tfh. Each differentiated subset produces its own cytokines and expresses certain transcription factors, corresponding to its immune regulatory functions. Figure taken and adapted from (138).

The expression of the TF T-bet directs cellular differentiation towards the Th1 phenotype, characterised by the production of IFNγ. Th1 cells are thought to be effective at the clearance of intracellular pathogens, and IFNγ production induces class switching to IgG2a/c and IgG2b in mice (152). However, Th2 subsets are associated with the resolution of extracellular or parasitic infections and promoted by IL-4 and the expression of the TF GATA-3, resulting in IgE and IgG1 class switching (153). Th17 cells have an important role in inflammatory responses, autoimmune diseases, and immunity against extracellular bacteria and fungi. They are expressing RORγτ, which stimulates the production of IL-17 (154).

Tfh subsets play an effective role in the formation of extrafollicular or germinal centre responses by expressing B cell lymphoma-6 (Bcl-6), along with IL-21, CD40L, and inducible T-cell co-stimulator (ICOS) (155). T-cells can also differentiate into T-reg cells that express the transcription factor Foxp3 and secrete cytokines like IL-10 and are associated with cell homeostasis and the suppression of autoimmunity (156).

1.6.2.2 B cell-mediated immune responses

B-cells can be categorised in mice as non-conventional B1 subsets and conventional B2 subsets. Both develop in the BM and undergo a series of steps for differentiation (Figure 1.12). These B cell populations are classified according to their migration site, markers that are expressed, and their contribution to TD or TI responses. B1 cells originate from foetal liver stem cells and are enriched in the peritoneum and pleural cavities. Unlike the conventional B2 cells, B1 subsets are not continually generated from bone marrow; instead, they are self-renewal and renew themselves (157). These unconventional cells are commonly involved in T-independent B cell responses (which is discussed in section 1.6.2.5). In mice, they can be

subdivided into B1a and B1b cells according to their expression of CD5, where the former express CD5 and the latter does not. However, their existence in humans is still unclear (158).

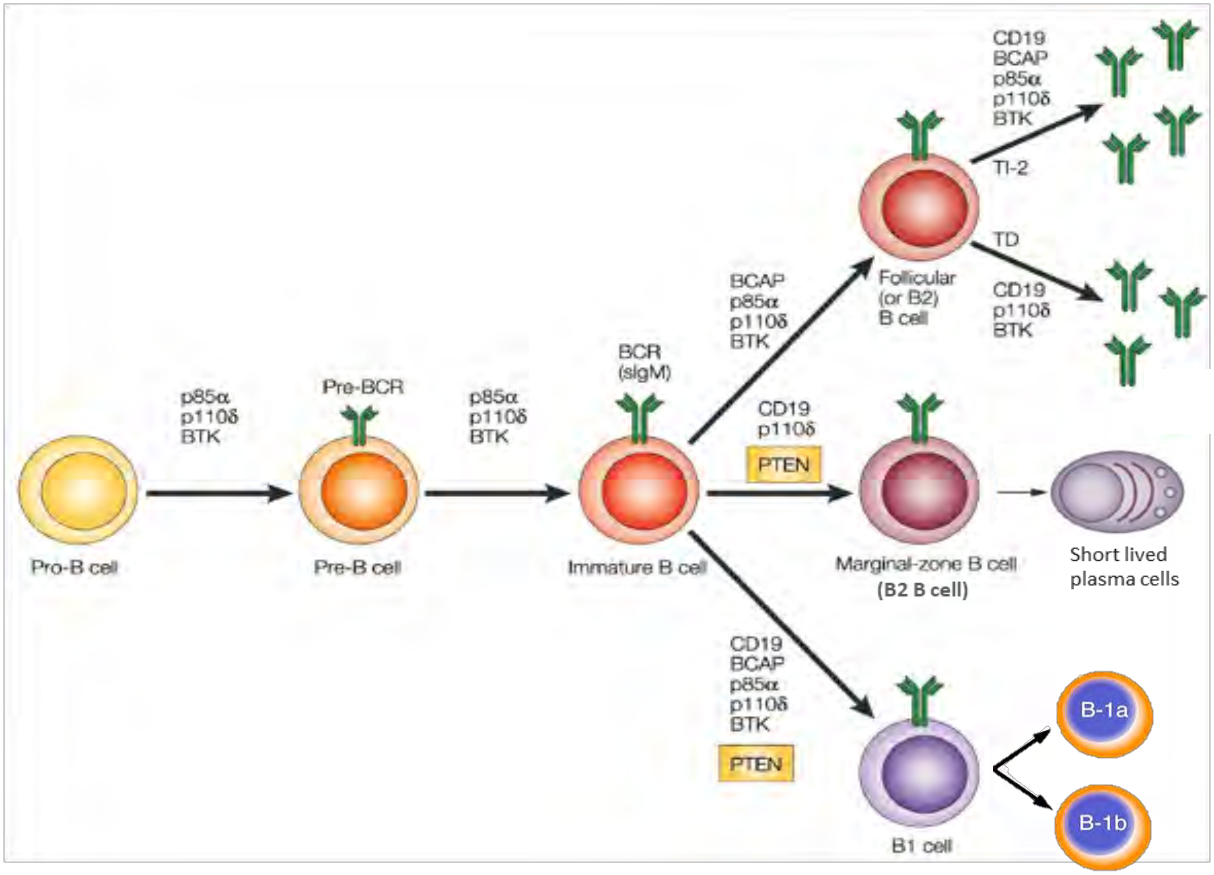


Figure 1.12 Development of B cell population.

B cells originate from the bone marrow and differentiate into B1 B-cells (B1a and B1 b) and B2 B-cells which include Marginal zone (MZ) and Follicular (FO) B cells. B1 cells reside mainly in the peritoneal and pleural cavities and are important in the TI responses. These subsets may also migrate to the spleen as short-lived plasma cells. Immature B2 progenitors migrate to the spleen, undergo transitional stages T1 and T2, and may then differentiate into mature MZ or FO B-cells. MZB cells may develop into short-lived plasma cells which secrete Abs in response to TI-antigens. FO B-cells may differentiate into plasma cells or memory B cells in TD manner through the extrafollicular or germinal centre responses. Figure taken and adapted from Yakimchuk K, T cell markers and B cell markers, 2016.

Conventional B cells include follicular B cells (FO B) and marginal zone B cells (MZ B), where FO B cells circulate through the blood or reside within B cell follicles in secondary lymphatic organs and MZ B cells remain in the MZ between the red and white pulp in the spleen (159). While MZ B cells can take part in T-Independent antibody responses, which are limited to extrafollicular responses, follicular B cells play a major role in T-dependent antibody responses. If antigens bind to B cells through their specific B-cell receptor (BCR), B cells may further differentiate into plasma cells (PCs) or memory B cells (B mem) and generate protective Ag-specific antibodies. This can occur via the extrafollicular (EF) response leading to mainly germ-line affinity antibodies or after the induction of germinal centres (GC) in the follicles. The GC response leads to the development of high-affinity antibody-secreting PC and Ag-specific memory B-cells (160). Extrafollicular and GC responses are further discussed in sections 1.6.2.4.1 and 1.6.2.4.2.

1.6.2.3 Antibodies

Antigen-induced antibodies derive from EF and GC responses. Antibodies are immunoglobulins (Ig) which consist of two heavy (H) protein and two light (L) protein chains joined by disulphide bonds, forming two distinct regions: the constant and variable regions (161). The variable region, also known as fragment antigen-binding (Fab), binds to Ag and determines the specificity of the Ab. On the other hand, the constant fragment crystallisable (Fc) binds to the Fc receptor (FcγR) on the surface of the effector cells and determines the antibody's class (isotype) and its effector function (122). Immunoglobulins are classified into five classes, including IgM, IgG, IgA, IgE, and IgD (Figure 1.13).

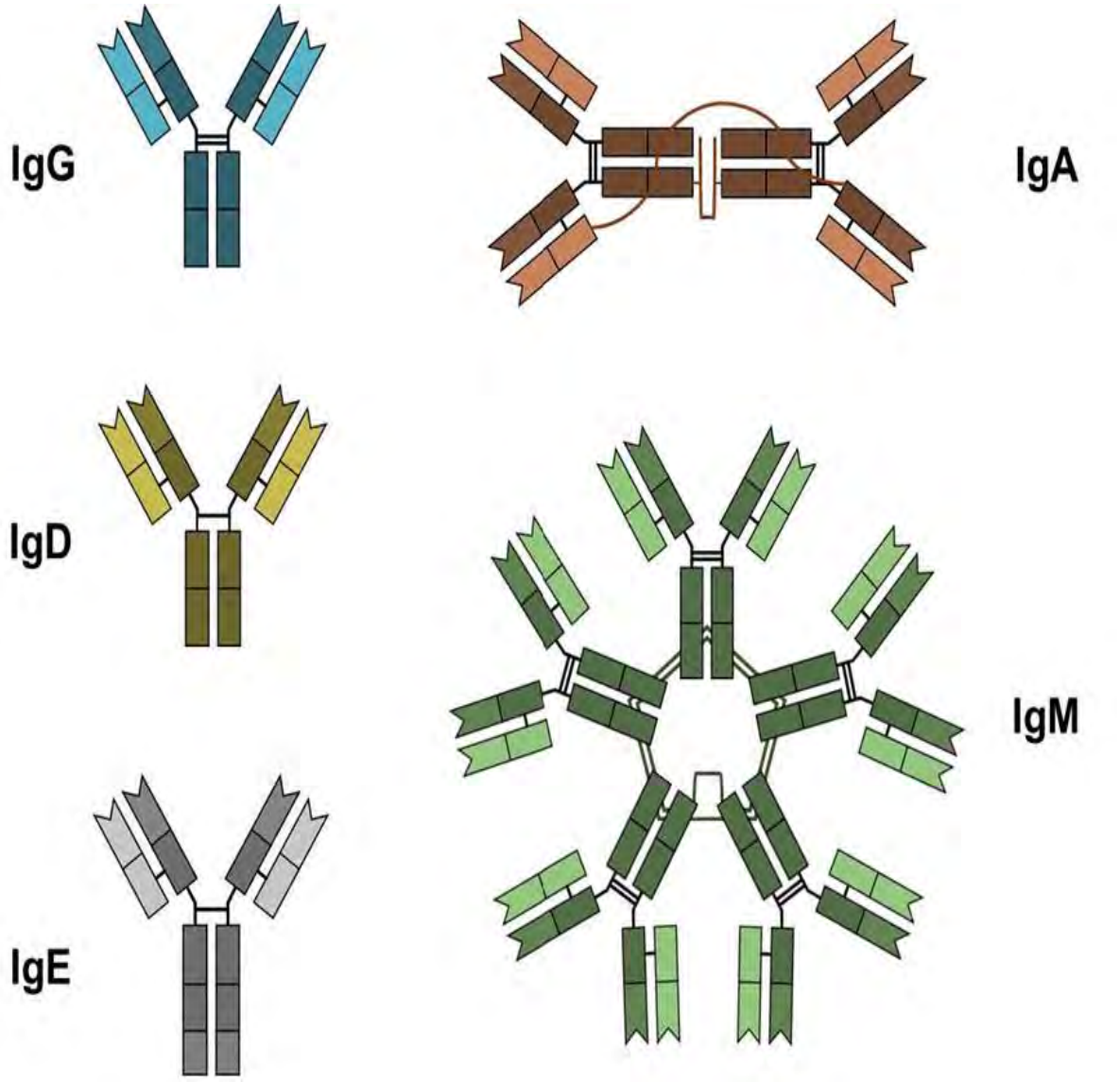


Figure 1.13 Structural differences of antibodies classes in mice.

There are five main classes of immunoglobulins: IgG, IgM, IgA, IgD, and IgE. Antibodies consist of 4 polypeptides; two identical heavy chains and two identical light chains connected by disulphide bonds. The upper part of the antibody is known as the variable region (Fab) which recognises the antigen and determines specificity. The lower part is called the constant region (Fc) which is responsible for the biological activity mediation. Secreted Abs can be found as monomer such as IgG, IgD, and IgE, dimers such as secretory IgA, or pentamers with five Ig units such as IgM. Figure taken from Encyclopaedia Britannica, Inc.,2023.

Antibodies have different functions, including neutralising bacterial toxins, mediating phagocytosis by opsonisation, and complement activation. IgM is the first Ab to be detected in sera following initial exposure to Ag, and titres quickly rise until reaching a peak a few days later. The Fc region of the IgM facilitates the linking of 5 IgM monomers, forming a pentameric structure. This characteristic of IgM leads to a significantly high avidity index and enables it to activate complement effectively (160, 162).

IgG is the most abundant type of antibody found in the bloodstream, comprising approximately 75% to 80% of all antibodies in the body. IgG antibodies have high affinity and can activate various immune responses such as opsonisation, complement activation, and antibody-dependent cellular cytotoxicity. It can also cross the placenta, providing passive immunity to newborns and offering protection against certain diseases during early life. IgG has a long half-life in the bloodstream (4-8 days in mice and 7-21 days in humans), allowing them to provide prolonged immune protection and contributing to the establishment of immunological memory (163). In mice, there are four IgG subclasses known as IgG1, IgG2a, IgG2b, and IgG3. However, in certain mouse strains, such as C57B/6 mice, IgG2c is considered equivalent to IgG2a (164). Generally, IgG1 and IgG3 are produced in response to proteins, while IgG2 are usually triggered by polysaccharides (161).

IgA is found in mucosal tissues, such as the respiratory and gastrointestinal tracts, and the primary function of IgA is to provide localised immunity and defence against pathogens at mucosal surfaces. IgA antibodies can exist in two forms: as a dimeric structure, known as secretory IgA, or as a monomer in the bloodstream. Secretory IgA plays a critical role in preventing the attachment and entry of pathogens into the mucosal cells by neutralising them and blocking their access. IgA antibodies also participate in immune exclusion, mucosal

immune tolerance, and the maintenance of healthy microbiota in mucosal environments (165). In humans, the half-life of IgA is around 6 days, while in mice, it is about 17-22 hours (163). IgE antibodies play a crucial role in allergic responses and defence against parasitic infections. The Fc region of IgE has a specific affinity for receptors located on mast cells and basophils. IgE antibodies have a relatively short half-life in the circulation, with a range of approximately 2-3 days in humans and about 12 hours in mice (163).

IgD is found primarily on the surface of B cells. The function of IgD antibodies is not fully understood, but they are believed to play a role in the early stages of B cell activation and maturation. IgD can act as co-receptors alongside the BCR to enhance antigen recognition and signalling. Compared to other antibody isotypes, soluble IgD antibodies are less common, but they can still bind to specific receptors on immune cells, such as basophils, and potentially participate in immune responses.

Antibodies have demonstrated significance in protecting against iNTS infections through cell-dependent mechanisms by promoting macrophage engulfment and subsequent killing through oxidative bursts (166). They also contribute to cell-free complement-mediated killing by promoting MAC (167). However, in mice, the efficiency of complement in promoting cell-free killing is limited and antibody-mediated killing of *S*. Typhimurium is primarily reliant on cell-dependent mechanisms (168).

1.6.2.4 T-dependent (TD) B cell responses

T and B cell interactions are necessary for TD responses, which are normally triggered by protein antigens or protein-based conjugates. Mature FO B cells, which are typically involved in TD responses, recirculate through blood and lymph to reside in the follicles of SLO following BCR-antigen binding (169). B cells then become fully activated by presenting antigens to T cells through MHC class II molecules. These MHC class II molecules are expressed not only in B cells but also in other cells like dendritic cells and macrophages. This interaction results in the formation of a complex involving the T-cell receptor, MHC class II molecules, and the antigen peptide, known as the TCR: MHC Class II-Peptide Complex (170) (Figure 1.14). Some activated B cells may then exit the follicle and drift to extrafollicular foci to initiate extrafollicular responses and differentiate into short-lived plasma cells, and secrete low-affinity antibodies. Alternatively, B cells may migrate to the B cell follicle to form germinal centres, resulting in the production of memory B cells and high-affinity class-switched antibodies (160, 171).

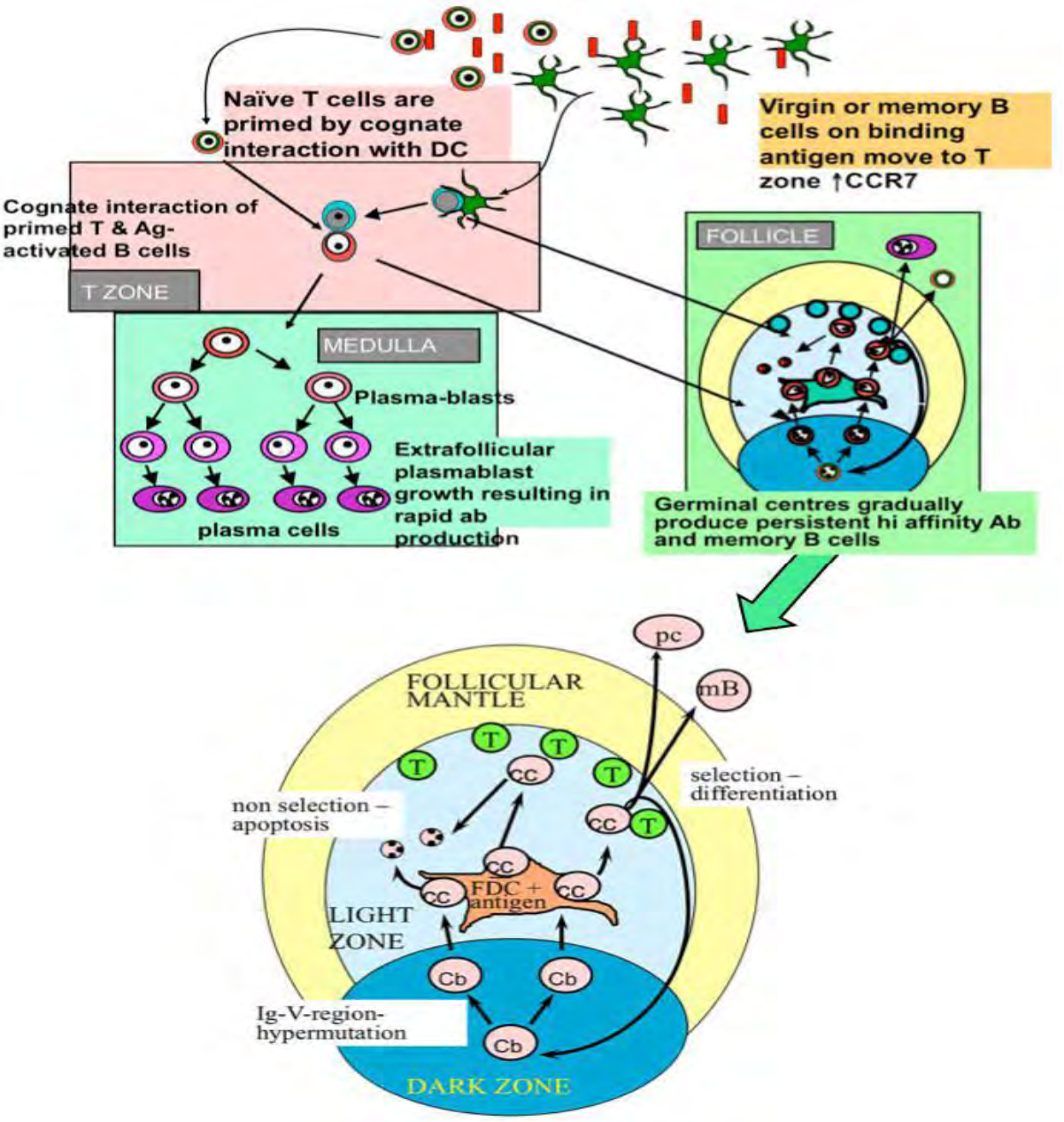


Figure 1.14 T-cell dependent antibody Responses; GC and EF responses.

Naïve CD4+ T cells can be activated by binding to MHC class II on the surface of APCs at the T zone. These activated CD4 T cells migrate towards the borders of the B cell follicles, where they interact with a cognate B cell that has already been activated by binding antigen to BCR. This interaction enables B cells to receive signals from CD4+ T cells and undergo proliferation in the follicle. Some proliferating B cells leave the follicle and differentiate into short-lived plasma cells that produce low affinity Abs or give rise to germinal centre independent memory B cells. The majority of the activated B cells migrate to the dark zone to undergo clonal expansion of antigen-specific B cells and BCR diversification, forming germinal centres (GC). GC B cells then migrate to the light zone and go through affinity selection through interaction with the follicular dendritic cells (FDCs) in conjunction with signalling from Ag-specific Tfh cells. Some of these B cells can re-enter another cycle of somatic hypermutation and affinity maturation. Other GC B cells may exit the germinal centre as memory B cells or long-lived plasma cells that secrete high affinity class switched antibodies. Figure adapted from I. C. M. MacLennan.

1.6.2.4.1 Extrafollicular Antibody Response

MZ B cells and some re-circulating B cells are recruited into the EF pathway following T and B cell interaction to generate plasmablasts (PB). These PBs start expressing syndecan-1 (CD138) and leave the interfollicular regions to migrate to the red pulp of the spleen. They then form extrafollicular foci and differentiate into low-affinity antibody-secreting PB through regulation of TF Blimp-1 expression in B cells and suppression of the TF PAX-5 (160). Blimp-1 expression is necessary for differentiation into plasma cells, while PAX-5 is associated with GC formation. Most of the PBs produce IgM early after infection, but they are short-lived and eventually undergo apoptosis. Some plasmablasts may undergo further differentiation into plasma cells, which are promoted with $CD11c^{Hi}$ DCs, leading to the expression of some markers, such as CD19 and B220 (172). During this stage of plasma cell differentiation, some of them may undergo class switch recombination to produce other isotypes (173). It should be noted that the type of isotype induced is influenced by the nature of the antigen and polarisation of the T cells interacting with B cells. For instance, in mice, Th1 responses associated with *S*. Typhimurium infection promote the development of IgG2a (IgG2c in C57BL/6 mice), while in response to extracellular bacteria, Th2 is associated with IgG1 production through IL-4 (174, 175). Only a limited percentage of these cells, nevertheless, can develop into memory B cells or long-lived plasma cells and reside in the spleen or BM.

1.6.2.4.2 The germinal centre (GC) response

Memory B cells and high-affinity class-switched antibodies are produced in GC, which form within follicles of SLOs (176). After activation and migration from the T zone into the follicle, B cells undergo somatic hypermutation (SHM) with upregulation of the TF BCL-6, which is a

key regulator of GC formation and suppressor of plasmablasts differentiation. This allows the massive expansion and proliferation of activated B cells (177) (Figure 1.14).

GC can be subdivided into two regions known as the dark zone and the light zone. In the dark zone, GC B cells undergo SHM and express activation induced cytidine deaminase (AID), allowing them to switch Ig subclasses (170). In addition, GC B cells express CD95 (Fas) and n-glycolylneuraminic acid (GL-7) and lose their expression of IgD. These cells then migrate to the light zone and undergo selection based on the affinity for antigen by follicular dendritic cells FDCs and Tfh cells. Positively selected B cells which have high Ag affinity can develop into memory B cells or high-affinity antibodies secreting plasma cells. These cells can migrate to the bone marrow and live for many years. To classical protein antigens like ovalbumin (OVA), GC typically peaks 10- 14 days after primary Ag encounter (178).

1.6.2.5 T-independent (TI) B cell responses

T-Independent (TI) antibody responses are immune responses that arise without T cell support and develop more rapidly than TD antibody responses, resulting in low to modest affinity germline polyreactive antibodies that offer an initial source of protection (160, 179). B1 and MZ B cells contribute to this response and their engagement based on the type of antigen. Antigens that induce TI antibody responses can be classified into TI type 1 (TI-1) and type 2 (TI-2). TI-1 Antigens, such as LPS and viral RNA, can directly bind to TLRs and induce B cell division (180). On the other hand, TI-2 Antigens, such as capsular polysaccharides of encapsulated bacteria like STy, viral capsids, and polysaccharides like Ficoll, have highly repetitive epitopes that can activate B cells by inducing multiple crosslinking of the BCR (181). B1 cells, which are mainly located in the peritoneal and pleural cavities, play an essential role in natural antibody production even in the absence of exogenous antigens (182). They can

secrete IgM and IgG3 in mice or IgM and IgG2 in humans (161, 183). TI antibody responses tend to have minimal memory, and antibodies are short-lived (184).

1.6.3 Immune responses to *S.* Typhimurium infection in mice

Initial control of *S*. Typhimurium infection in mice is predominantly regulated by innate immunity and T cell-independent immune responses. During infection, neutrophils and macrophages migrate to infected areas, such as the Peyer's patches, to limit the systemic dissemination of the infection through phagocytosis and releasing reactive oxygen species (ROS) (185, 186). Through TLR stimulation, macrophages initiate the production of IL-12 and TNF (187). IL-12 is a key cytokine that induces a Th1 response, stimulates the activity of NK cells for early defence against intracellular *salmonella* by directly killing infected cells, and promotes the production of IFN-γ to further activate macrophages. TNF helps recruit immune cells to the site of infection, promoting an inflammatory environment essential for combating the pathogen. It also activates macrophages, as these activated cells are more efficient in clearing the infection. Phagocytes located in the spleen and liver effectively eliminate bacteria from the bloodstream. The ability of the innate immune system to eliminate the infection is limited by the ability of *Salmonella* to reside within vacuoles in macrophages and DCs, which provides an intracellular niche for *Salmonella* replication and survival (188). Hiding within macrophages and DCs enables the bacteria to evade the innate inflammatory response in the gut and helps spread *Salmonella* to systemic sites. In these sites, there is rapid recruitment of monocytes, macrophages, neutrophils, and DCs in a further attempt at early control through phagocytosis and the release of cytokines like IL-12, IL-15, and IL-18 (189, 190). IL-18 works synergistically with IL-12 to induce IFNγ production and enhance the activity of NK cells and T cells, promoting an effective immune response against intracellular bacteria. These cytokines,

together with TLR4 activation, allow neutrophils, macrophages, and NK cells to release IFNγ, which promotes intracellular bacterial killing. IFNγ promotes the intracellular killing of *S*. Typhimurium by activating macrophages, stimulating the production of reactive oxygen and nitrogen intermediates, enhancing phagosome maturation, and facilitating efficient antigen presentation. Activated macrophages exhibit increased phagocytic and bactericidal capabilities, generating reactive species that contribute to bacterial destruction. Additionally, IFNγ-mediated maturation of phagosomes and improved antigen presentation enhance the overall immune response, enabling a coordinated effort involving both innate and adaptive immunity to effectively clear intracellular S. Typhimurium infections.

If the innate immune response fails to control *S*. Typhimurium infection locally, a cell-mediated immune response is required to resolve the infection. CD4 Th1 cells are vital for the clearance of *Salmonella* infections (191). T-bet cells produce IFNγ, which in conjunction with TNF, facilitates the disruption of the SCV and the disintegration of intracellular *S*. Typhimurium. This is achieved by activating macrophages to express iNOS and NADPH oxidase enzymes. These enzymes catalyse the production of ROS and RNS, which alongside Th1 dependent activation of macrophages, results in the disruption of the SCV and the destruction of intracellular *Salmonella* (192, 193). The development of a strong Th1 response thus enables clearance of *S*. Typhimurium around 5 weeks after infection (174). In a study conducted on mice lacking TCR α-β, MHC class II or T-bet showed that they are unable to clear attenuated *S*. Typhimurium infection (194, 195). Moreover, mice lacking IFNγ receptors or receiving anti-IFNγ are more susceptible to *Salmonella* infection (196), demonstrating the significance of CD4+ Th1 cells and the production of IFNγ in protection against *Salmonella*. However, the role of CD8+ T cells is less clear, and studies in mice showed that CD8+ T cells are not required for

protection against primary infection with attenuated *S*. Typhimurium (197) and showed only a minimum effect in the clearance of *Salmonella* during a secondary exposure in mice with depleted CD8+ T cells (198, 199).

B cell antibody-mediated responses have an important role in the protection of *S*. Typhimurium during secondary infection but are dispensable during primary infection. Some studies showed that B cells are not required for controlling primary infection with attenuated *Salmonella* in B-cell-deficient mice (200, 201). However, B cells are required for clearance of a virulent *S*. Typhimurium infection, and antibodies help limit the spread of *Salmonella* systematically, lowering the prevalence of bacteraemia (202). It has been demonstrated that the antibodies produced from EF and GC in resistant mice assist in preventing *S*. Typhimurium from infecting macrophages in the spleen; thus, both humoral and cell-mediated immune responses are required for protection (174). Passively administered antibodies have proven their key role in conferring protection against infection with the virulent strain of *S*. Typhimurium (199, 203).

1.6.3.1 Targets of protective immunity after *S*. Typhimurium infection

Antibodies against a variety of *S*. Typhimurium antigens, including LPS, OMPs, and flagellin, are produced in mice after *S*. Typhimurium infection, and some of these antigens are targets for protective antibodies and possible candidates for vaccines (34, 94, 174, 204, 205). However, natural *S*. Typhimurium infection induces different immunological responses. *S*. Typhimurium O-Antigen is a prime target, and antibodies specific to O-antigen are associated with protective immunity against *S*. Typhimurium (206, 207). In addition, monoclonal antibodies (mAbs) raised in response to O-antigen demonstrated the ability to kill invasive *Salmonella* in mice (204, 208). Immunisation with flagellin alone or conjugated with O-antigen

induces significant protective immune responses after lethal *Salmonella* challenge in mice (39, 94, 209). Antibodies induced in response to some porins, such as OmpD, can be protective after *S*. Typhimurium challenge in mice (12). *S*. Typhimurium-OmpD is a potential candidate and can induce antibodies to prevent systemic *S*. Typhimurium infection (34). Thus, understanding the antigenic targets in *Salmonella* could offer the possibility of developing an effective vaccine against invasive salmonellosis.

1.7 Mouse model of *S*. Typhimurium infection

Mice can be used as a model to study the immune responses to iNTS by infecting them with *S*.Typhimurium or other *Salmonella* serovars. Unlike S.Typhi, which is restricted to humans and higher primates, *S*. Typhimurium can infect mice and induce systemic infection with symptoms similar to those reported in infected humans, making this an appropriate model to investigate iNTS (210). Bacteria can be administered orally, intraperitoneally (i.p.) or intravenously (i.v.), and the latter two routes can be used to ensure proper dosage and synchronised infection. Virulent *Salmonella* serovars can be given to resistant mice to enable the control and clearance of the infection (211). Mouse strains that are hyper-susceptible to wild-type (WT) *S*. Typhimurium, such as C57BL/6 and BALB/c, are often used. These strains have a point mutation in the natural resistance associated with macrophage protein 1 (Nramp-1), which affects the ability of macrophages to control phagolysosomal killing (212, 213). However, these strains do not survive for long after the challenge with virulent *Salmonella*; instead, attenuated *S*. Typhimurium strains such as Δ*aroA* mutants can be used. The ΔaroA mutants have impaired aromatic amino acid synthesis, which reduces the virulence of the bacteria (214), allowing susceptible mice to clear a systemic infection in a few weeks (174).

CD1 mice, which are mostly utilised to model iNTS throughout this project, are Nramp-1-competent mice and can survive virulent *Salmonella* challenge and handle the infection. Therefore, they are suitable for studying chronic infection (215).

1.8 Aims of this thesis

The main aim of this project is to examine the immune response induced in mice after OMV-based vaccine against *S*. Typhimurium and *S*. Enteritidis and assess the potential of using OMV with different O-antigen expression levels as a vaccine against iNTS. This thesis has a strong focus on optimising the O-antigen level and understanding how altering O-antigen expression in OMV vaccines affects responses to the vaccine antigens tested. The hypothesis tested is that reducing the O-antigen expression of the OMV vaccine enhances access to other OMV antigens and improves protection against homologous and heterologous serovars of *Salmonella*.

Objectives of this project are:

1. To characterise the immune responses elicited by *S*. Typhimurium-OMVs with different O-antigen expressions.
2. To assess and compare the ability of the vaccines to protect against *S*. Typhimurium and *S*. Enteritidis.
3. To characterise the reactivity and bactericidal activity of monoclonal antibodies (mAbs) raised against *S*. Typhimurium-OMV and *S*. Typhimurium-OmpD.

Chapter 2. MATERIALS AND METHODS

2.1 Media and buffer

All buffers and details of all the media used throughout this study are listed in Table 2.1

Table 2.1: Media and buffers

Media/Buffer	Ingredients	Source
Luria-Bertani (LB) broth (Lennox)	20 g/L of LB broth powder dissolved in dH2O, autoclaved at 121 ºC.	Sigma-Aldrich
Luria-Bertani (LB) Agar (Lennox)	35 g/L of LB agar dissolved in dH_2O, autoclaved at 121 ºC.	Sigma-Aldrich
Phosphate Buffered Saline (PBS)	5x PBS tablet dissolved in 1 L dH_2O.	Sigma-Aldrich
ELISA carbonate-bicarbonate coating buffer	1.66 g/L of Na_2CO_3 2.84 g/L of NaHCO3 dissolved in dH2O	Sigma-Aldrich
FACS Buffer	2 % Heat-inactivated Foetal Bovine Serum (Hi-FBS) 5 mM EDTA In sterile 1x PBS	Sigma-Aldrich
R-10 media	10 % Heat-inactivated Foetal Bovine Serum (Hi-FBS) 1 % Penicillin-Streptomycin (1mg/mL) In sterile RPMI media	Sigma-Aldrich
Tris buffer pH 6.8	Make 1L of 200 mM Tris Base (6.075g) in dH2O Add 1.5L of 154 mM NaCl The pH was then adjusted by adding 0.1 N HCl.	Sigma Aldrich

0.5% Sodium Azide	0.5% Sodium Azide 0.5 mg Sodium Azide crystals Dissolved in 10 mL dH2O Diluted 1:10 into PBS on use	Sigma Aldrich

2.2 Bacterial strain

Human and mouse strains of virulent *S*. Typhimurium and *S*. Enteritidis are commonly used throughout this thesis, and the bacteria are typically administrated intraperitoneally. *S*. Typhimurium and Enteritidis were used for *in vivo* and in vitro experiments. Wild type and attenuated forms of both serovars were used throughout this project as well as the laboratory strain *S*. Typhimurium SL1344. These strains are outlined in Table 2.2. *S*. Typhimurium 14028 were used to construct OMV-producing strains, which have been described in Table 2.3

Table 2.2: *Salmonella* wild-type strains and mutated strains

Strain	Characteristics and Source
***S*. Typhimurium**	
***S*. Typhimurium** **D23580**	Virulent invasive *S*. Typhimurium, human isolates from a Malawian child with malaria and anaemia. source: Queen Elizabeth Central Hospital (QECH) (216). Resistant to: Chloramphenicol 30 µg/ml and Ampicillin 100 µg/ml

S*. Typhimurium D23580 Δ*wbaP	The strain was generated by disrupting the *wbaP* gene, which inhibits the synthesis of O-antigen in the LPS. The λ red recombination method is employed to generate this strain. The parent strain from which it originates is STm D23580. Source: Prof. Calman A. MacLennan. Resistant to: Kanamycin 50 µg/ml
***S*. Typhimurium SL1344**	Virulent aroA+ *S.* Typhimurium strain, animal isolates. Parent strain: *S*. Typhimurium S2337 (214). No resistance cassette
***S*. Typhimurium SL3261**	Attenuated *S*. Typhimurium strain with AroA- deficient. These animal isolates were attenuated by the deletion of aroA from a transposon insertion using aroA554::Tn10. Parent strain: STm SL1346 (214). No resistance cassette
S. Enteritidis	
***S*.Enteritidis D24954**	Wild-type virulent invasive strain of *S*. Enteritidis, human isolate from Malawian child. Source: Prof. Calman A. MacLennan. Resistant to: Chloramphenicol 30 µg/ml, Tetracycline 15 µg/ml, and Ampicillin 100 µg/ml
S*.Enteritidis D24954 Δ*wbaP	Mutated from a wild-type invasive human isolate *S*.Enteritidis D24954. No expression of O-antigen due to a deletion of *wbaP*. Source: University of Birmingham. Resistant to: Chloramphenicol 30 µg/ml

Table 2.3: *Salmonella* Typhimurium OMV-producing strains.

Strain	Characteristics and Source
S*. Typhimurium 14028 Δ*tolR	*S*. Typhimurium strain mutated from wild-type LT8-like animal isolates with deletion of the *tolR* gene. OMV-producing strain due to *tolR* deletion (217). Resistant to: Kanamycin 50 μg/ml
***S*. Typhimurium 14028 Δ*wzy* (rfc)**	*S*. Typhimurium strain mutated from wild-type LT8-like animal isolates with deletion of the *wzy* gene, affecting O-antigen polymerase producing 1 O-antigen/LPS (217). Resistant to: Chloramphenicol 50 μg/ml
***S*. Typhimurium 14028 Δ*wbaP* (rfbP)**	*S*. Typhimurium strain with deletion of *wbaP* gene, which inhibits synthesis of O-antigen producing LPS with no O-antigen (217). Resistant to: Chloramphenicol 50 μg/ml

2.3 Antigen

All antigens were prepared in a coating buffer for ELISA and PBS for ELISPOT and immunisation. Antigens used for analysis in vitro in this study were:

2.3.1 LPS derived from *Salmonella* Typhimurium. *S*. Typhimurium -LPS was purchased from Enzo Life Sciences LTD.

2.3.2 Purified porins of *Salmonella* Typhimurium.

S. Typhimurium -porins were kindly provided by Prof. Constantino López-Macías (Medical Research Unit on Immunochemistry, Specialties Hospital, Mexico City, Mexico). Preparations

were obtained from *S*. Typhimurium strain ATCC 14028, and the extraction and purification of these have been outlined elsewhere (31).

2.3.3 Purified OmpD from *Salmonella* Typhimurium.

Purified OmpD from *Salmonella* Typhimurium. The *S*. Typhimurium -OmpD was also kindly provided by Dr López-Macías. OmpD was extracted and purified from the *S*. Typhimurium strain SL3261 ompC::aph ompF::cat according to the protocol described previously (34). OmpD has also been used for immunisation according to the concentration stated in section 2.16.

2.3.4 Outer membrane vesicles (OMV) from *Salmonella* Typhimurium.

OMVs were isolated from *S*. Typhimurium 14028 Δ*tolR*, *S*. Typhimurium 14028 Δ*wzy* Δ*tolR*, and *S*. Typhimurium 14028 Δ*wbaP* Δ*tolR,* prepared as described in section 2.7. These OMVs were also used for immunisation. Mice were immunised intraperitoneally (*i.p.*) with 1 µg *S*. Typhimurium -OMVs, where mass is based on protein content. The immunisation protocols employed in each experiment within this project are detailed in the results section.

2.3.5 Bacterial strains (Table 2.2 and 2.3)

Cultures from *Salmonella* strains were inoculated in 10 ml LB broth in a loosely capped universal tube and incubated overnight at 37°C. At OD600 = 0.8-1, the culture was washed in PBS before finally being resuspended in sterile PBS containing 0.05% sodium azide (NaN3) to prevent further bacterial growth. The concentration was determined via the bicinchoninic acid assay (Pierce, Thermo Fischer) according to the manufacturer's instructions, and then diluted to 10µg/ml using coating buffer for ELISA.

2.4 Preparation of bacteria for mice challenge

Salmonella was grown by inoculating a single colony in 10ml LB broth supplemented with appropriate antibiotics as needed and incubating overnight at 37 °C in a rotatory incubator. Overnight cultures were diluted 1:5 into LB and incubated at 37 °C until OD_{600} 0.8-1. At this OD, 1 ml of culture was harvested and centrifuged at 6000 xg for 8 minutes and then washed twice with PBS before finally being diluted in 1 ml of PBS. For infection doses, mice were infected intraperitoneally i.p. as specified for each experiment in the results section. Bacteria were diluted in a final volume of 200 µl of sterile PBS per mouse. The right infection doses were confirmed by plating serial dilution.

2.5 Construction of OMV-producing *S*. Typhimurium mutants

2.5.1 Bacterial strains used

S. Typhimurium 14028 Δ*tolR*, *S*. Typhimurium 14028 Δ*wzy*, and *S*. Typhimurium 14028 Δ*wbaP* (Table 2.3) were obtained from an ordered single gene deletion library (217). *S*. Typhimurium 14028 Δ*tolR* was prepared by introducing a kanamycin resistance cassette (kan), which is an antibiotic resistance gene, into the *tolR* gene in *S*. Typhimurium 14028. *S*. Typhimurium 14028 Δ*wzy* and *S*. Typhimurium 14028 Δ*wbaP* were also prepared by replacing *wzy* and *wbaP* genes with a chloramphenicol resistance cassette (cat).

To generate OMV-producing strains from *S*. Typhimurium 14028 Δ*wzy* and *S*. Typhimurium 14028 Δ*wbaP,* the *tolR* gene needs to be disrupted in both strains, resulting in *S*. Typhimurium strains with two mutations. *TolR* genes were deleted in both strains following a method described in detail elsewhere (218). This gene was replaced by a kanamycin resistance

cassette produced by PCR amplification of the relevant gene segments in *S*. Typhimurium Δ*tolR*.

2.5.2 Polymerase chain reaction (PCR)

The Kanamycin resistance gene in *S*. Typhimurium Δ*tolR* strain was amplified by using gene-specific forward and reverse primers composed of approximately 40 bp homologous to the flanking regions of the *tolR* gene (to be deleted) and approximately 20 bp at the 3' end of homology to the kanamycin cassette (table 2.5). MyTaq Red Mix 2X (Bioline) was used according to the manufacturer's instructions. A freshly isolated colony of *S*. Typhimurium Δ*tolR* was suspended in a master mix which contained 0.1 µm of primers, MyTaq Red Mix 2X, and sterile deionised water. The PCR reaction was performed under the following condition: 95°C for 5 min; 5 cycles of 95°C for 30 sec, 52°C for 15 sec, 72°C for 2 min; 30 cycles of 95°C for 30 sec, 55°C for 15 sec, 72°C for 2 min. The PCR product was confirmed by gel electrophoresis using 1% agarose with DNA Midori Green Stain. PCR products were then purified by using a QIAquick PCR purification kit in accordance with the manufacturer's instructions and were ready to be inserted into *Salmonella* recipient cells.

Table 2.4 Primers names and sequences

Primer Name	Primer Sequence
***tolR* forward**	CCAGGCGTTTACCGTAAGCGAAAGCAACAAGGGGTAAGCCCTCTGGTAAGGTTGGGAA
***tolR* reverse**	CCTGTTACTCGCCGTCTTTCAAGCCAACGGGACGCAGACTTCAGAAGAACTCGTCAAG

2.5.3 Preparation and transformation of electro-competent cells

To prepare competent cells from *S*. Typhimurium Δ*wzy* and *S*. Typhimurium Δ*wbaP*, cultures were grown overnight in 5 ml LB broth supplemented with Chloramphenicol at 37°C. The next day, 1 ml of overnight culture was inoculated into 100 ml LB broth and allowed to reach an OD_{600} ≈ 0.6 at the same conditions. Cultures were placed on ice for 10 minutes and then centrifuged for 10 minutes at 3000 g at 4°C. Pellets were resuspended with sterile ice-cold H_2O and washed three times. Cells were then used immediately for electroporation. For electroporation, electrocompetent cells were transferred into a pre-chilled electroporation cuvette (1 mm, Geneflow) along with helper pKD46. pKD46 is a plasmid for RED-recombineering encodes lambda red gene and is ampicillin-resistant. This plasmid also shows temperature-sensitive replication, and the bacteria containing the plasmid should be grown at no greater than 30 °C to maintain the plasmid (218). Electroporation was done by Eppendorf Eporator System according to the manufacturer's instructions. Transformed cells were recovered in super-optimal broth (SOC) LB media (Invitrogen) for 1 hour with aeration at 30°C to maintain the plasmid. The cultures were then centrifuged, resuspended with 100 µl SOC broth, and transferred to LB agar supplemented with ampicillin and incubated overnight at 30°C. The colonies that grew were the transformants which carry pKD46 only.

2.5.4 Deletion of tolR gene and removal of pKD46 plasmid

Transformed *Salmonella* carrying pKD46 plasmid were grown in 5 ml SOC LB supplemented with Ampicillin and 20% L-arabinose at 30°C to an OD600 ≈ 0.6 and then made electrocompetent cells by following the same procedure in section 2.6.3. Electroporation was done this time by adding 100 µl of electrocompetent cells to 12 µl of 88 ng/ml PCR product (Kanamycin resistance gene). This was done to replace the *tolR* gene in *S*. Typhimurium Δ*wzy*

and *S*. Typhimurium Δ*wbaP* with a kanamycin-resistant gene. Cells were then recovered in SOC media for 2 hours at 30°C and then selected by plating on an LB agar plate with 50 µg/ml kanamycin and incubated overnight at 30°C. At that point, mutant bacteria potentially contained a *tolR* deletion due to the insertion of Kanamycin resistance cassette, but still also contained pKD46 plasmid. This plasmid is a temperature-sensitive replicon, and to be cured from a strain, bacteria were grown on LB agar at 41 °C to ensure getting rid of the plasmid. The following day, different colonies were selected and plated in a 1 cm line in the same location onto two LB agar plates (as shown in Figure 2.1), one with Kanamycin and the other with Ampicillin, and both plates were incubated overnight at 37°C. Colonies that only grew on Kanamycin agar but not on Ampicillin agar indicated that the plasmid was lost. These kanamycin transformants were chosen and grown overnight in 5 ml LB broth at 37 ˚C and were then stored in 10% glycerol at -80 ˚C. Successful *tolR* gene deletion was confirmed by PCR of relevant gene segments, which was done in the same way as previously described in section 2.6.2 and followed by gel-electrophoresis.

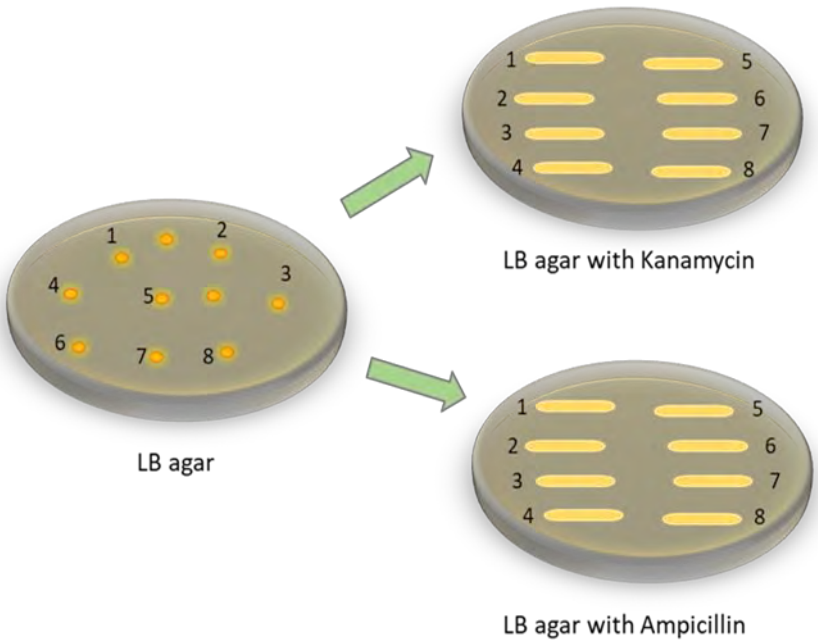


Figure 2.1 Schematic of resultant colonies being patch-plated for selection.

Colonies are patch-plated onto LB agar containing the appropriate antibiotics by touching the top of each colony with a sterile loop and streaking a 1 cm line on the corresponding agar plates. This technique can be used when screening for a specific phenotype such as antibiotic resistance.

2.6 Preparation of *Salmonella* Typhimurium Outer Membrane vesicles (*S.* Typhimurium -OMV)

OMVs were prepared from WT *S*. Typhimurium 14028 Δ*tolR*, *S*. Typhimurium 14028 Δ*wzy* Δ*tolR*, and *S*. Typhimurium 14028 Δ*wbaP* Δ*tolR* (Figure 2.2). OMV-producing strains were grown overnight in 5 ml LB broth (supplemented with 50 µg/ml of kanamycin for *S*. Typhimurium 14028 Δ*tolR* and 50 µg/ml of both antibiotics, chloramphenicol and kanamycin for the other two strains) at 37°C in shaking incubator. 300 ml of sterile LB were inoculated with overnight culture to an OD_{600} ≈ 0.05 using 1000 ml sterile vented flask and incubated at

37°C in shaking incubator. When bacteria grew to the desired Optical density, which is 1, centrifugation at 4000 xg for 10 minutes at 4°C was done (118). Supernatants were filtered using 0.22 µm 500 ml filter unit. For purification, the filtrate was ultra-centrifuged one time for 2 hours at 4°C, at 186,000 xg, using 70-Ti Beckman and Coulter rotor and 26.3 ml capped polycarbonated tubes. Pellets were then washed with cold, sterile PBS and ultra-centrifuged again for 1 hour at the same conditions. Finally, pellets were resuspended in 1 ml cold PBS and filtered with a small 0.22 µm filter for sterility. The concentration of OMVs was quantified using BCA assay and stored at 4°C. The OMVs were assessed by Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis and visualised with Coomassie blue stain.

2.7 Lipopolysaccharide (LPS) profile of OMVs derived from *S*. Typhimurium mutants

Extraction of LPS from OMVs and examination were done in the same method used previously for the whole *Salmonella* cells with some modification (219). 50 µl of OMV sample was mixed with 50 µl of cracking buffer (1 M Tris, 2% SDS, 4% β-mercaptoethanol, pH 6.8), boiled for 10 minutes, and cooled. Samples were then centrifuged at 14,000 x g for 8 minutes to remove any insoluble materials. Then, samples were treated with 5 mg/ml of Proteinase K (Sigma-Aldrich) for one hour at 37°C In order to eliminate contaminating protein. 10 µg of each Sample were loaded onto a 12% SDS-PAGE gel (Invitrogen) and visualised with a Pierce Silver Stain Kit (Thermo Scientific) following the manufacturer's instructions.

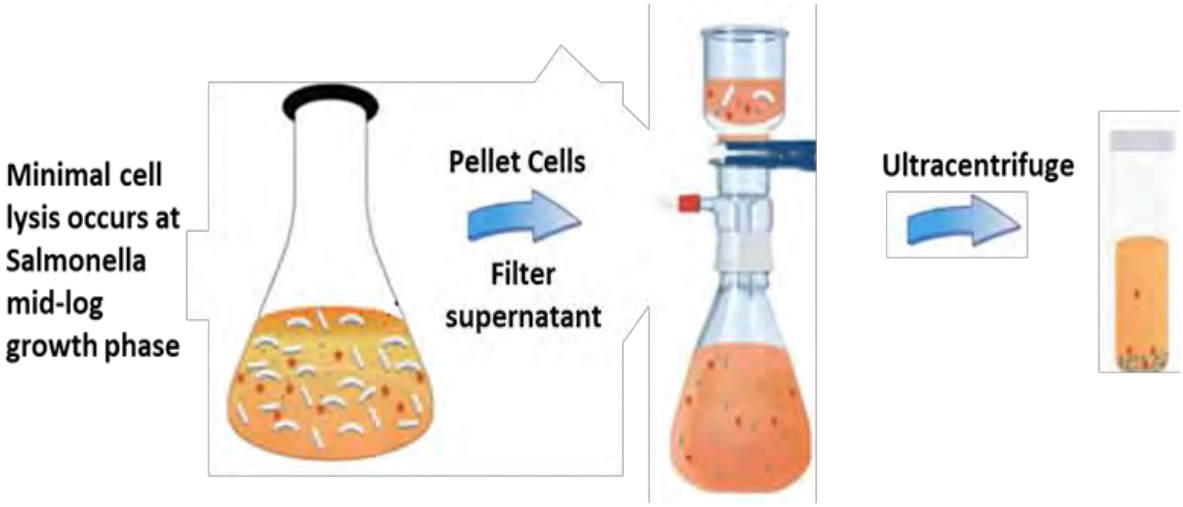


Figure 2.2 Isolation of bacterial OMV workflow.

OMVs isolation is relatively simple process. It was generated by growing bacteria and purified straight from the culture by multiple steps of filtration and centrifugation.

2.8 Mouse studies

All Mice used in this study were aged 6-12 weeks. WT CD1, 6-8 weeks, were purchased from Charles River (Italy) or Harlan (UK). BALB/c and C57BL/6 mice, 6-12 weeks, were obtained from HO Harlan OLAC Ltd (Bicester, UK) and used as wild-type (WT) controls in some experiments. Genetically modified mice used throughout this thesis were bred and maintained under specific-pathogen free conditions at the Biomedical Service Unit (BMSU) at the University of Birmingham and are listed in Table 2.4. All animal experiments were performed in accordance with Home Office regulations and with approval from the UK Research Animal Ethics Committees.

Table 2.5: Genetically modified mice used in this study.

Mouse strain	Characteristics and Source
T-bet-/- **(C57BL/6 background)**	Disruption of the T-bet gene by homologous recombination resulted in Impaired development of Th1 cells (220).
sIgM -/- **(C5q7BL/6 background)**	Mice lacking secretory IgM (still expresses membrane-bound IgM) due to replacing the *μs* splice region with a cDNA fragment encoding the Cμ4 and μm exons (221).

Mice were immunised *i.p*. with 1 μg OMVs in 200 μL PBS and challenged *i.p*. with $1x10^4$ CFU of virulent *S*. Typhimurium D23580, *S*. Typhimurium SL1344, or *S*. Enteritidis D24954 in 200 μL sterile PBS. Attenuated *S*. Typhimurium SL3261 were administrated at $5x10^5$ CFU to T-bet KO mice and IgMs KO mice in 200 μL PBS. Mice were exsanguinated by cardiac puncture at the end of the experiment after being anesthetised with Isoflurane. Mice were then sacrificed by cervical dislocation following the schedule 1 method according to the Home Office Regulations. About 1 mL of blood was collected through cardiac puncture and left for one hour at 37 °C to clot. Blood was then centrifuged at 6000 x g for 8 minutes to separate serum before being aliquoted into Eppendorf's and stored at -80 ° C. Tissues were also collected post-mortem in order to process them for Bacterial culture, ELISPOT, and flow cytometry.

2.9 Bacterial burden assessment in tissues

Spleens, livers, and kidneys were taken and weighed, and a piece from the organ was mashed through a 70 μm cell strainer (Falcon) in 1 ml sterile PBS. The cell suspensions were then

serially diluted (1:10,1:100 and 1:1000) in sterile PBS, and 100 µl (sometimes 200 µl) was plated on LB agar plates using an L-shaped cell spreader. Plates were incubated at 37 °C overnight, and colonies were then counted and adjusted for dilutions and the weight of the organ. The colony-forming unit (CFU) per organ was calculated as follows:

Total CFU = CFU per plate x 10 x Dilution Factor x (Organ Mass/Bacterial Culture Mass).

For the bacterial culture of the blood, about 100-200 µl was plated immediately during blood collection and bacterial numbers were obtained according to the volume of the sample plated.

2.10 Preparation of single cell suspensions from the spleen and bone marrow

Cell suspensions were prepared from spleens and bone marrows to be used in ELISPOT and flowcytometry. A part of the spleens was weighed and mashed through a 70 µm cell strainer in 4 ml R-10 media (RPMI from Sigma Aldrich supplemented with 10% heat-inactivated fetal bovine sera (FBS) and 1% penicillin/streptomycin). Bone marrow cells were also prepared by flushing both the tibia and femur of one hind leg with R-10 media. Cells were transferred to a 15 ml falcon tube and pelleted in a centrifuge at 375 x g for 5 minutes. Cells were then re-suspended in 500 µl of ACK lysis buffer (gibco) for 3 minutes in order to lyse erythrocytes. Cells were then washed again and re-suspended in R-10 media. Live cells were counted using a haemocytometer by diluting 10 µl of the cell suspension with trypan blue. Total number of cells per sample was calculated using the following equation:

Cells/ml= Cell count x Dilution in trypan blue (1:10) x square factor of haemocytometer (10^4) x Volume of sample

Cell suspensions were washed again and diluted in R-10 media and adjusted to $1x10^7$ cells/ml. Cells were always kept on ice.

2.11 B cell Enzyme-Linked ImmunoSpot (ELISPOT)

Antigen-specific antibody-secreting cells (ASC) in the spleen and bone marrow were assessed by B cell Enzyme-Linked ImmunoSpot (ELISPOT). LPS or porins were prepared in PBS and used to coat MultiScreen 96-well filtration plates (Merck Millipore) at a concentration of 5 µg/ml with a total volume of 50-100 µl per well. Plates were then incubated overnight at 4 °C. The following day, wells were washed with PBS to remove unbound antigens and blocked with R-10 media for one hour at 37 °C. Blocked plates were then washed one more time with PBS, and about 50 µl from the prepared spleen or bone marrow cells (as described in section 2.11) were loaded to the wells in triplicate in 200 µl R-10 media giving a total of $5x10^5$ cells per well. Cells were then incubated at 37 °C, 0.05% CO_2 incubator for six hours to stimulate antigen-secreting cells. After incubation, plates were washed with PBS-0.05% Tween 20 to lyse and wash away the cells. AP-conjugated anti-IgG and anti-IgM at 1:1000 (Southern Biotech) diluted in PBS were used to detect LPS or porin-specific antibodies and incubated overnight at 4°C. Plates were then washed 3 times in 0.05% PBS-Tween and one time in PBS prior to adding SIGMA FAST 5-Bromo-4-chloro-3-indolyl phosphate / Nitro blue tetrazolium (BCIP/NBT) tablets (Sigma) in order to develop spots. BCIP/NBT tablets were diluted in sterile water according to manufacturer instructions, and spots were developed for 5-9 minutes. Spots were counted using an AID ELISPOT plate reader and AID software version 7.0 (AID GmbH). ASC counts were represented as spot-forming units (SFUs) per $1x10^6$ cells.

2.12 Flow cytometry

The previously adjusted splenocytes were resuspended with FACS buffer in 96-well V-bottom plates at a concentration of $2.5x10^6$ cells per well and spun at 370 x g for 5 minutes at 4 °C. Cells were then resuspended with FC blocking agent (anti-CD16:CD32 Ab) to block cell CD32/FcγIII and CD16/FcγII receptors for 15 minutes at 4 °C. After blocking, the cells were centrifuged at the same condition, and 50 µl of the extracellular antibody mix prepared in FACS buffer was added to each well and incubated on ice for 25 minutes in the dark. These Antibodies were used in order to identify different lymphocyte populations by targeting different cell surface markers (Table 2.6). Splenocytes were washed with FACS buffer in order to wash out the unbound antibodies and then incubated with 100 µl of viability dye diluted in PBS for 15 minutes at 4°C in the dark. Samples that were only stained for extracellular markers were fixed in FACS buffer with 0.1% v/v paraformaldehyde PFA to be acquired the next day. For intracellular staining, 50 µl of the intracellular antibody mix (Table 2.6) diluted in permeabilisation buffer (BD biosciences) was added to the cells and incubated for half an hour at 4 °C protected from light. Cells were then washed twice with perm buffer and re-suspended in 200 µl 0.1 % PFA FACS buffer and kept at 4°C for acquisition the next day. For Sample acquisition, cells were acquired on BD LSRFortessa^{TM} X-20 Cell Analyzer and analysed using FlowJo 10.1 software (TreeStar). The gating strategy utilised is outlined in Appendix B

Table 2.6 Antibodies used for FACS

Specificity and Fluorochrome	Stock Concentration	Dilution	Supplier and Clone	Staining panel/ Application
Fcγ Receptor Blocking				
CD16:CD32 Purified	0.5 mg/ml	1:150	eBioscience 93	Block FcγII and FcγIII receptors
Extracellular Staining				
B220 Brilliant Violet 510	100 µg/ml	1:200	BioLegend RA3-6B2	B cells Plasma cells
B220 APC eF-780	0.2 mg/ml	1:300	eBioscience RA3-6B2	GC B cells
CD 19 Brilliant Violet 786	0.2 mg/ml	1:400	BD Biosciences 1D3	B cells GC B cells Plasma cells
CD138 Brilliant Violet 650	0.2 mg/ml	1:200	BD Biosciences 281-2	Plasma cells
CD38 Alexa Fluor 700	0.2 mg/ml	1:200	eBioscience HIT2	Plasma cells GC B cells panel
TACI Alexa Fluor 647	0.2 mg/ml	1:200	BD Biosciences 8F10	B cells panel
IgD Brilliant Violet 421	0.2 mg/ml	1:150	BioLegend 11-26c.2a	B cells panel
GL7 FITC	0.6 mg/ml	1:500	BD Biosciences GL7	GC B cells panel
FAS (CD95) Brilliant Violet 605	0.2 mg/ml	1:100	BD Biosciences Jo2	GC B cells panel
CD3e Brilliant Violet 510	100 µg/ml	1:100	BioLegend 145-2C11	T cells
CD4 PE-CF594	0.2 mg/ml	1:200	BD Biosciences RM4-5	T cells
Intracellular staining				
IgG FITC	0.5 mg/ml	1:500	Southern Biotech Polyclonal	Plasma cells
IgM APC eF780	0.2 mg/ml	1:300	eBioscience 11/41	Plasma cells
BCL6 PE	0.2 mg/ml	1:500	eBioscience K112-91	GC B cells panel
Ki-67 PE-Cy7	0.2 mg/ml	1:200	BD Biosciences B56	GC B cells panel

2.13 Enzyme-linked immunosorbent assay (ELISA)

This assay was done to measure the relative titre antigen-specific antibodies after immunisation with *S*. Typhimurium -OMVs and *S*. Typhimurium/ *S*. Enteritidis post-infection. The specificity of the antibodies was tested against different generated *S*. Typhimurium -OMVs, *S*. Typhimurium porins, *S*. Typhimurium LPS and whole bacteria of *Salmonella* Typhimurium or *Salmonella* Enteritidis. Flat-bottomed Maxisorp 96 well NUNC plates (Thermo Fisher Scientific) were coated with 5 μg/ml of antigens or bacteria diluted in coating buffer and incubated overnight at 4 °C. After incubation, plates were washed and then blocked with 1% Bovine Serum Albumin (BSA)(Sigma-Aldrich) dissolved in 1x PBS and incubated for one hour at 37°C. Plates were washed with 0.05% Tween 20 in 1x PBS. Sera (primary antibodies) were diluted 1:100 or 1:50 in blocking buffer with 0.05% Tween and serially diluted in 3-fold steps and incubated as before. Plates were washed, and goat anti-mouse antibodies conjugated to alkaline phosphatase (AP) were added with either IgG, IgG1, IgG2b, IgG2c, IgG3 (1:1000) or IgM (1:2000), (Southern Biotech). After washing, colour was developed with Sigma FAST P-nitrophenyl phosphate (pNPP) tablets (Sigma-Aldrich) dissolved in water according to manufacturer instructions and plates were read by measuring the absorbance of each well at OD_{405} nm on a SpectraMax ABS plus plate reader (Molecular Devices) using SoftMax pro software version 6.5. Ab titres were determined by the inversion of the dilution of sera generating an OD value at the point where ELISA curves were approximately parallel. The relative IgG concentration (μg/mL) was determined according to the dynamic range of the standard curve by interpolating the concentration of the standards that corresponds to the absorbance value at which the test sample gave approximately half of the O.D of the 95% of

the maximum O.D. of the standard. Ab titre was mostly determined by the last dilution that gave an average O.D. value of 1.5 for IgG Ab titre and 1 for IgM Ab titre.

2.14 Serum bactericidal assay (SBA)

2.14.1 Growth of bacteria to stationary and log phases

An overnight culture of *Salmonella* was prepared by adding one colony to 10 ml of LB broth and incubating at 37 °C in a tube with a loosened cap to reach the stationary phase. 100 µl of overnight culture were transferred to pre-warmed LB broth in a tightly capped and incubated on a rocker plate at 37°C for about two hours until OD_{600} ~ 0.2 was reached. After incubation, 1.5 ml was transferred to two Eppendorf tubes and cell pellets were washed 3 times in PBS, centrifuged for 5 minutes at 3,300 x g and finally re-suspended both Eppendorf tubes in 1 ml of PBS to create a stock inoculum. The final concentration of the inoculum was determined by serial plating and counting the colonies using Miles and Misra method (222).

2.14.2 Preparation of sera used in SBAs

In this assay, human serum with known *S*. Typhimurium anti-bactericidal Ab was used as a positive control, heat-inactivated (HI) human serum (Abs only), and depleted human serum with *Salmonella* (complement only) were used as negative controls. Human serum was heat-inactivated to inhibit complement function, and This was done by heat-inactivation (HI) at 56 °C in a water bath or hot plate for one hour prior to SBA. Depletion of *Salmonella*-specific antibodies in human serum was done by growing the same *Salmonella* serovar used for the assay in 100 ml LB broth at 37 °C overnight. The next day, bacteria were centrifuged, washed twice, and re-suspended in 1 ml PBS. For the depletion, 100 µl of the concentrated bacteria were added to 900 µl of human serum and incubated on a tube rotator at 40 rpm for 1 hour

at 4 °C. Serum was centrifuged at 3000 x g for 5 minutes, and the supernatant was then collected to repeat the same cycle for two more times. After the last cycle, serum was filtered using a small 0.22 µm filter unit and stored at -80 °C to be used later. HI serum and antibody-depleted human serum were added in a 1:1 proportion with PBS and used as a positive control because it contains both antibodies and complement source.

2.14.3 Analysis of mice sera in SBAs

Sera to be tested (immunised mice sera) were heat inactivated for 1 hour at 56 °C to inactivate complement and supplemented with antibody-depleted human serum as a complement source 1:1 ratio to give a final volume of 45 µl. This process was conducted due to the insufficient bactericidal activity of mice complement, requiring the supplementation of human complement to induce bacterial killing (168). Non-immunised mice sera were also inactivated and supplemented with human complement to indicate that anti- *S*. Typhimurium -OMV Abs were responsible for inducing killing after immunisation and not human complement. Stock inoculum of bacteria (prepared in section 2.15.1) was added to a concentration of 1:10 to human serum and the other samples in a round-bottomed 96 wells plate to a final volume of 50 µl per well. Plates were then incubated at 37°C on a rocker plate for time points 45, 90 and 180 min. At each point, 10 µl from each well was serially diluted and plated on LB agar plates. Agar plates were incubated at 37 °C overnight, and colonies were counted the day after and compared the starting inoculum concentration of the bacteria to the CFU concentration established at the different time points to determine the killing trend of sera.

2.15 Generation of monoclonal antibodies

Monoclonal antibodies (MAbs) were raised against outer membrane protein D (OmpD) of *Salmonella* Typhimurium in conjunction with Dr Margaret Goodall, Monoclonal antibody production unit, University of Birmingham. Female, young adult BALB/c mice were immunised and boosted intraperitoneally *(i.p)* with 20 µg of *S*. Typhimurium -OmpD on days 1 and 14. Each mouse was boosted for a second time with OmpD on day 21. Three days later, spleen cells were harvested, and cell fusions were carried out using NS0 cells (an immortal cell line derived from the non-secreting murine myeloma) using 50% polyethylene glycol (PEG) as described previously (223) with some modifications. Selection of these fused cells was done by using hypoxanthine-aminopterin-thymine (HAT) selective media and seeded into 96-well cell culture plates for 14 days. Plates were screened for viable clones, and hybridoma supernatants were screened by enzyme-linked immunosorbent assay (ELISA) against *S*. Typhimurium -OMPs, LPS and whole cells. Hybridomas of interest were selected for expansion and cloning, and supernatants were tested with western blot, ELISA, and SBA.

Monoclonal antibodies (MAbs) were also raised against a mixture of *S*. Typhimurium -OMVs from each of the three strains generated earlier and *S*. Typhimurium -OmpD. Female Balb/c mice were immunised and boosted i.p with 0.5 µg of *S*. Typhimurium -OMVs from each strain and 5 µg *S*. Typhimurium -OmpD on day 1 and day 14. Each mouse was boosted for a second time on day 21. Three days later, spleen cells were processed with the same method discussed above. Supernatants of the growth-positive wells were screened by ELISA for the production of IgG, IgM, and IgA against OMVs and OmpD.

2.16 SDS-PAGE and Western blot

For SDS-PAGE analysis, OMVs from the three strains (WT *S*. Typhimurium, *S*. Typhimurium Δ*wzy*, and *S*. Typhimurium Δ*wbaP*) were boiled for 10 minutes at 70 °C with NuPAGE LDS sample buffer and sample reducing agent (Invitrogen) on a heat block. Samples were loaded to a NuPAGE 4-12% gradient Bis-Tris gel (Invitrogen)and run at 200 V in NuPAGE MOPS SDS running buffer for 30 minutes. Gels were then washed in ultrapure water and stained with Coomassie Blue stain (Thermo Fisher) following the manufacturer's protocol or transferred to PVDF Immobilon-P transfer membrane (Merck) for Western Blotting using wet transfer System (Invitrogen) following manufacturer's guidelines. The blot was run at 30 V for 2 hours. Membranes were washed and then blocked with TBS (Tris-buffered saline) containing 5% powder milk (Marvel) for at least 1 hour at room temperature on a rotary platform. Membranes were probed with monoclonal antibodies raised against the *S*. Typhimurium OmpD, diluted 1:100 in blocking buffer and incubated at the same condition. After incubation, goat anti-mouse IRDye 800 IgG secondary antibody (Abcam) diluted in 1:15,000 was added and incubated at RT for another hour. Bands were then visualised by fluorescent detection using the ChemiDoc Imaging System from BIO-RAD, according to the manufacturer's instructions.

2.17 Statistical analysis

GraphPad Prism version 8 was used for data analysis. A normality test using the D'Agostino & Pearson test was conducted on each set of samples. Mann-Whitney U test was used every time, unless otherwise stated, to determine differences between two groups. Kruskall-

Wallace ANOVA with Dunn's post-hoc multiple comparisons test was used for comparison between multiple groups. $p \leq 0.05$ was used as the determinant of significance.

Chapter 3. IMMUNE RESPONSES TO OUTER MEMBRANE VESICLES WITH DIFFERENT LEVELS OF O-ANTIGEN EXPRESSION

3.1 Introduction

In Africa, invasive Non-Typhoidal *Salmonella* (iNTS) poses a substantial threat, contributing significantly to both mortality and morbidity, with patient death rates reaching approximately 20%. Deaths usually correlate with people with impaired immune systems, like those with HIV, and children under three (58). The primary culprits behind iNTS are *Salmonella* enterica serovars Typhimurium and Enteritidis, capable of infecting both humans and animals. While antibiotics prove effective in preventing *Salmonella* infections, challenges arise due to limited accessibility in certain regions and the diminishing efficacy caused by multidrug resistance. Consequently, there is an ongoing and pressing need to develop vaccines aimed at reducing the prevalence of this illness (216, 224). Despite the absence of licensed vaccines for iNTS prevention, promising strides are being made, with several vaccines currently in development and undergoing thorough investigation (225, 226).

Live attenuated *Salmonella* vaccines have been demonstrated to provide protection in mice against lethal infection with both S. Typhimurium and S. Enteritidis (227, 228). However, these are not appropriate for use in immune-compromised or elderly individuals (58). Protein subunit vaccines, such as porins or flagella, can induce protection against NTS infections in mice (12, 34). In addition, O-antigen-based conjugate vaccines have been tested in mice and shown to be protective against virulent *Salmonella* (209). However, O-antigen-based conjugate and protein subunit vaccines provide minimal protection against heterologous *Salmonella* serovars and have not yet been developed as human vaccines (12, 72).

Outer membrane vesicles (OMVs) are an alternative option for *Salmonella* vaccines, and they can include O-antigen. OMVs are spherical, nanoscale vesicles derived naturally as blebs from the surface of gram-negative bacteria (226). To increase blebbing, bacteria can be genetically engineered by deleting the *tolR* gene, which affects the stability of the linkage between the inner and the outer membrane (98, 108). OMVs are potent auto-adjuvants because they include a variety of antigens from the bacterial outer membrane, including membrane proteins and LPS. Several studies have been conducted on OMV-based vaccines derived from different Gram-negative bacteria and their ability to induce strong immunity and confer protection against bacterial challenges in animal models is established (229, 230, 231, 232, 233). In addition, OMVs from *Neisseria meningitidis* have been approved worldwide as a vaccine to prevent meningococcal serogroup B infection in humans (234). Furthermore, when OMVs from *S*. Enteritidis or *S*. Typhimurium were administered intraperitoneally or intranasally to mice, they could significantly activate humoral and mucosal immune responses and provide protection during iNTS (120, 235). Therefore, OMV-based vaccines are considered a possible candidate against multiple *Salmonella* serovars.

OMVs contain LPS, and O-antigen is the primary component in LPS that is the target for protective antibody responses in infections such as Shigellosis and NTS (72, 236). Nevertheless, in some cases, antibodies against O-Antigen are only effective in protecting and mediating killing if they do not exceed a certain level. In some HIV patients, excess anti-LPS antibodies are present and paradoxically may block and inhibit bacterial killing, and this has been observed for many Gram-negative pathogens (61). Therefore, controlling the extent of antibody levels to O-antigen may be important for vaccine development (61, 237).

O-antigen chain length is a potential parameter that can affect and alter the immunological response induced by vaccines (238). It has been shown that *Shigella sonnei* conjugates with low molecular mass O-specific polysaccharides (O-SP) induced significantly higher antibody levels in mice than the full-length O-antigen (239). Furthermore, better protection and increased anti-LPS Ab titres were seen in both mice and rabbits with *S*. Typhimurium O-SP conjugates of two or three repeating units compared to one repeating unit (240). These results indicate that any structural modifications of such components may impact the immunogenicity of the glycoconjugate vaccines. However, the effect of O-Antigen chain length on the Immunogenicity of *Salmonella* OMVs vaccines is still unclear. Therefore, the optimal length of the O-antigen chain should be assessed, which can be achieved through altering antigen expression levels. This can be achieved through the genetic deletion of enzymes such as *wbaP* which is responsible for the transfer of the first sugar residue by catalyses the transfer of galactose-1-phosphate onto undecaprenyl phosphate (Und-P). Deletion of this gene results in rough LPS consisting of a complete core and lacking O-antigen (18, 241, 242). The subsequent step in O antigen synthesis is dependent on *Wzx/Wzy* pathway, where sugars are sequentially added to form a complete Und-P-P-linked O antigen subunit. O antigen subunits are subsequently polymerised by the O-antigen ligase, encoded by the *wzy* gene to form multiunit O-antigen molecules. The *wzy* mutant results in LPS with a single O-antigen unit known as semi-rough LPS (243).

The overall aim of the work presented in this chapter is to examine the impact of O-antigen chain length in *S*. Typhimurium -OMVs on the immune responses induced with the antibody specificity, isotype, longevity, and serum bactericidal activity assessed in vitro. In addition, germinal centre formation (GC) and splenic antibody-secreting cells (ASC) were also analysed

and compared. For clarity throughout this project, the OMVs generated from *tolR* mutants are referred to as wild-type (WT)-OMVs, as they express LPS O-antigens of variable lengths. Additionally, wzy-OMVs or wbap-OMVs refers to OMVs purified from bacterial cultures of Δ*tolR* Δ*wzy* or Δ*tolR* Δ*wbaP* double-mutants respectively, which express LPS containing either a single or no O-antigen repeat.

3.2 Results

3.2.1 Construction of OMVs-producing *Salmonella* mutants

To control antibody levels to O-antigen as well as assess the potential of the O-antigen chain length of the OMV on the induced immune responses, OMVs were generated from WT *S*. Typhimurium (STm 14028 Δ*tolR*), *S*. Typhimurium 14028 Δ*wzy*, and *S*. Typhimurium 14028 Δ*wbaP*. WT *S*. Typhimurium carries a natural LPS structure, while other mutants have single O-antigen LPS and O-antigen deficient LPS, respectively. To increase OMV production through increased blebbing, these mutants were engineered by deleting the *tolR* gene. The *tolR* gene was deleted from both *S*. Typhimurium Δ*wzy* and *S*. Typhimurium Δ*wbaP* by replacing the open reading frame of the coding region with a kanamycin resistance gene flanked by Flippase recognition target sequences (Figure 3.1 A). The successful deletion of the *tolR* gene was confirmed by PCR amplification of the newly inserted gene. PCR products were then examined by gel electrophoresis along with the amplified *tolR* gene from the original mutants (STm Δ*wzy* and STm Δ*wbaP*) (Figure 3.1 B). The results showed that *S*. Typhimurium Δ*wzy* Δ*tolR* and *S*. Typhimurium Δ*wbaP* Δ*tolR* had different band sizes than the original mutants due to the replacement of the *tolR* gene with a kanamycin-resistant cassette.

OMVs from the different mutants were purified from cultures of OMV-producing strains and harvested from the culture supernatant. The filtrate was purified to separate the OMVs from other soluble proteins in the supernatant. Protein concentration in the OMVs was quantified by a bicinchoninic acid assay. The protein contents of the purified OMVs isolated from these mutants were detected by 12% SDS-PAGE, and the protein content was visualised by Coomassie blue staining of SDS-PAGE gels (Figure 3.1 C). The presence of the LPS in *S*. Typhimurium -OMVs was examined by silver staining (Figure 3.1 D). The LPS profile showed distinct LPS lengths that ranged from variable length O-antigen chains in the wild-type strain to single O-antigen units in the *wzy* mutant, to no O-antigen in the *wbaP* mutant.

Protein identification from the OMVs from each mutant was determined using mass spectrometry by our collaborators (Daniel Larsen and Jakub Kaczmarek, Institute of Biochemistry and Molecular Biology, University of Southern Denmark). Above 65% of the proteins identified, were found in all three mutants, while 78% were found in two out of three mutants. Thereby, showing a high level of conservation of proteins between the different OMV types, with OmpA and the trimeric porins included amongst commonly detected proteins in all OMV types. The details of these analyses are presented in Appendix C.

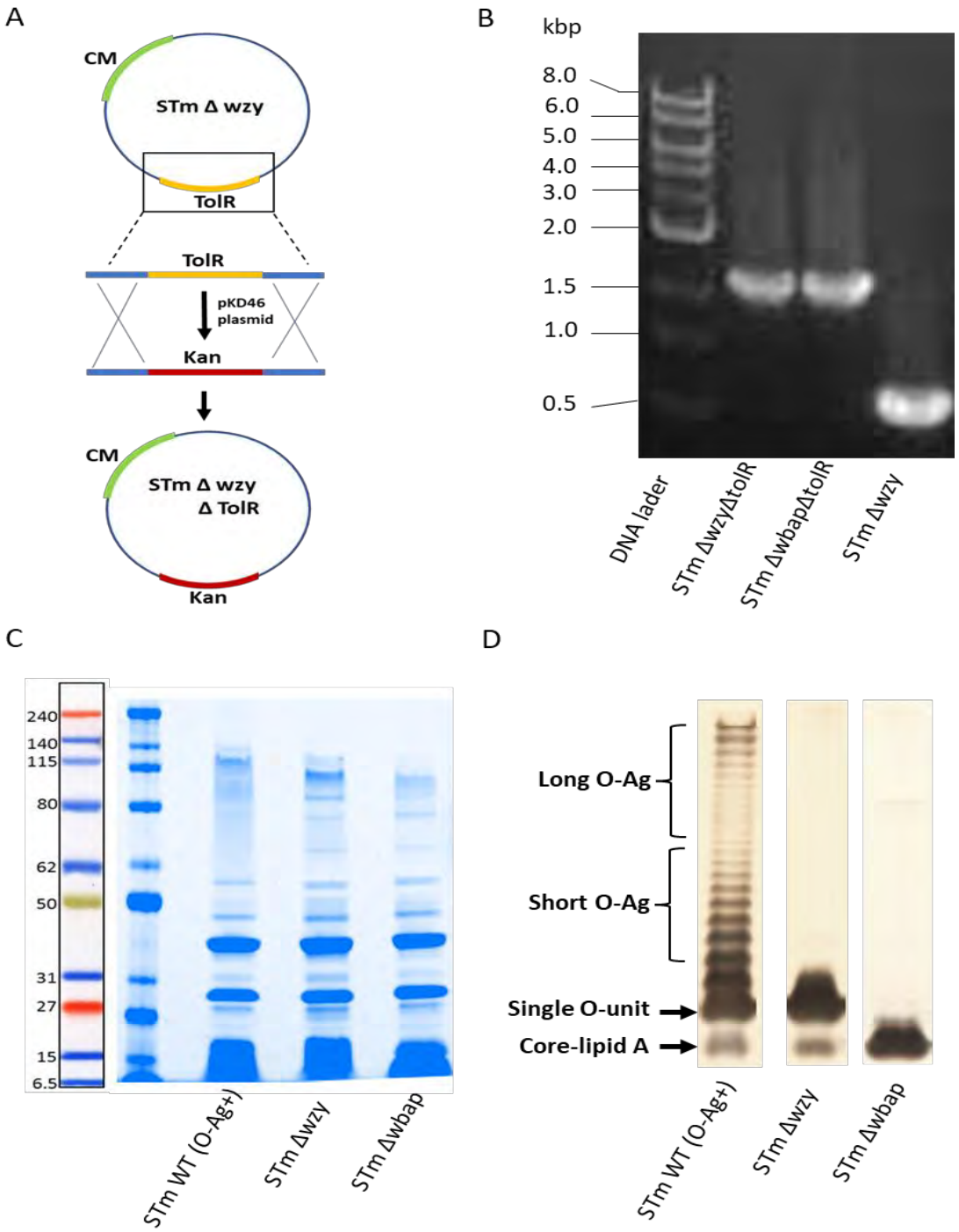


Figure 3.1 Construction of OMV-producing STm mutants and Characterization of OMVs from different STm mutants.

(A) Schematic overview of the process for generating mutants by deletion of the *tolR* gene to generate OMV-producing mutants and exchanging the gene of interest with a kanamycin resistance cassette (Kan) using the helper plasmid pKD46. (B) Confirmation of *tolR* gene deletion. Successful removal of *tolR* was verified by gel electrophoresis of PCR amplicons of the gene of interest. Sizes of internal kan cassette is 1500 bp in both STm ΔwzyΔtolR and STm ΔwbaPΔtolR. The size of *tolR* is 510 bp for STm Δwzy. (C) Proteins in each OMV samples from mutant strains separated by SDS-PAGE and stained with Coomassie blue. (D) Silver staining of lipopolysaccharide (LPS) derived from OMVs of the wild-type strain of STm and STm mutants separated on a 12% SDS-PAGE gel.

3.2.2 Assessment of immunogenicity of OMVs with different O-antigen Lengths

In vivo studies were conducted to evaluate and compare the immunogenicity of OMVs displaying O-antigen of varying lengths. Each experiment was repeated at least twice to ensure consistency. Purified OMVs were administered *i.p.* to CD1 mice at the same dose of 1 µg protein content, once or twice, at different times. A non-immunised control group was included as a negative control in each experiment.

3.2.2.1 Assessment of immune responses induced 21 days post-primary immunisation with *S*. Typhimurium -OMV

Female CD1 mice were immunised once with 1 µg WT, wzy, or wbaP-OMV, or PBS as a negative control for 21 days. Serum IgM and IgG specific for different *S*. Typhimurium antigens and splenic/bone marrow antibody-secreting cells (ASCs) were quantified. Germinal centre and extrafollicular responses to OMV vaccines were also assessed.

3.2.2.1.1 Serum antibody responses vary depending on the O-antigen chain length of the OMV

Serum IgM and IgG specific for LPS, porins, and the three OMVs (derived from WT, *wzy* mutant, and *wbaP* mutant) were assessed by ELISA and presented as relative titres (Figure 3.2). After immunisation with WT and wzy-OMV, significantly more anti-LPS IgM was induced than after immunisation with wbaP-OMV (Figure 3.2 A). The reverse was observed for IgM to porins. Typically, after immunisation, IgM responses were highest to the matching OMV type but were most similar for the WT and wzy groups. The matching IgG responses showed some key differences (Figure 3.2 B). The pattern of IgG responses induced to WT-OMV matched that for IgM. In contrast, after immunisation with wzy-OMV, IgG responses to LPS O-Ag containing

antigens remained similar to those induced to WT-OMV, but to porins and wbap-OMV, IgG responses were more similar between the wzy-OMV and wbap-OMV groups. Thus, a single O-antigen repeat (*wzy*-OMV) can induce a comparable anti-LPS response as WT-OMV.

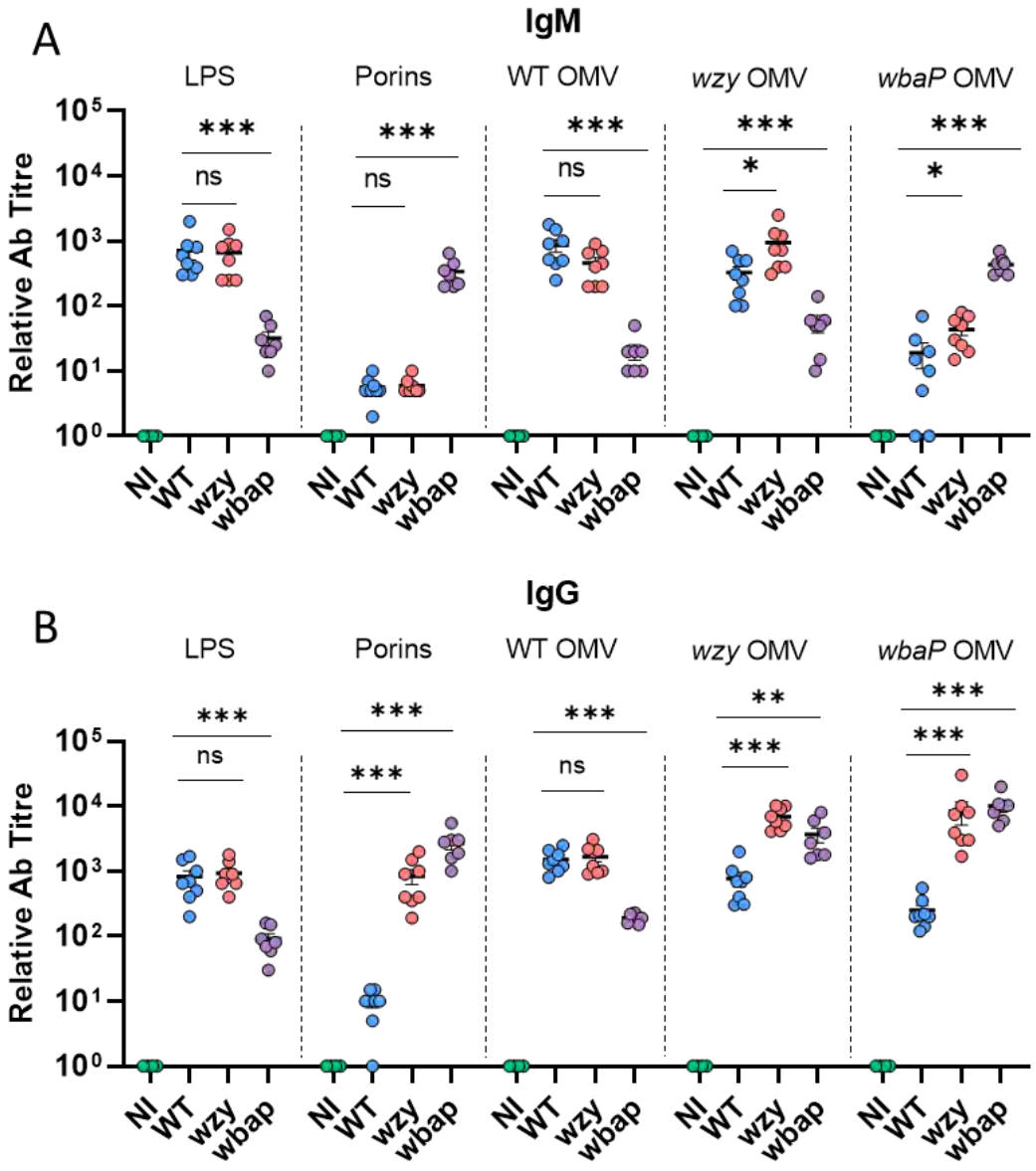


Figure 3.2 Serum IgG and IgM antibody titres 21 days after primary immunisation with WT, wzy, or wbaP-OMV.

CD1 mice were immunised i.p. with 1 µg WT, wzy, wbaP-OMV or PBS for 21 days. (A) Relative IgM-antibody titres specific for LPS, porins, or OMVs (tolR/wzy/wbap), quantified by ELISA in sera from each group. (B) Relative IgG- antibody titres specific for LPS, porins, or OMVs (tolR/wzy/wbap), quantified by ELISA in sera from each group. Representative of 2 experiments where n=4 mice/group. *= p≤0.05, ** = p<0.01 and *** = p<0.005.

To evaluate the effect of O-Antigen length on the IgG isotype distribution, IgG subclasses were assessed against wzy-OMV and wbaP-OMV (Figure 3.3). All IgG isotypes were detected at day 21 against wzy-OMV and wbaP-OMV in all mice groups and the isotype distribution was similar to both antigens (Figure 3.3 A and B).

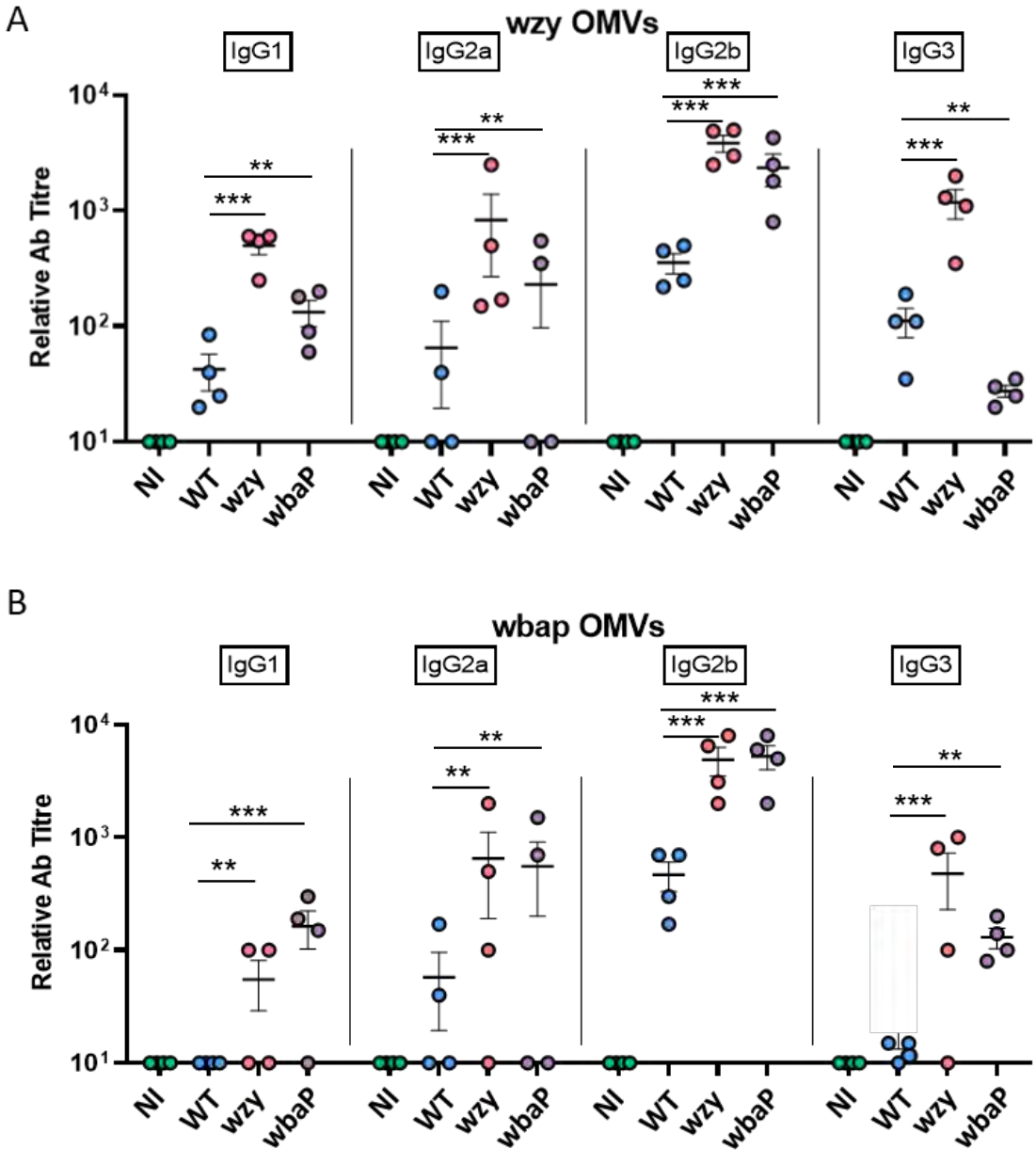


Figure 3.3 Serum antibody IgG isotype distribution of the different STm-OMVs immunised mice.

Antibody titres for IgG subclasses specific to (A) wzy-OMV and (B) wbaP-OMVs was detected in sera from non-immunised (NI) mice or mice immunised with WT, wzy, or wbaP-OMVs. Each dot represents one mouse with n=4 mice/group.

To complement the serum antibody analysis, IgM+ and IgG+ antibody-secreting cells (ASC) specific to LPS and porin were determined by ELISPOT in both spleen and bone marrow post-immunisation (Figure 3.4). The results here paralleled the serum antibody responses. Anti-LPS IgM and IgG ASCs were detected at a higher level in WT and wzy-OMV immunised mice in both spleen and bone marrow than after wbaP-OMV immunisation (Figure 3.4 A and B). However, WT-OMV immunised mice had lower anti-porin IgM and IgG ASCs in both spleen and bone marrow than wzy and wbaP-OMV immunised mice. Collectively, these results indicate that altering the O-antigen chain length of the OMV can affect the anti-LPS or anti-surface porins responses.

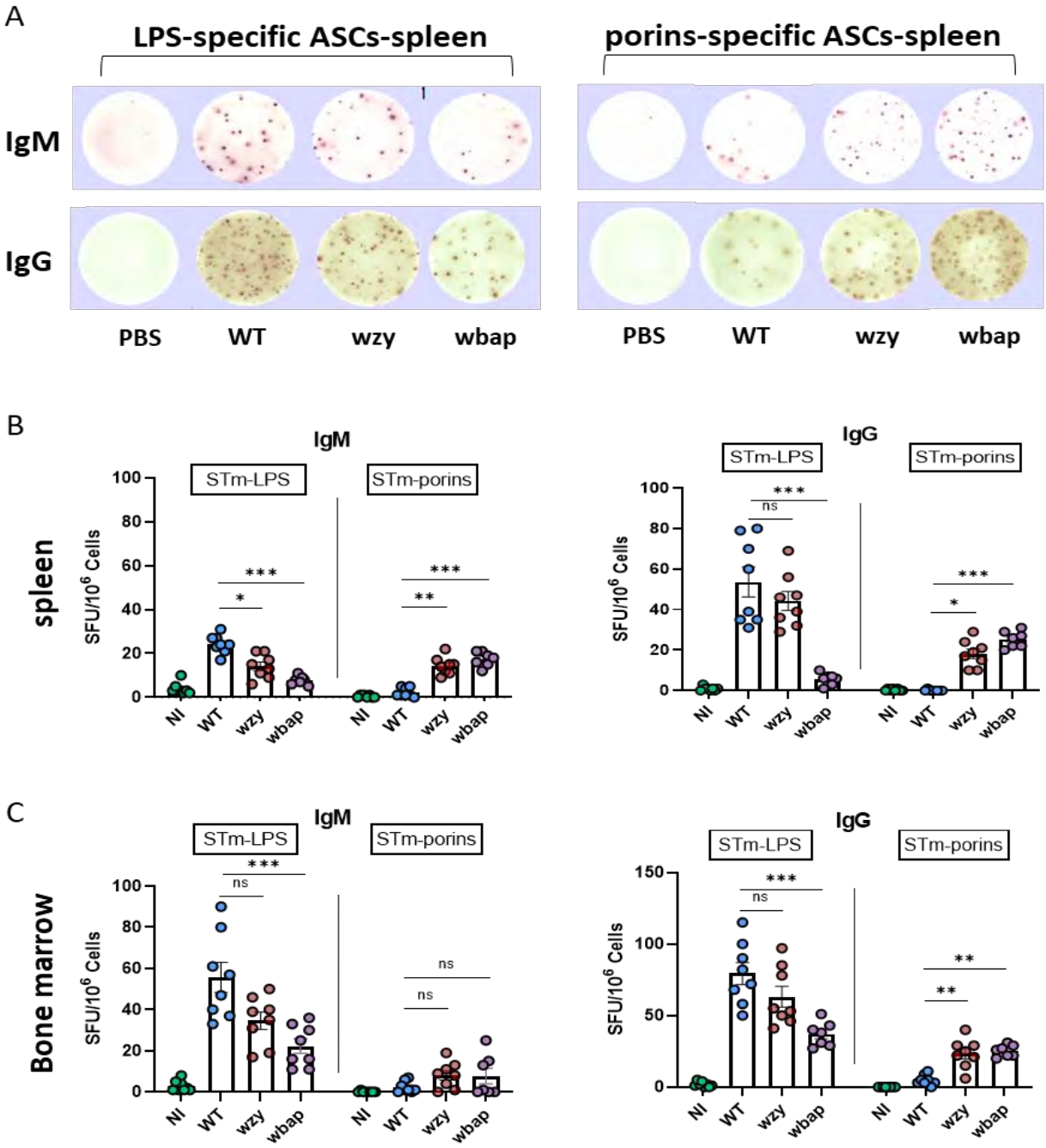


Figure 3.4 IgM+ and IgG+ antibody-secreting cells (ASC) specific to LPS and porins 21 days after STm-OMVs immunisation in mice.

Anti-LPS and anti-porins ASCs were quantified by ELISPOT in the spleen and bone marrow. (A) representative ELISPOT wells of the assessed spleen for anti-LPS and anti-porins IgM ASC (top), and anti-LPS and anti-porins IgG ASC (bottom). (B) Splenic IgM+ (left) IgG+ (right) ASC against LPS and porins in non-immunised (NI) and immunised mice with the three OMVs of different O-Ag structures. (C) Bone marrow IgM+ (left) IgG+ (right) ASC against LPS and porins in the same mice group. Each dot represents one mouse. Data is representative of two independent experiments where n=4 in each experiment. SFU= spot-forming units.

3.2.2.1.2 Reduced O-antigen expression in OMV enhances induction of IgM and IgG cross-reactive antibodies

Besides assessing antibody responses against individual antigens, serum antibody responses to the whole organism of *S*. Typhimurium were also measured (Figure 3.5). Intact inactivated *S*. Typhimurium was used to coat an ELISA plate, and the binding of antibodies in sera from the immunised mice was assessed. Results showed significantly higher IgM Abs levels were induced to WT-OMV immunisation compared to wzy-OMV, although IgG responses against whole *S*. Typhimurium bacteria were similar between these groups (Figure 3.5 A). There was minimal IgM and IgG binding of antibodies in sera from the wbaP-OMV immunised group. These results indicated that shortening O-antigen length may affect IgM binding to *S*. Typhimurium. The level of cross-reactivity of antibodies against *S*. Enteritidis, which expresses O9 rather than the O4, was also assessed (Figure 3.5 B). The binding of IgM and IgG to the whole *S*. Enteritidis was considerably higher when the O-antigen had been reduced or removed (wzy or wbaP) compared to the full-length O-antigen chain in the WT-OMV. Therefore, reduced O-antigen expression in OMVs can alter the Abs binding level and enhance the cross-reactivity level with different serovars.

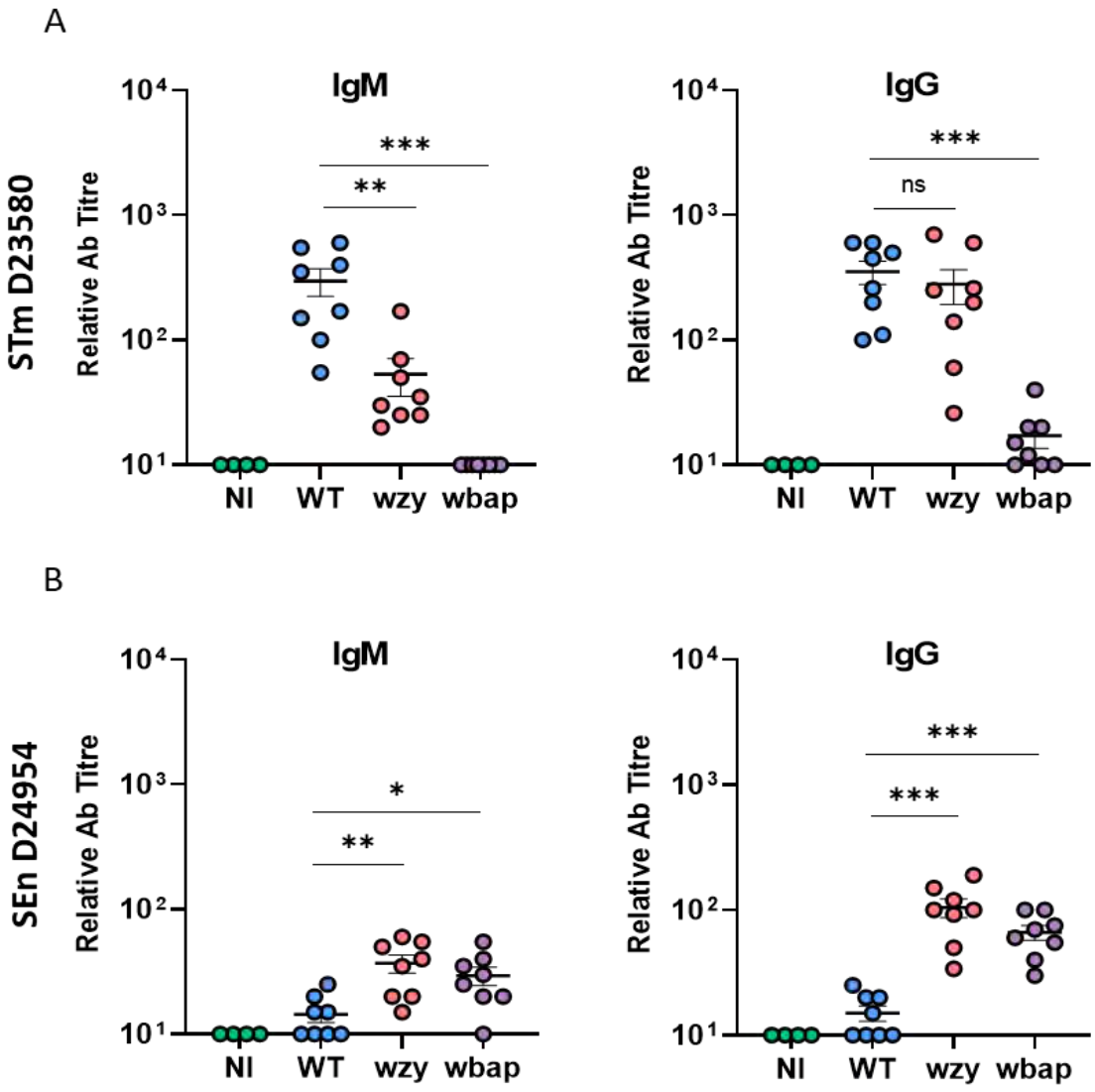


Figure 3.5 Serum antibody titres against whole bacteria of *Salmonella* Typhimurium (STm) and *Salmonella* Enteritidis (SEn).

Serum antibody responses were assessed by ELISA. (A) IgM and IgG antibody responses to *S*. Typhimurium in sera from mice immunised with the OMVs or Non immunised mice (NI). (B) IgM and IgG antibody responses to *S*. Enteritidis in sera from mice immunised with the OMVs or Non immunised mice. Representative of 2 experiments where n=4 mice/group. *= p≤ 0.05, ** = p ≤ 0.01, and *** = p ≤ 0.005.

3.2.2.1.3 All OMVs induce similar germinal centre and plasma cell responses 21 days after immunisation

As a part of the investigations into the effect of a reduced or absent O-antigen level on the primary immune response to OMVs, the B cell germinal centre and total IgM+ and IgG+ plasma responses in the spleen were examined by flow cytometry (Figure 3.6). As shown in the gating strategy, B cells were identified as CD19+ B220+ cells (Figure 3.6 A). After immunisation with the three different OMVs, there were similar proportions of total B cells in the spleen between the groups (Figure 3.6 B). After assessing germinal centre responses induced post-immunisation, it was found that the proportion and number of GC cells increased significantly after immunisation with WT, wzy, or wbaP-OMV compared to non-immunised controls. (Figure 3.6 C and D).

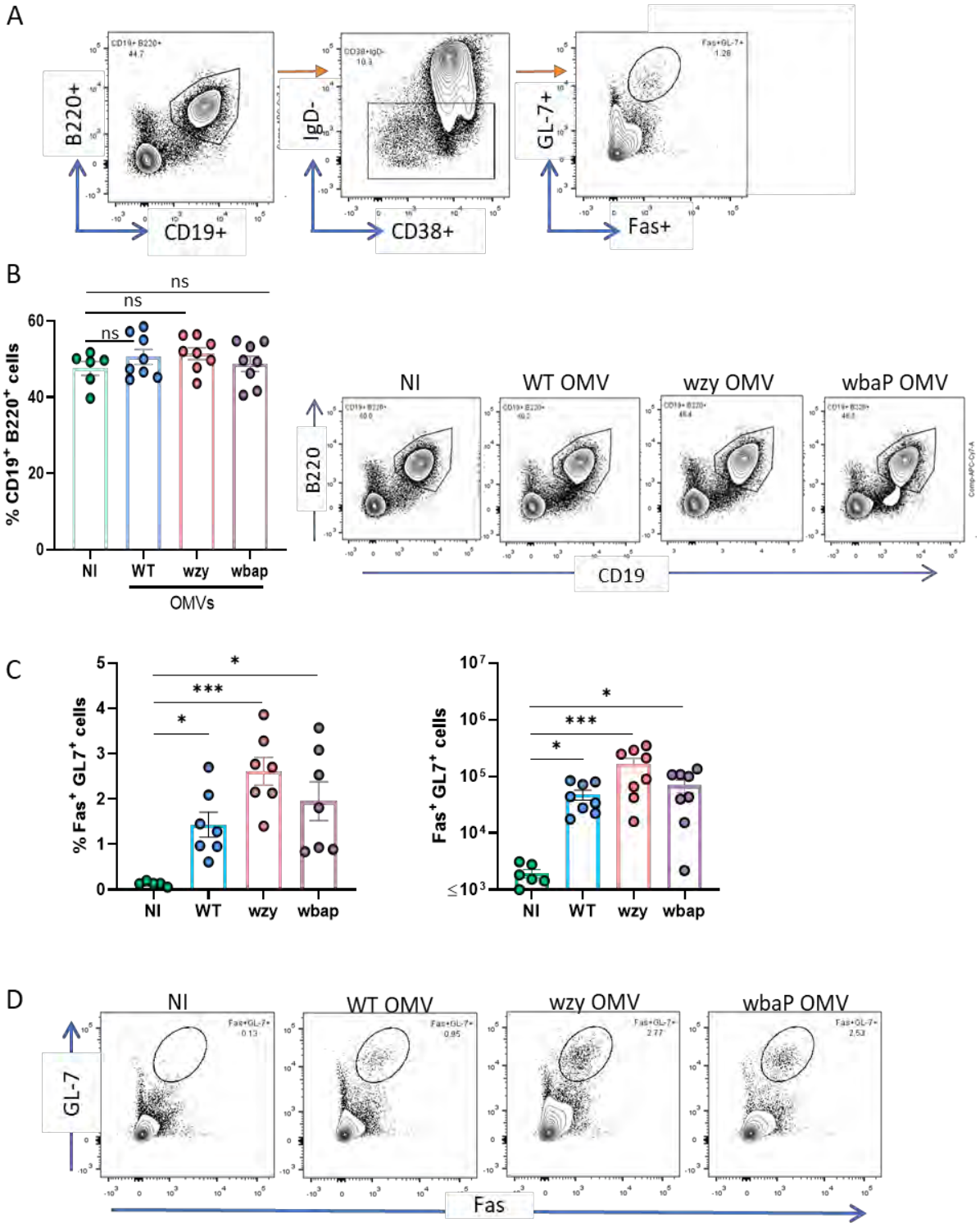


Figure 3.6 Immunisation with WT, wzy, or wbaP-OMV induces a B cell response and formation of germinal centre (GC) B cells after 21 days.

(A) Representative FACS Gating for B cells and GC. GC B cells were gated on Fas+ and GL7+. (B) Proportion of CD19+ B220+ B cells in the spleen post immunisation (left) and representative FACS plots the B cell population in the spleen from immunised and NI mice. (C) Proportion (left) and total number (right) of FAS+ GL7+ GC B cells. (D) Representative FACS plots for Fas+ GL7+ GC B cells in the spleen from immunised and NI mice. Representative of 2 experiments where n=4 mice/group. *= p≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.005, ns= non-significant.

The total ASCs (plasmablasts and plasma cells gated on CD138+ TACI+ cells) were also analysed (Figure 3.7 A). Results showed significant differences in the proportion of total ASCs between immunised and non-immunised mice, with no differences between the three vaccinated groups (Figure 3.7 B). Furthermore, immunisation with OMVs did not result in significant changes in total intracellular IgM+ and IgG+ plasma cells compared to non-immunised mice (Figure 3.7 C), but there was a notable change in the proportion of plasmablast cells that highly expressed intracellular IgM and IgG compared with the non-immunised controls (Figure 3.7 D and E). Thus, all OMVs induce similar GC and plasma cell responses, regardless of their O-antigen level.

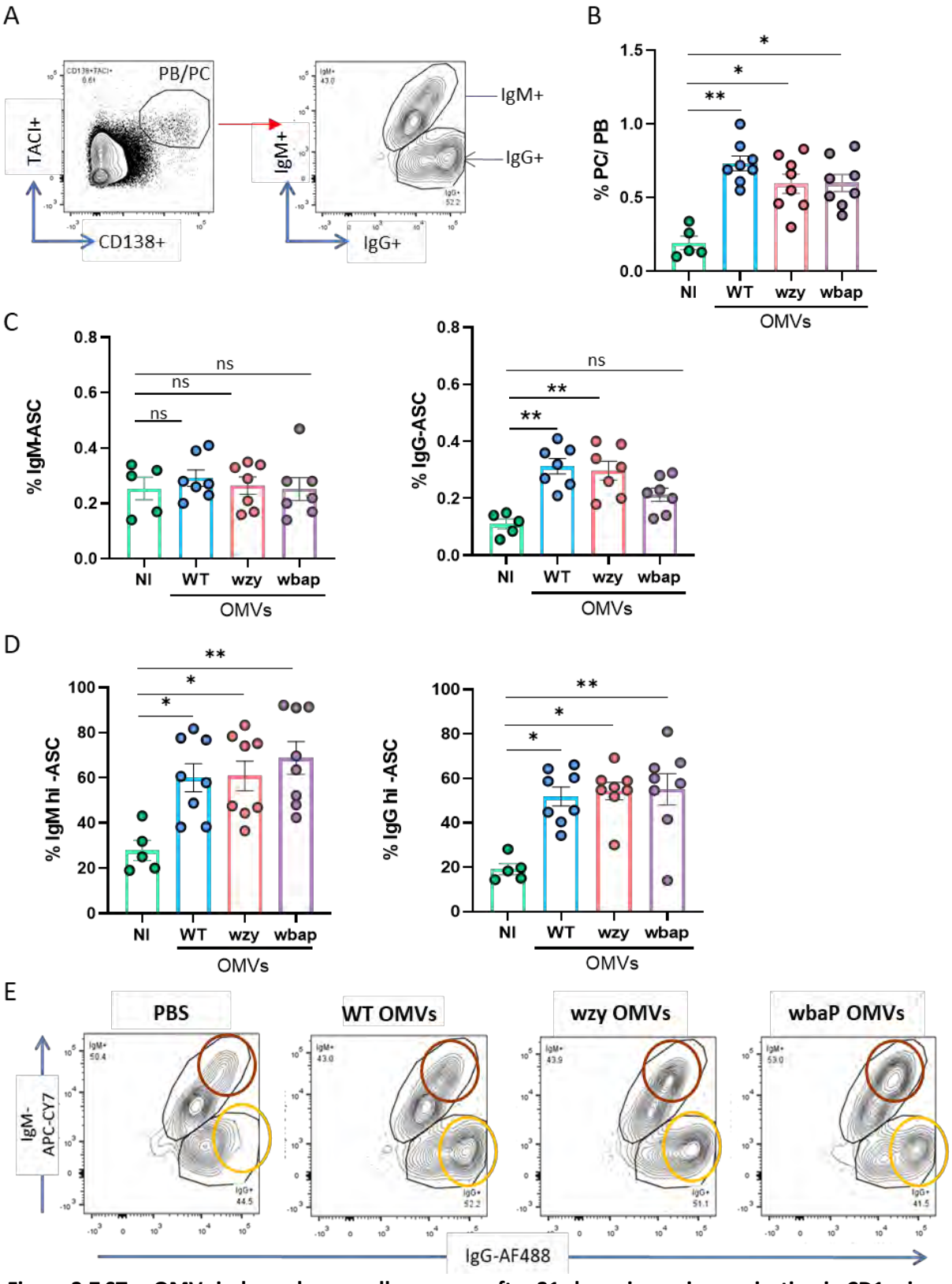


Figure 3.7 STm-OMVs induce plasma cell response after 21-day primary immunisation in CD1 mice.

(A) Plasmablast (PB)/plasma cells (PC) were gated on CD138+ TACI+ and stained for intracellular IgM and IgG. (B) proportion of PB /PCs after immunisation. (C) Proportion of intracellular IgM and IgG. (D) Proportion of IgM and IgG intracellular high population. (E) Representative FACS plots for Plasma cells stained for intracellular IgM and IgG which were gated on plasma cells in panel A. *= p≤ 0.05 and ** = p ≤ 0.01.

3.2.2.2 Assessment of the immune responses induced 35 days post-primary immunisation with *S*. Typhimurium -OMV

The kinetics of the immune response to OMV is unclear. Understanding how the OMV vaccination schedule affects the immune responses elicited to improve vaccine immunogenicity is essential. For this reason, female CD1 mice were immunised once for 35 days with 1 µg WT, wzy, or wbaP-OMV, or PBS as a negative control. Serum IgM and IgG specific for different *S*. Typhimurium antigens and splenic/bone marrow antibody-secreting cells (ASCs) were quantified. Germinal centre and extrafollicular responses to OMV vaccines were also assessed.

3.2.2.2.1 Serum antibody and Ag-specific plasma cell responses at day 35 following OMV immunisation are comparable to those at day 21

Serum IgM and IgG specific for LPS, porins, and the three OMVs (derived from WT, *wzy* mutant, and *wbaP* mutant) were assessed by ELISA as was shown in section 3.2.2.1.1. The results showed that immunisation with the WT and wzy-OMVs induced significantly more anti-LPS IgM compared to wbaP-OMVs immunisation, while anti-porin IgM was more induced in response to wzy and wbaP-OMVs immunisation (Figure 3.8 A). Moreover, WT and wzy-immunised mice induced more anti-WT OMV and anti-way OMV compared to wbaP-immunised mice. In contrast, wbaP-immunised mice induced more anti-wbaP OMV than the other two immunised groups. Immunisation with WT and wzy-OMVs also led to higher anti-LPS IgG responses than wbaP-OMVs immunisation (Figure 3.8 B) whereas anti-porin IgG was higher in the wzy and wbaP-OMV groups. It is also notable that immunisation with wzy-OMV was able to induce IgG antibodies against the three OMVs.

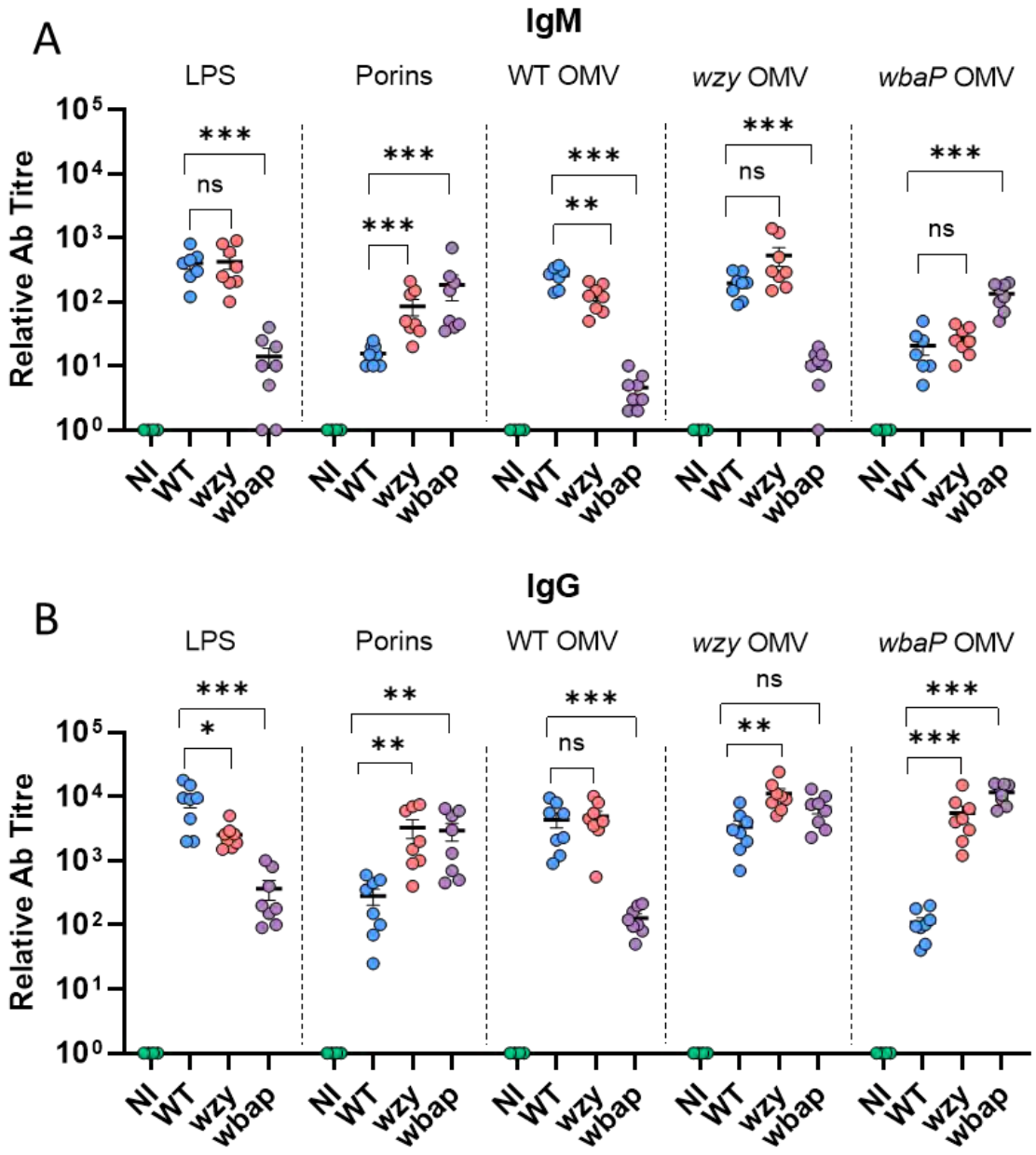


Figure 3.8 Serum IgG and IgM antibody titres 35 days after primary immunisation with WT, wzy, or wbaP-OMV.

CD1 mice were immunised i.p. with 1 µg WT, wzy, wbaP-OMV or PBS for 35 days. (A) Relative IgM antibody titres specific for LPS, porins, or OMVs (WT/wzy/wbap), quantified by ELISA in sera from each group. (B) Relative IgG antibody titres specific for LPS, porins, or OMVs (WT/wzy/wbap), quantified by ELISA in sera from each group. Representative of 2 experiments where n=4 mice/group. * = $p \leq 0.05$, ** = $p \leq 0.01$ and *** = $p \leq 0.005$.

Additionally, ELISPOT analysis was performed to determine the number of IgM+ and IgG+ antibody-secreting cells (ASCs) specific to LPS and porin in spleen and bone marrow post-immunisation with the three different OMVs (Figure 3.9). Results showed that immunisation with WT and wzy-OMVs led to a higher level of anti-LPS IgM and IgG ASCs in both spleen and bone marrow compared to wbaP-OMVs immunised mice. In contrast, immunisation with WT-OMVs resulted in lower anti-porin IgM and IgG ASCs than wzy and wbaP-OMVs in the spleen and bone marrow. Overall, the results were comparable to those obtained following a 21-day immunisation.

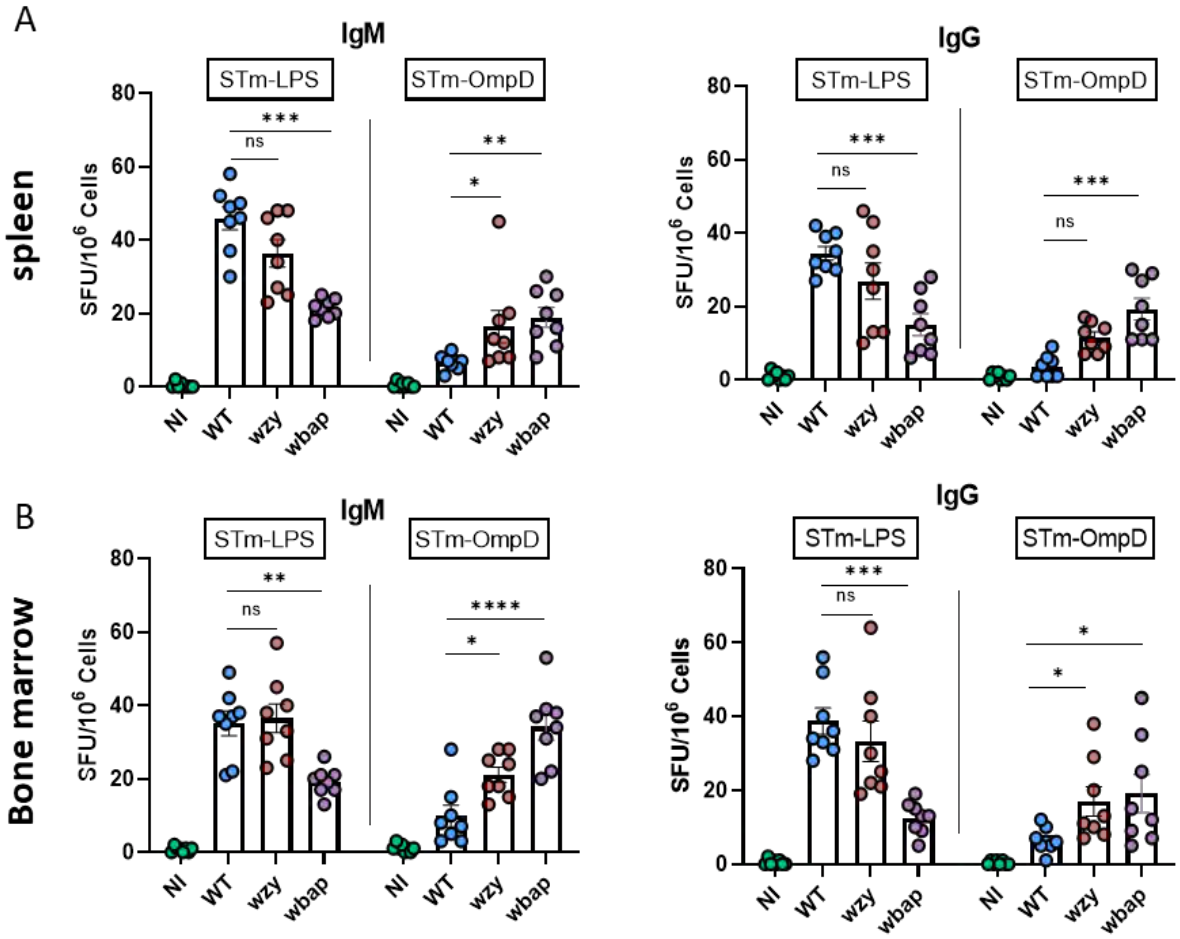


Figure 3.9 IgM+ and IgG+ antibody-secreting cells (ASC) specific to LPS and porins 35 days after primary immunisation with OMVs.

Anti-LPS and anti-porins ASCs were quantified by ELISPOT in the spleen and bone marrow. (A) Splenic IgM+ (left) IgG+ (right) ASC against LPS and porins in non-immunised (NI) and immunised mice with the three OMVs of different O-Ag structures. (B) Bone marrow IgM+ (left) IgG+ (right) ASC against LPS and porins in the same mice group. Each dot represents one mouse. Data is representative of two independent experiments where n=4 in each experiment. SFU= spot-forming units.

3.2.2.2.2 All OMVs induce similar germinal centre and plasma cell responses 35 days after of immunisation

To investigate the impact of extending the OMV- immunisation time on the primary immune response to the different OMVs, the B cell germinal centre and total IgM+ and IgG+ plasma responses in the spleen were examined by flow cytometry (Figure 3.10). Following

immunisation with any of the three distinct OMVs, the proportion of total B cells in the spleen did not differ between immunised and non-immunised mice. (Figure 3.10 A). However, the proportion and number of GC cells increased significantly after immunisation with WT, wzy, or wbaP-OMV compared to non-immunised controls (Figure 3.6 B). Immunisation with OMV for 35 days induced no or minor changes in total intracellular IgM+ and IgG+ plasma cell proportions (Figure 3.10 D), but a more noticeable difference in the proportion of plasma (blast) cell numbers that were high in the expression of IgM or IgG was observed (Figure 3.10 E). Therefore, GC and plasma cell responses are similar after 21 and 35 days of immunisation.

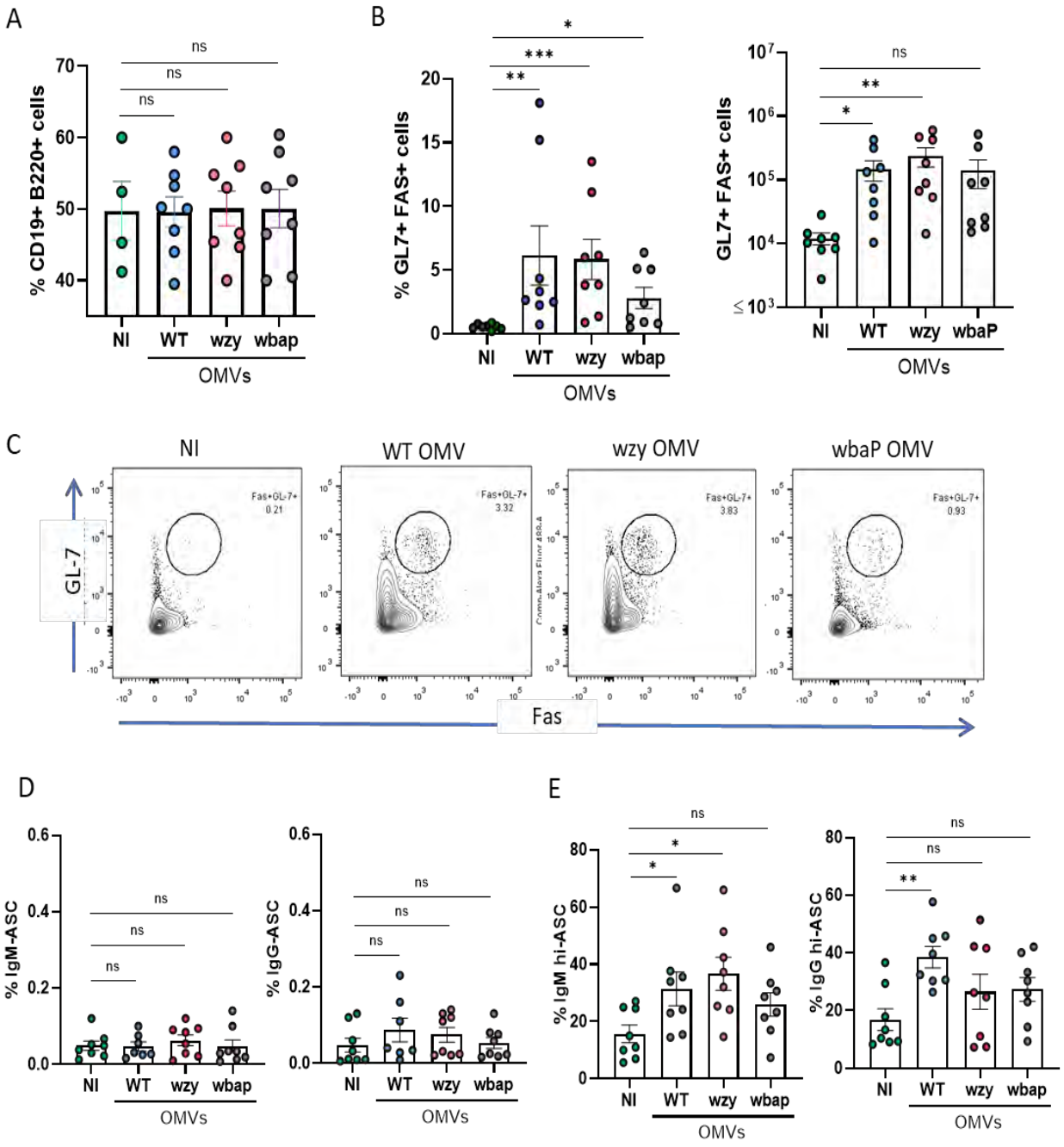


Figure 3.10 Immunisation with WT, wzy, or wbaP-OMV induces a B cell response and formation of germinal centre (GC) B cells after 35 days.

(A) Proportion of CD19+ B220+ B cells in the spleen post immunisation. (B) Proportion (left) and total number (right) of FAS+ GL7+ GC B cells. (C) Representative FACS plots for Fas+ GL7+ GC B cells in the spleen from immunised and NI mice. (D) Proportion of intracellular IgM and IgG. (D) Proportion of IgM and IgG intracellular high population. Representative of 2 experiments where n=4 mice/group.

3.2.2.3 Assessment of the immune responses induced 21 days post-secondary immunisation with different OMVs

Several vaccine-related factors, such as the number of vaccinations, are known to impact the overall adaptive immune response. To optimise OMV, immunisation schedules require a deeper understanding of how the immune system responds to multiple vaccine encounters. Multiple doses may generate longer-lasting protective immunity, no least through boosting antibody responses. In this experiment, female CD1 mice were primed and boosted intraperitoneally with 1 μg WT, wzy, or wbaP-OMV, or PBS as a negative control, as described below (Figure 3.11 A). Serum IgM and IgG specific for different *S*. Typhimurium antigens and splenic/bone marrow antibody-secreting cells (ASCs) were quantified. Germinal centre and extrafollicular responses to OMVs vaccines were also assessed. Moreover, the immunogenicity of OMVs following primary and secondary immunisations was compared.

3.2.2.3.1 Enhanced antibody responses are induced in mice primed and boosted with all different OMVs

Serum responses to LPS, porins, and OMVs in mice immunised twice with the different OMVs were examined. ELISAs showed that after boosting there was a significant increase in anti-LPS IgM and IgG Ab titres even with the wbaP immunised mice, but the responses to LPS in the latter group remained less than in the two other OMV groups (Figure 3.11 B and C). Results also showed that anti-porins IgM and IgG levels were remarkably higher in response to wzy and wbaP-OMV immunisation compared to WT-OMV immunisation (Figure 3.11 B and C). Furthermore, there were enhanced levels of IgM and IgG against all OMVs in immunised mice.

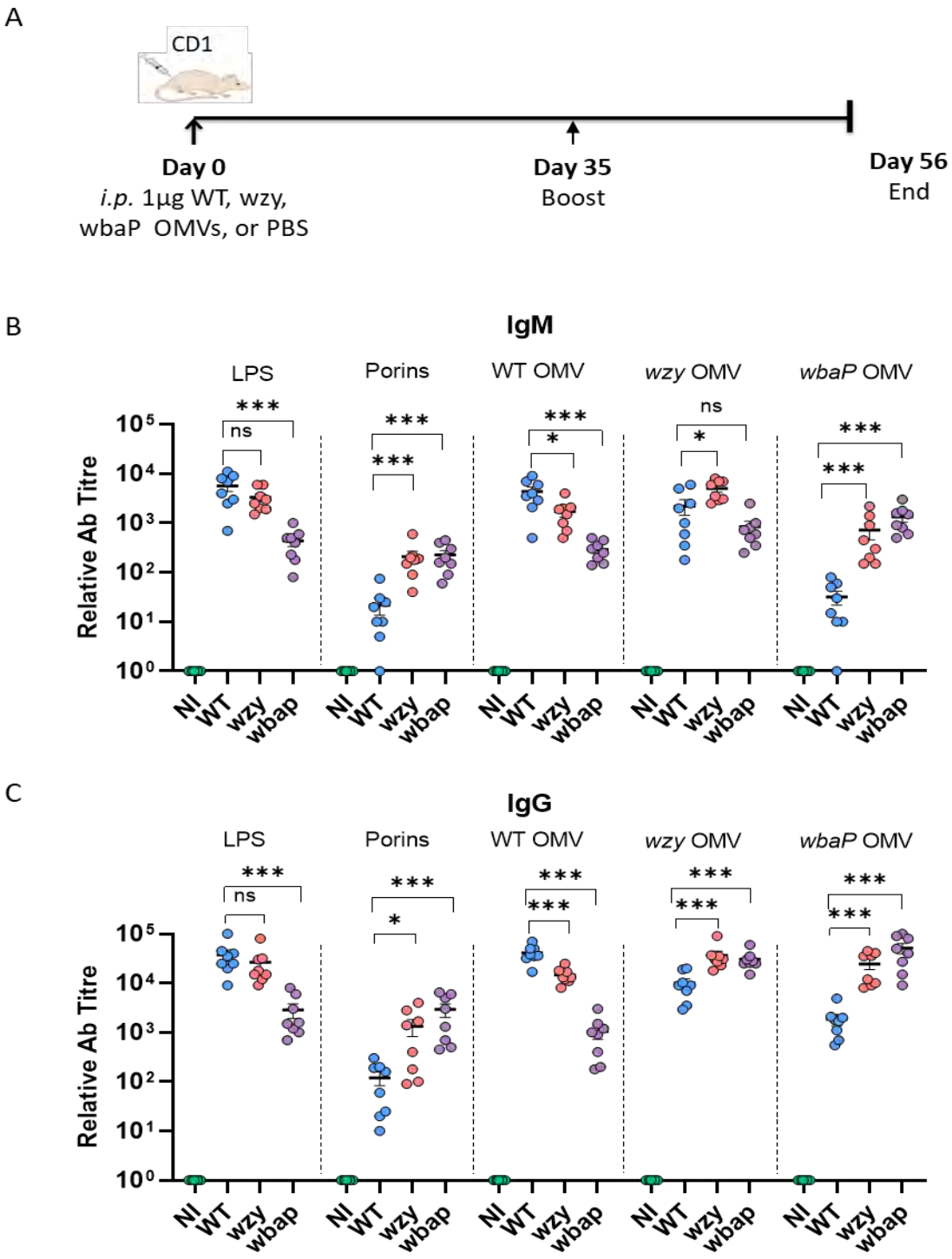


Figure 3.11 Serum IgM and IgG antibody titres 21 days after secondary immunisation with WT, wzy, or wbaP-OMV.

(A) Schematic representation of immunisation protocol. CD1 mice were immunised i.p. at days 0 and 35 with 1 µg WT, wzy, or wbaP-OMV. Mice were sacrificed 21 days post-secondary immunisation. (B) Relative IgM antibody titres specific for LPS, porins, or OMVs (WT/wzy/wbap), quantified by ELISA in sera from each group. B) Relative IgG antibody titres specific for LPS, porins, or OMVs (WT/wzy/wbaP), quantified by ELISA in sera from each group. Representative of 2 experiments where n=4 mice/group. * = $p \leq 0.05$ and *** = $p \leq 0.005$

When serum antibody responses to LPS and porins were compared between primary and secondary immunisations (Figure 3.12), it was noted that similar anti-LPS IgM or IgG responses were detected after immunisation with wt or wzy-OMV (Figure 3.12 A and C) and these were higher than after immunisation with wbaP-OMV. In contrast to the anti-LPS response, the anti-porin IgM and IgG responses were more similar between the wzy and wbaP-OMV groups than to the WT-OMV group (Figure 3.12 B and D). Thus, wzy-OMV induce anti-LPS responses similar to those induced by WT-OMV, but anti- porin responses more similar to those induced by wbaP-OMV.

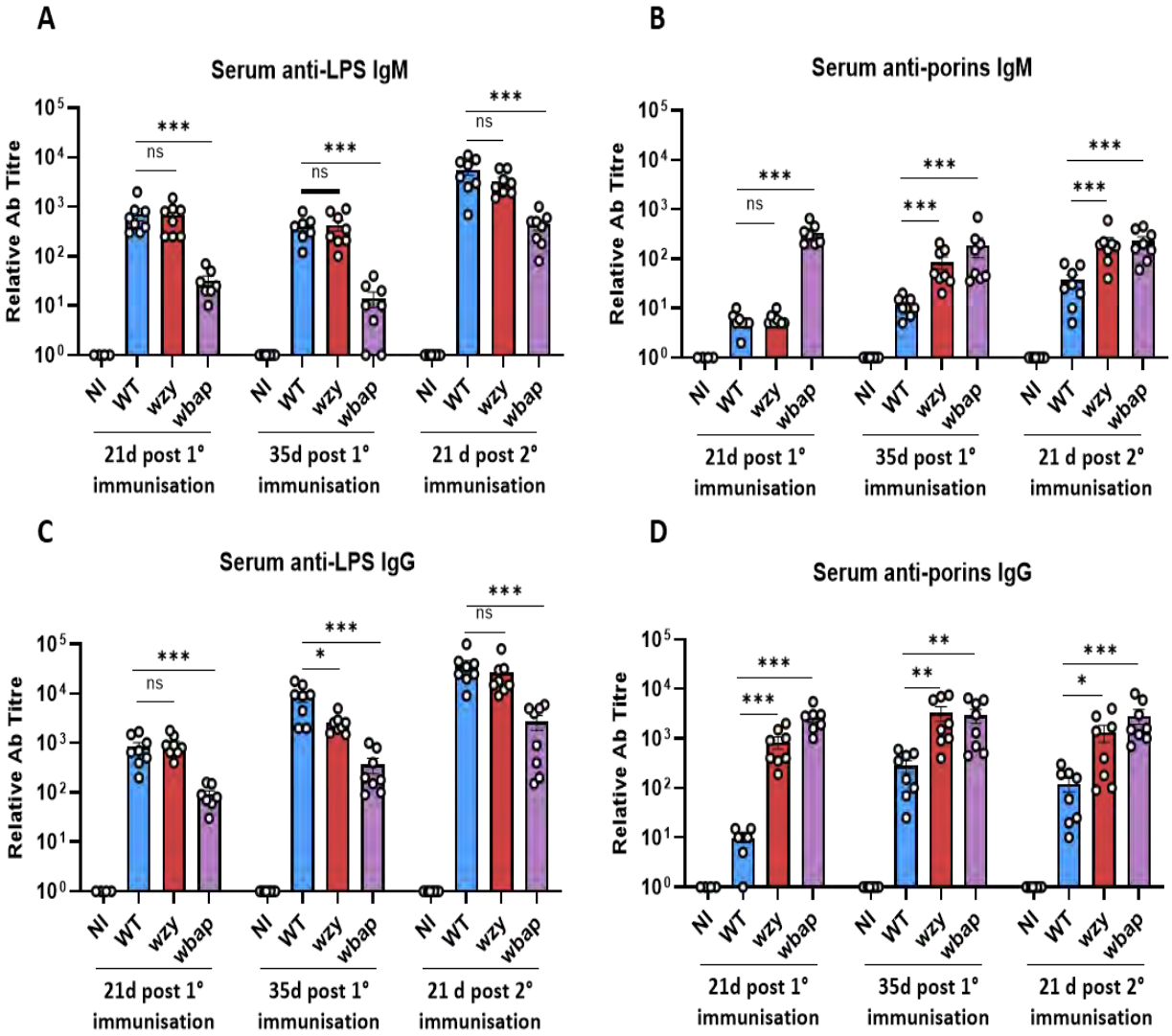


Figure 3.12 Comparison of serum antibody titres against STm-LPS and STm-porins between mice immunised once or twice with OMVs.

CD1 WT mice were immunised *i.p*. once or twice with 1 µg STm-OMVs of different O-Ag structures. Serum IgM responses were assessed by ELISA against STm-LPS and STm-porins (A and B). Relative serum anti-LPS and porin IgG titres (C and D) were assessed in sera from non-immunised and immunised mice. Each dot represents one mouse and data is representative of 2 experiments where n=4 mice/group.

Frequencies of LPS and porin-specific IgM and IgG-expressing antibody-secreting cells (ASC) were determined by ELISPOT in the spleen and bone marrow post-secondary immunisation (Figure 3.13). In both the spleen and BM, mice immunised with wbaP-OMV typically had lower levels of anti-LPS IgM+ and IgG+ ASC than mice immunised with WT and wzy-OMV, which induced more similar anti-LPS ASC responses. Conversely, mice immunised with wzy-OMV or wbaP-OMV typically had a higher level of anti-porins IgM+ and IgG+ ASC.

Antigen-specific plasma cell responses to LPS and porins were compared after primary and secondary immunisations (Figure 3.14). In both the spleen and the bone marrow, IgM+ and IgG+ ASC were detected against LPS and porins post-immunisation with any of the OMV types. Mice immunised with wbaP-OMV typically had lower median frequencies of anti-LPS IgM+ and IgG+ ASC than mice immunised with WT and wzy-OMV, which induced more similar median anti-LPS ASC responses. Mice immunised with wzy-OMV or wbaP-OMV typically had a higher frequency of anti-porin IgM+ and IgG+ ASC. In all cases, the highest median ASC responses against LPS or porin were observed after boosting.

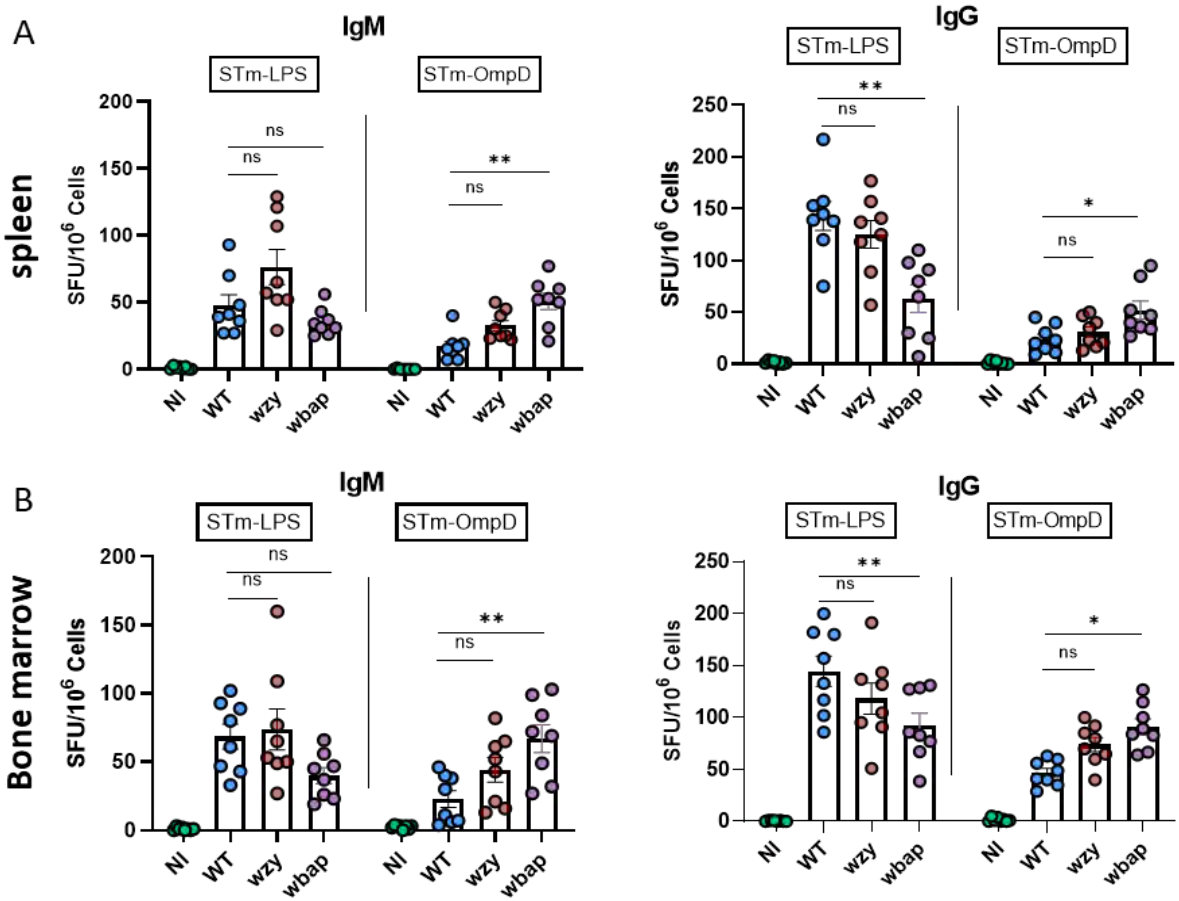


Figure 3.13 IgM+ and IgG+ antibody-secreting cells (ASC) specific to LPS and porins 21 days after secondary immunisation with OMVs.

Anti-LPS and anti-porins ASCs were quantified by ELISPOT in the spleen and bone marrow. (A) Splenic IgM+ (left) IgG+ (right) ASC against LPS and porins in non-immunised (NI) and immunised mice with the three OMVs of different O-Ag structures. (B) Bone marrow IgM+ (left) IgG+ (right) ASC against LPS and porins in the same mice group. Each dot represents one mouse. Data is representative of two independent experiments where n=4 in each experiment. SFU= spot-forming units.

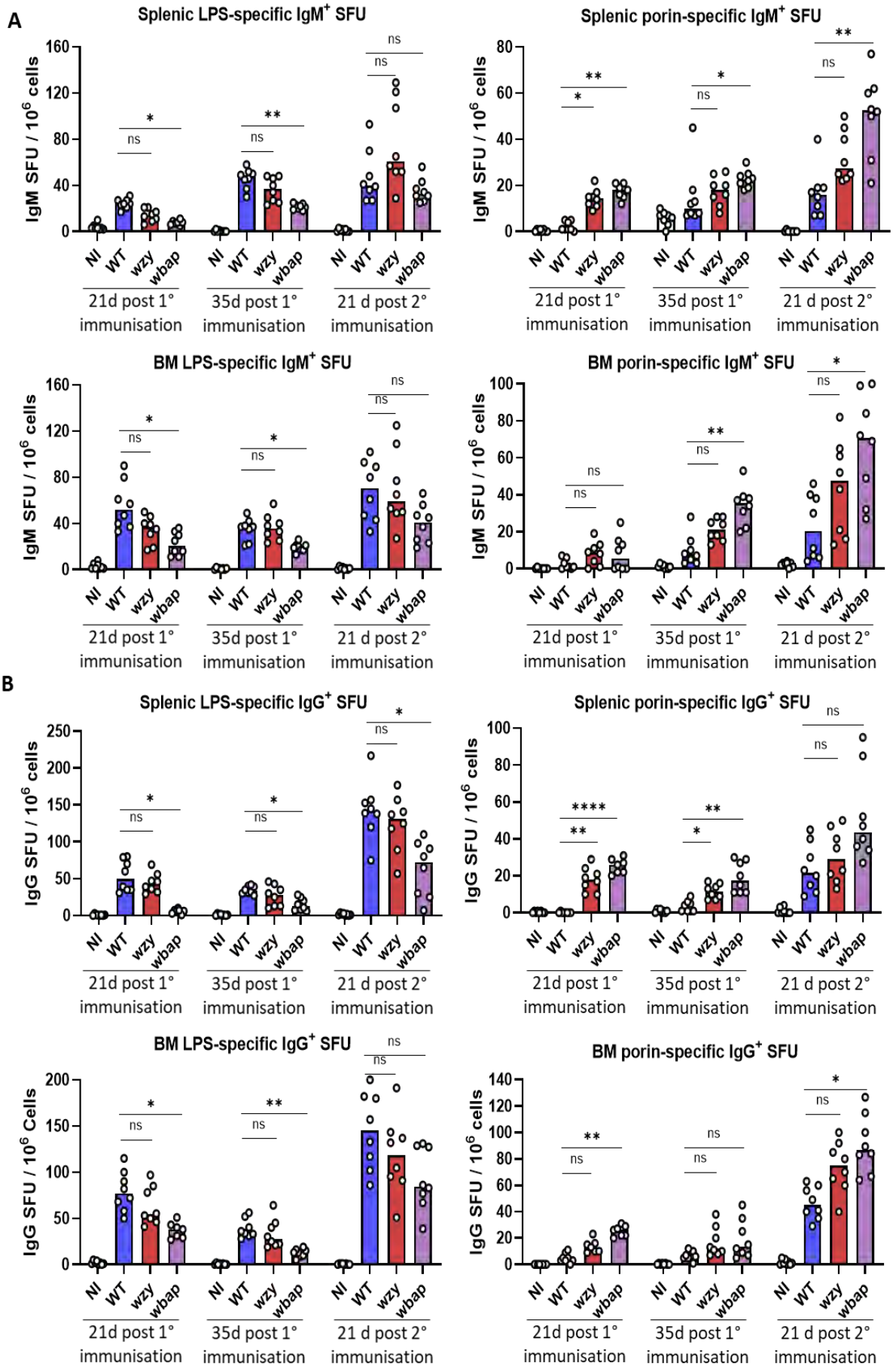

A
Splenic LPS-specific IgM+ SFU
IgM SFU / 10^6 cells
Splenic porin-specific IgM+ SFU
IgM SFU / 10^6 cells
BM LPS-specific IgM+ SFU
IgM SFU / 10^6 cells
BM porin-specific IgM+ SFU
IgM SFU / 10^6 cells
B
Splenic LPS-specific IgG+ SFU
IgG SFU / 10^6 cells
Splenic porin-specific IgG+ SFU
IgG SFU / 10^6 cells
BM LPS-specific IgG+ SFU
IgG SFU / 10^6 Cells
BM porin-specific IgG+ SFU
IgG SFU / 10^6 cells
NI
WT
wzy
wbap
21d post 1° immunisation
35d post 1° immunisation
21 d post 2° immunisation
ns

Figure 3.14 Anti-LPS and anti-porin IgM and IgG antibody-secreting cells induced to OMVs.

WT mice were immunised i.p. with 1 µg of OMV for 21 or 35 days or boosted at 35 days for a further 21 days. (A) Frequencies of splenic (top row) or bone marrow (bottom row) LPS or porin-specific IgM antibody-secreting cells were determined by ELISPOT. (B) Frequencies of splenic (top row) or bone marrow (bottom row) LPS or porin-specific IgG antibody-secreting cells. Bars show medians and individual points represent spot forming units (SFU) from a single mouse. NI = Non-immunised, WT = WT-OMV, wzy = wzy-OMV, wbaP = wbaP-OMV. ns = non-significant, * = p≤ 0.05, ** = p≤ 0.01, *** = p≤ 0.005, and **** = p≤ 0.001. Graphs show the combined data from 2 independent experiments.

3.2.2.3.2 Boosting OMVs enhances Ab binding to intact *S*. Typhimurium and *S*. Enteritidis

We assessed the binding of serum antibodies to *S*. Typhimurium bacteria or the related serovar *S*. Enteritidis. IgG binding to *S*. Typhimurium was similar between the WT or wzy-OMV groups, although less IgM binding was detected by sera from wzy-OMV-immunised mice (Figure 3.15 A). However, wbaP-OMV sera showed lower IgM and IgG binding to *S*. Typhimurium than sera from the WT and wzy-OMV groups. The same sera were also tested against *S*. Enteritidis (Figure 3.15 B). Results showed that the reduction or elimination of the O-antigen can increase the level of cross-reactive IgM and IgG antibodies. When results from each time-point were compared (Figure 3.16), it was found that relative IgM and IgG titres to *S*. Typhimurium were lower for sera from the wbaP-OMV immunised groups at each time-point, although binding did increase with time and after boosting (Figure 3.16 A and B). The increase in anti-*S*. Typhimurium in the wbaP-OMV immunised groups suggested the non-O-antigen antibody response could evolve over time and be enhanced by boosting. Against S. Enteritidis, there was little change in IgM and IgG titres between days 21 and 35 after primary immunisation, although anti-*S*. Enteritidis median titres were higher for the sera from the wzy-OMV and wbaP-OMV immunised mice than for the WT-OMV group (Figure 3.16 C and D). After

boosting there was an augmentation in titres, but again the median titres were highest in the wzy and wbaP-OMV groups. Therefore, immunisation with WT and wzy-OMV induces similar levels of anti-*S*. Typhimurium antibodies and reduced O-antigen expression in OMV lead to higher levels of cross-reactive IgM and IgG antibodies.

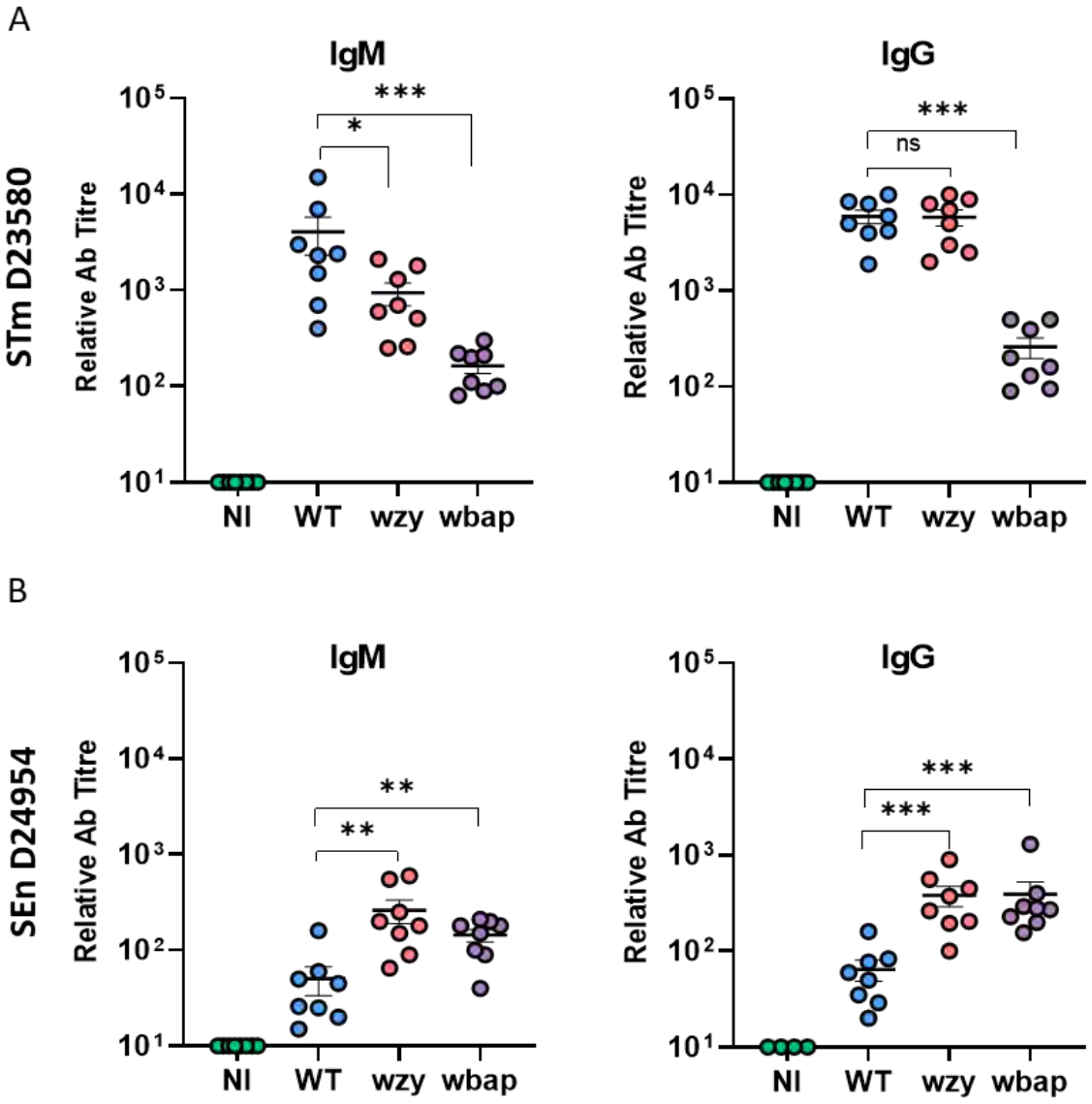


Figure 3.15 Serum antibody titres against whole bacteria of *Salmonella* Typhimurium (STm) and *Salmonella* Enteritidis (SEn) after secondary immunisation with OMVs.

Serum antibody responses were assessed by ELISA. (A) IgM and IgG antibody responses to *S*. Typhimurium in sera from mice immunised with the OMVs or Non immunised mice (NI). (B) IgM and IgG antibody responses to *S*. Enteritidis in sera from mice immunised with the OMVs or Non immunised mice. Representative of 2 experiments where n=4 mice/group. * = $p \leq 0.05$, ** = $p \leq 0.01$ and *** = $p \leq 0.005$.

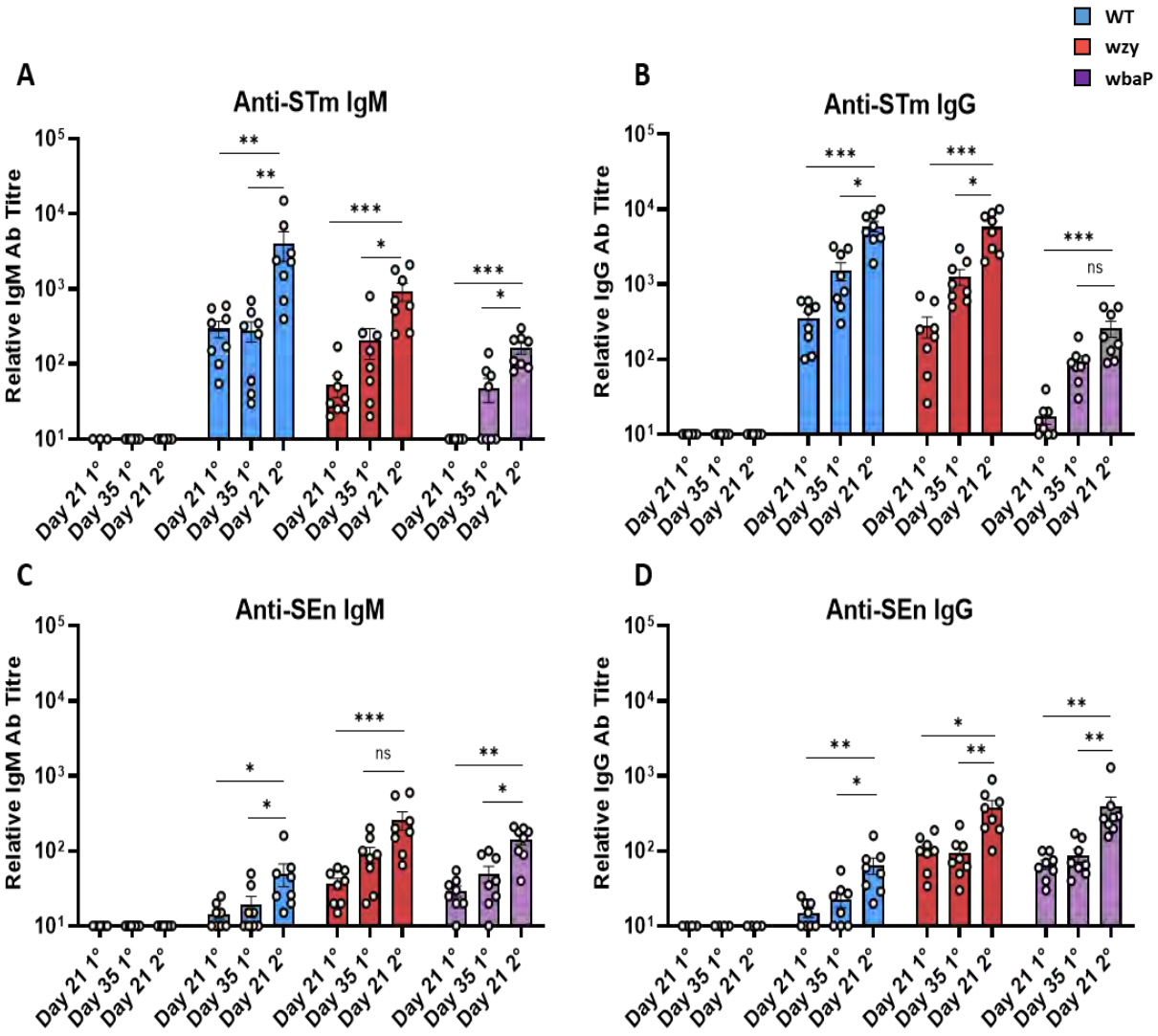


Figure 3.16 Comparison of serum antibody titres against whole bacteria of *Salmonella* Typhimurium (STm) and *Salmonella* Enteritidis (SEn) after primary and secondary immunisation with OMVs.

Serum antibody responses were assessed by ELISA. IgM and IgG antibody responses to *S*. Typhimurium in sera from mice immunised once or twice with the OMVs for different time-point (A and B). IgM and IgG antibody responses to *S*. Enteritidis in sera in the same mice groups (C and D). Representative of 2 experiments where n=4 mice/group. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

3.2.2.3.3 A second dose of WT, wzy and wbaP-OMV Induce similar germinal centre and plasma cell responses

Splenic germinal centre and total IgM+ and IgG+ plasma(blast) responses were assessed after secondary immunisation with the different OMVs. Immunising twice with WT, wzy or wbaP OMV resulted in increases in the proportion and number of germinal centre cells compared to non-immunised controls (Figure 3.17 A). These results were similar at all three time points (Figure 3.17 B). Immunisation with OMV induced no or minor changes in total IgM+ and IgG+ plasma(blast) proportions at all time points assessed compared with non-immunised mice (Figure 3.18 A). There was an increase in the proportion of plasma(blast) cells with the highest expression of IgM or IgG most notably at day 21 post-primary immunisation (Figure 3.18 B). Thus, all OMVs, independent of O-antigen content, induce similar GC and plasma cell responses. Findings presented in this chapter are summarised in table 3.1.

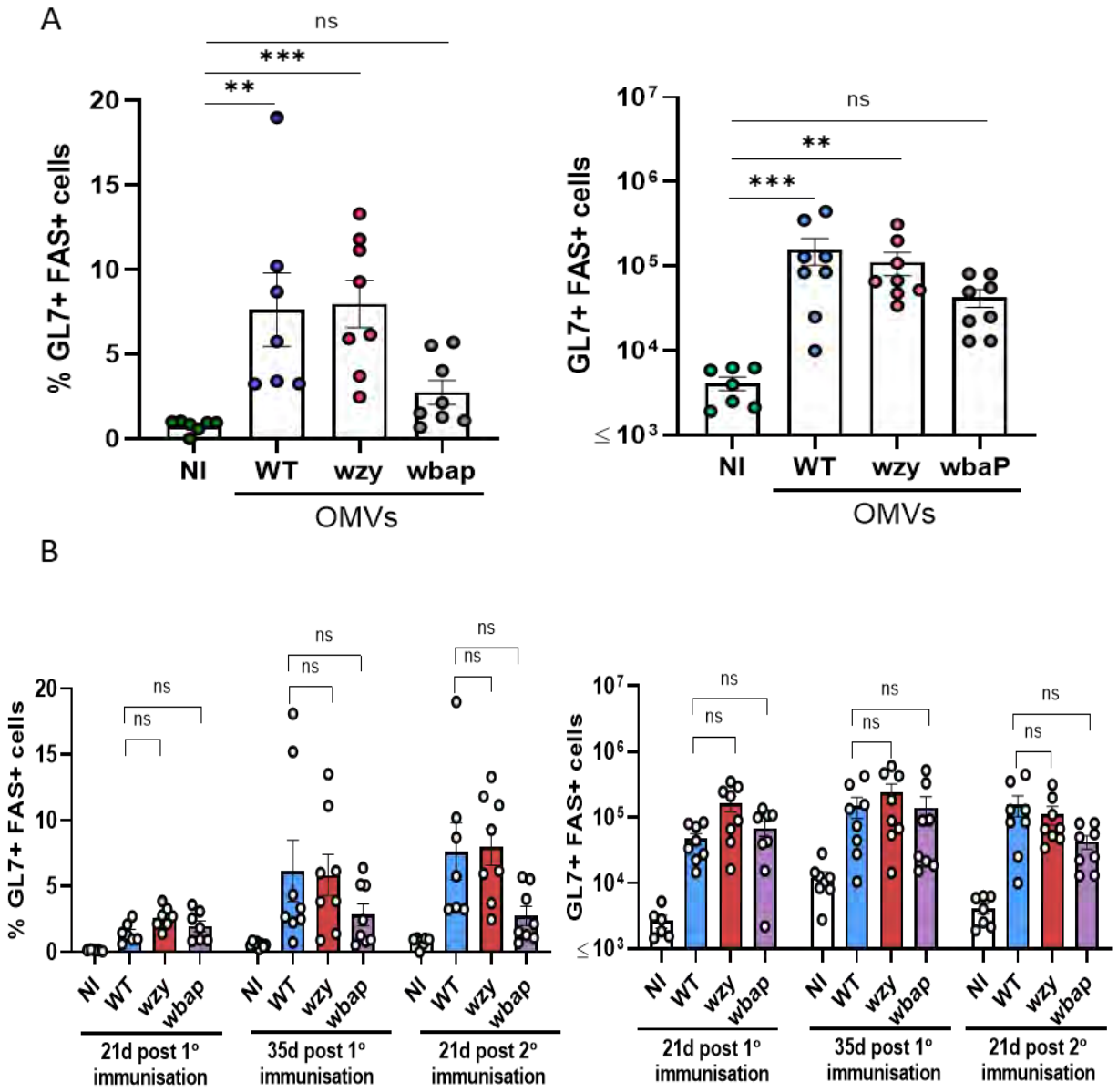


Figure 3.17 A second dose of WT, wzy, or wbaP-OMV induces a germinal centre (GC) B cell formation.

(A) Proportion (left) and total number (right) of FAS+ GL7+ GC B cells. (B) Proportion of intracellular IgM and IgG. (B) Comparison of the proportion (left) and total number (right) of FAS+ GL7+ GC B cells after primary immunisation for 21 or 35 days, or secondary immunisation at day 35 for further 21 days

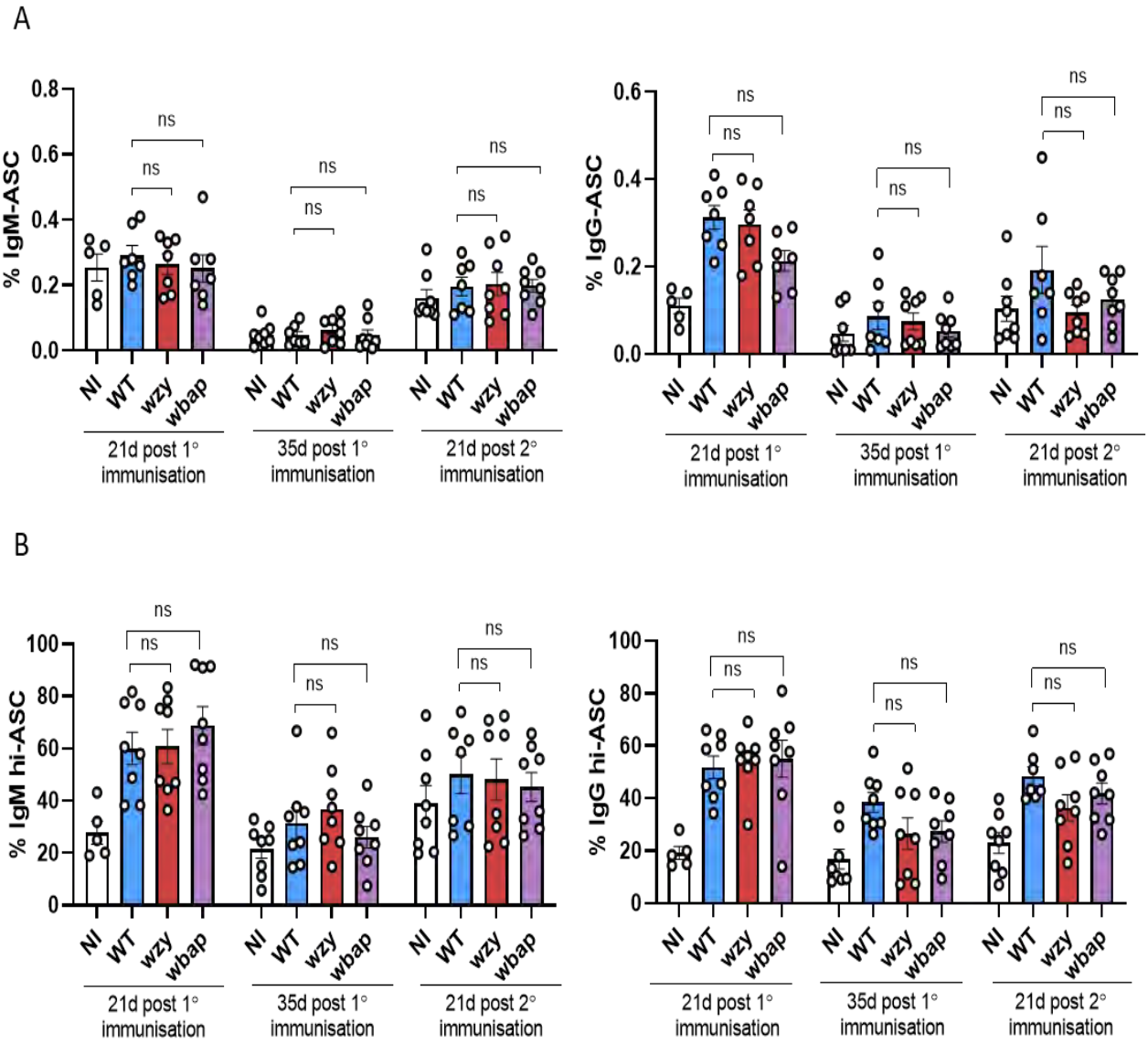


Figure 3.18 Comparison of plasma cell responses after primary or secondary immunisation with WT, wzy, or wbaP-OMV.

CD1 mice were immunised once with 1µg of each OMV for 21 or 35 days. Some mice were boosted at 35 days for a further 21 days. (A) Proportion of intracellular IgM and IgG for the different time points. (B) Proportion of IgM and IgG intracellular high population for the different time-points. Representative of 2 experiments where n=4 mice/group.

Table 3.1: Results summary of immunogenicity induced by the OMVs with different O-Ag lengths *.

	21 d post 1° immunisation			35 d post 1° immunisation			21 d post 2° immunisation		
	WT	**wzy**	**wbap**	**WT**	**wzy**	**wbap**	**WT**	**wzy**	**wbap**
Serum anti-LPS IgM	********	********	****	*******	*******	***	*********	*********	*******
Serum anti-LPS IgG	********	********	*****	*********	********	******	**********	**********	********
Serum anti-porins IgM	**	**	******	***	*****	******	****	******	******
Serum anti-porins IgG	***	*******	********	******	********	********	*****	********	********
Splenic LPS-specific IgG ASCs	****	****	*	***	***	*	*********	********	*****
Splenic porin-specific IgG ASCs	*	***	***	*	**	**	***	****	*****
BM LPS-specific IgG ASCs	******	****	***	***	***	*	*********	********	******
BM porin-specific IgG ASCs	*	**	***	*	**	**	****	******	*******
Anti-STm IgM	******	***	*	******	******	***	*********	********	*****
Anti-STm IgG	******	******	**	********	********	****	**********	**********	******
Anti-SEn IgM	*	***	**	**	****	***	***	******	*****
Anti-SEn IgG	*	****	***	**	****	****	***	******	******
GC B cells	********	*********	********	*********	*********	*********	*********	*********	********

*Arbitrary scale, where asterisks (minimum of 1, maximum of 10) represent the degree of response detected.

3.3 Discussion

The work in this chapter characterised and compared the immunogenicity and the antibody responses to OMV vaccines derived from different *S*. Typhimurium mutants expressing wild-type length O-antigens (WT-OMV), where it was restricted to a maximum length of one O-antigen repeat (wzy-OMV), or lacking O-antigen (wbaP-OMV). In OMV-based vaccine development, removing or modifying such components may alter immunogenicity. Although immunisation with OMV from Gram-negative bacteria is being studied for its immunogenicity and potential to protect against many infections (112, 118, 120, 244, 245), the effect of altering or removing the immunodominant O-antigen on the virulence and immunogenicity of *Salmonella* enterica serovar Typhimurium has not been fully elucidated. Results from this study will help to understand how these structural differences in *S*. Typhimurium -OMV vaccines result in different immunological responses and how antibody responses to bacterial antigens develop.

In this chapter, natural OMVs derived from *S*. Typhimurium (WT-OMV) and from mutant *S*. Typhimurium (wzy-OMV and wbaP-OMV) were successfully obtained, which were confirmed using MS analysis, Coomassie staining, and silver stain. MS analysis revealed distinct *S*. Typhimurium -OMVs components such as OMPs. In addition, the Coomassie stain of the protein bands Showed that OMVs contain various proteins, including OmpA, OmpC, and OmpD, which have molecular sizes of 37.5, 41.2, and 39.6 kDa, respectively. Proteomic investigation of *Shigella*-derived OMVs has revealed that outer membrane proteins make up a significant portion of the overall protein content (78%) (246), and it is most likely the same for *S*. Typhimurium -derived OMVs.

There is limited understanding of how antibodies interact with O-antigen, including the diversity of the antibody repertoire to the specific O-antigen used by a bacterial species. This is significant because incorporating O-antigens into next-generation vaccines is crucial for many Gram-negative bacteria, such as *Salmonella* and *Shigella* (99, 117, 247, 248, 249, 250, 251). Furthermore, antibody targeting of O-antigen can be paradoxical because although it can be protective, excessive levels can inhibit bacterial killing in both cell-dependent and independent systems, which has been demonstrated for several Gram-negative bacteria, including *Salmonella*, *Escherichia coli*, and *Pseudomonas aeruginosa*, leading to worse clinical outcomes(61, 237, 252, 253). One of the typical aims of vaccination is maximising the antibody response to a vaccine. This makes understanding why some individuals generate excess antibodies and how to maintain antibody levels within the "Goldilocks" zone necessary to exploit O-antigen as a vaccine target.

Our study aimed to investigate the level of anti-LPS antibodies induced by the different OMV types. Surprisingly, the levels of IgG induction to LPS were similar between the full-length O-antigen chain and when the expression of O-antigen was limited to one per LPS molecule. This finding suggests that a single O-antigen repeat is roughly the same size as the Fab region of an antibody. Moreover, the WT and wzy-OMV induced similar frequencies of ASC in the spleens and BM of the mice, indicating that the response to these immunogens is likely to persist and decay at similar rates, at least in the short term. These results were in line with those of previous studies, which showed that O-antigen length does not play a significant role on the immune response elicited by *S. flexneri* 6, *S. sonnei*, and *S*. Typhimurium OMV and that even short OAg chains can induce high and functional anti-LPS responses, like that generated by long O-antigen chains(254, 255). In contrast, infection with a live wzy-deficient *Salmonella*

mutant induced less anti-LPS antibody than the WT control, possibly because of differences in bacterial persistence (256). The reasons for the comparable responses to LPS between the two OMV types are uncertain, but there are some possibilities. First, it could mean that one O-antigen repeat is a large enough epitope, so longer O-antigen lengths may not be necessary. Second, if this is true, there may be comparable numbers of O-antigen-containing LPS molecules, instead of O-antigen molecules alone, in each OMV, which leads to equal responses and uptake by B cells. Third, it is possible that the amount of LPS molecules present in the given antigen is enough to prompt a maximal B cell response to O-antigen, and the detected levels show a natural limit in this response. Although it was unsurprising to observe the lowest responses after immunisation with wbaP-OMV, the detected levels were still higher than anticipated. This finding implies that core oligosaccharide and lipid A can prompt significant levels of responses even in the absence of O-antigen.

Restricting O-antigen expression resulted in increased levels of antibody responses to non-O-antigen components of the OMV. This was evident when wzy and wbaP-OMV immunised mice induced higher levels of anti-porins IgG and ASC frequencies in the spleens and BM than in WT-OMV immunised mice. This illustrates the effectiveness of O-antigen at shielding the bacterial surface and the role of longer O-antigen chains in limiting the Ab-response generated towards other antigens. Furthermore, the reduction in the binding of anti- *S*. Typhimurium WT-OMV antibodies to *S*. Enteritidis might be because most antibodies target the O-antigen, which varies between the two serovars, and the O-antigen may also limit access of antibodies to other surface conserved antigens such as porins.

Our results showed that OMVs, particularly those derived from *wzy* and *wbaP* mutants, could promote cross-reactive antibodies against *S*. Enteritidis intact bacteria. This increase in the

cross-reactivity level confirms what was previously reported that minimising the O-antigen expression may provide access through LPS to other conserved epitopes and broaden the range of antigens targeted within the proteinaceous component of the OMV, such as porins (12). This also accords with earlier observations, which showed that immunisation with OMVs derived from the *wbaP* mutants elicited significantly higher cross-reactive IgG responses against *S*. Choleraesuis and *S*. Enteritidis challenge than the WT-OMVs (235). Furthermore, it was found that boosting dramatically impacted levels of cross-reactive protection, despite only modest increases in anti- *S*. Typhimurium, LPS or porin-specific antibodies from day 35 after priming and day 21 after boosting. One possibility is that boosting with the two O-antigen-containing OMVs increases antibodies against other O-antigen species common to *S*. Typhimurium and *S*. Enteritidis (O1 and O12). However, reports are inconsistent in describing changes in levels of cross-reactive antibodies induced after immunisation or infection with LPS mutant bacteria, OMVs and OMPs that differ in how deeply the mutations shorten the LPS (235, 256, 257, 258). Interestingly, despite the O-antigen length, all OMVs induced significant germinal centre B cell responses after immunisation, which suggests that the level of O-antigen has no effect on the germinal centre induced.

Chapter 4. ASSESSMENT OF PROTECTIVE EFFICACY PROVIDED BY OMV OF DIFFERENT O-ANTIGEN EXPRESSIONS AGAINST *SALMONELLA* SEROVARS

4.1 Introduction

OMV vaccines hold promise as vaccine vectors as they contain multiple antigen components, including LPS, in their native conformation. In animal models, robust immunity and protection against bacterial challenge are observed when immunised with OMVs derived from *Shigella spp, Vibrio cholerae, E. coli, Burkholderia pseudomallei,* and *Acinetobacter baumannii* (229, 230, 231, 233). Furthermore, a global license had been approved for a vaccine utilising OMVs derived from *Neisseria meningitidis* to prevent meningococcal B disease in children and adults (234). Additionally, OMVs generated from *Haemophilus influenzae* have been found to confer cross-protective immunity against other serotypes (259). Therefore, developing an OMV-based vaccine is a practical strategy for generating immune responses that can protect against both homologous and heterologous serotypes of *Salmonella*.

Antibody responses are an essential part of providing optimal protection against *Salmonella* (49), and to achieve this, the antibodies must specifically target surface antigens (260). After infection with Gram-negative bacteria, antibodies can be induced to the immunodominant O-antigen sub-component of LPS, which can be protective (261). Such antibodies provide serovar-specific protection that typically offers limited cross-protection after infection with similar pathogens that differ in the O-antigen type expressed. For example, the O-antigen types expressed by different *Salmonella* serovars can vary, and this variation is sufficient to markedly reduce cross-protection afforded by one serovar against another. This has been

most clearly demonstrated by classical experiments whereby mice vaccinated with attenuated *S*. Enteritidis and challenged with isogenic strains of SEn-expressing a different O-antigen are far less protected than after challenge with bacteria expressing the identical parent O-antigen (228). Other studies have also demonstrated the importance of anti-O-antigen antibodies. Immunisation with O-antigen conjugate vaccines is sufficient to protect against infection, and immunisation with *Salmonella* mutants or outer membrane vesicles (OMVs) that lack O-antigen typically induces less protection than their O-antigen-containing counterparts (12, 117, 118). Similar observations have been made on the importance of antibodies to O-antigen for protection against infections caused by other Gram-negative pathogens such as *Shigella sonnei* and *S. flexneri* (247, 249, 251).

Nevertheless, although antibodies to O-antigen are sufficient to protect from infection, other non-LPS antigens may also contribute to protection. Examples of such antigens in *Salmonella* include the trimeric porin proteins and the trimeric autotransporter SadA from *S*. Typhimurium (30, 34, 36, 262, 263). Furthermore, protein antigens from other Gram-negative bacteria are included in vaccines licensed for humans, such as the acellular pertussis vaccine and two vaccines against disease caused by group B meningococcus (264, 265). Immunisation schedules for many bacterial vaccines are based on giving multiple boosts. Boosting with proteinaceous antigens induces memory germinal centre responses resulting in higher levels of total anti-vaccine, long-lived, high-affinity antibodies (266, 267, 268). Over time, boosting antibody responses can help maintain or even increase the overall functionality of the antibodies induced, even as antibody levels naturally drop over time (30). Therefore, the combination of antigen targets and the persistence of the response combine to influence overall vaccine efficacy.

In this chapter, the effect of multiple doses on protection was examined, and how levels of LPS O-antigen in OMVs affect their capacity to control bacterial infection after challenge with *S*. Typhimurium (which expresses O4-type LPS) or cross-protection against infection by *S*. Enteritidis (which expresses O9-type LPS) was assessed.

4.2 Results

4.2.1 OMVs derived from LPS-mutant *S.* Typhimurium provide protection against *S.* Typhimurium infection at a comparable level as WT-OMV

To examine if OMVs were able to impair a live infection, CD1 mice were immunised once with 1 µg WT, wzy, or wbaP-OMV *i.p.* for 21 days before being challenged *i.p.* with 1 x 10^4 CFU of *S*. Typhimurium D23580 strain (an invasive African isolate) for 24 hours (Figure 4.1 A). Immunised mice lost about 10-15% of their weight after vaccination compared to the unvaccinated mice. But these mice quickly regained weight in the next two days (Figure 4.1 B). On the other hand, after the infection, the vaccinated mice did not lose any weight, as did the unvaccinated mice (Figure 4.1 C).

Bacterial burdens in blood, spleens, and livers were assessed by direct culturing 1 day after infection. As expected, mice immunised once for 21 days with the different OMVs and challenged with invasive *S*. Typhimurium had lower bacterial burdens in organs after challenge when compared to non-immunised mice, and no bacteria were detected in the blood of any of the OMV-immunised groups (Figure 4.1 D, E, and F). The reduction in bacterial numbers in the spleens and livers was similar between the WT- and wzy-OMV groups. The decline in bacterial numbers in the spleens and livers of the wbaP-OMV immunised group was not as pronounced as in the O-antigen-containing OMV groups, consistent with O-antigen being a major determinant of antibody-mediated protection.

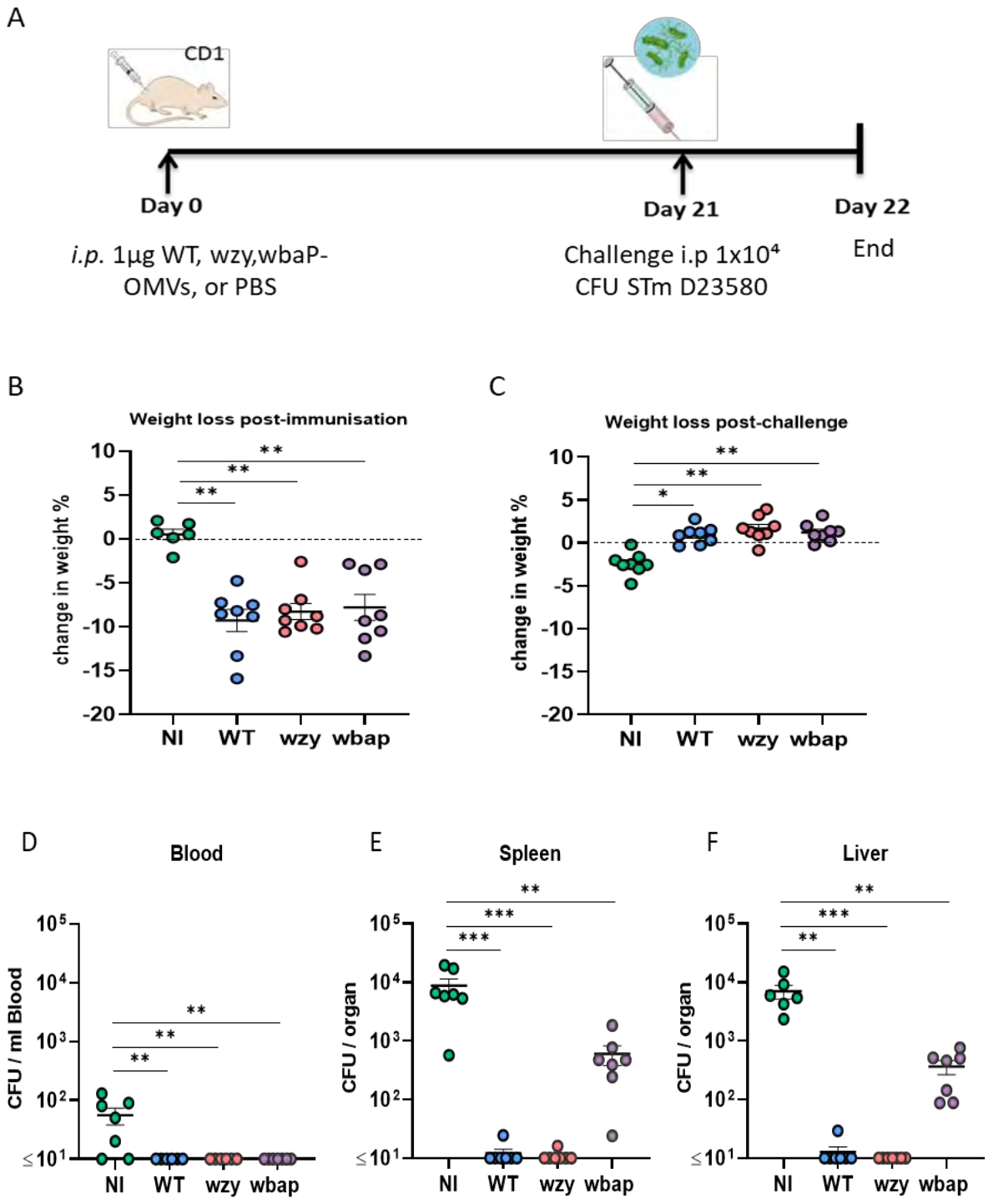


Figure 4.1 Primary immunisation with STm-OMVs having O-chain impairs virulent STm infection.

(A) CD1 mice were immunised once with 1µg of OMV from the different mutants for 21 days and infected with STm D23580 for 24 hours. (B) Percentage of change in weight post-immunisation in immunised and non-immunised mice. (C) Percentage of change in weight post-infection in immunised and non-immunised mice. Bacteria were counted 1 day after infection for the non-immunised and immunised group in the blood (D), spleen (E), and liver (F). Representative of 2 experiments where n=4 mice/group. ** = $p \leq 0.01$, *** = $p \leq 0.005$, and **** = $p \leq 0.001$.

When mice were immunised twice with the different OMVs at days 0 and 35 and infected with the virulent strain of *S*. Typhimurium 21 days later for 24 hours (Figure 4.2 A), Similar results were observed in WT and wzy-OMV immunised mice in organs and blood (Figure 4.2 B, C, and D). However, boosting mice with wbaP-OMV induced enhanced protection in the liver and spleen compared to primary immunisation. (Comparisons between primary and secondary immunisation are shown in Figure 4.3).

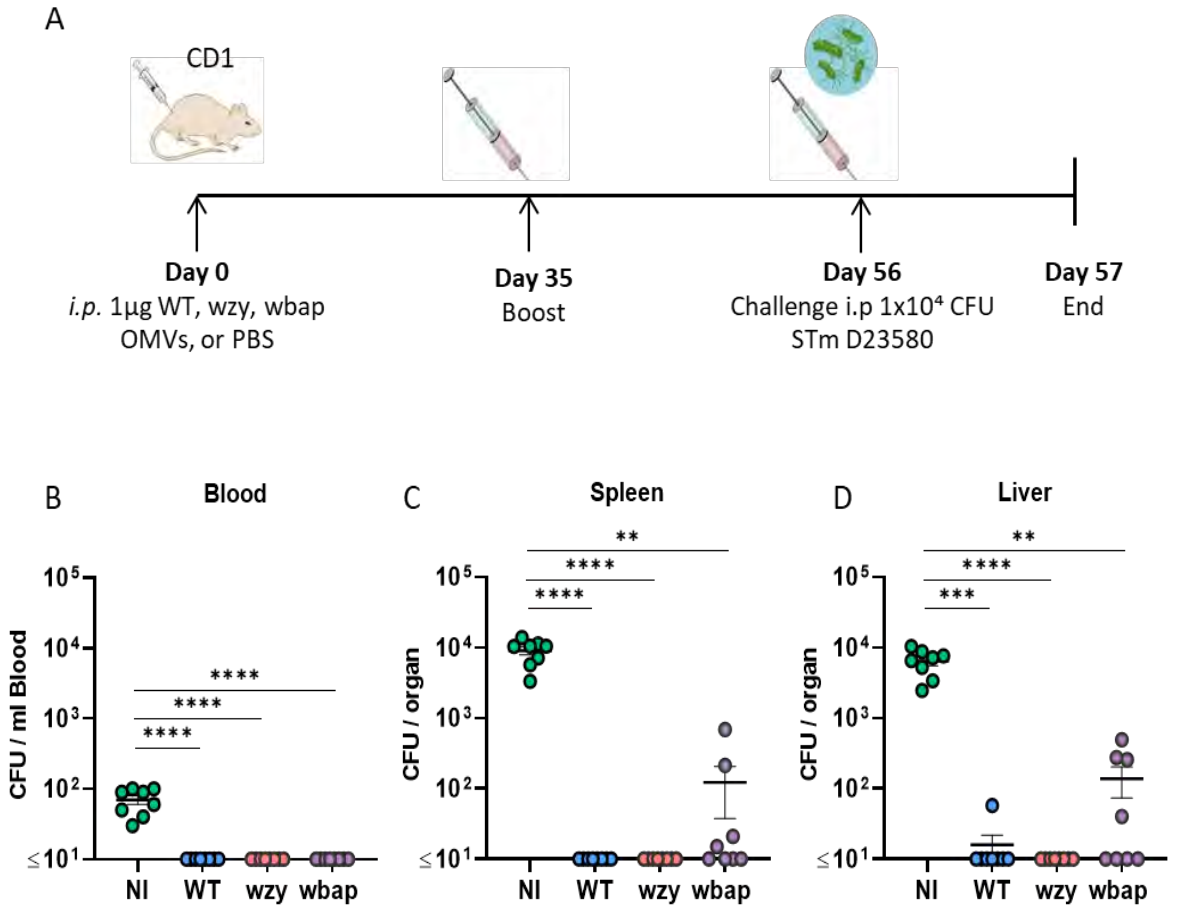


Figure 4.2 Bacterial burden after virulent STm challenge of mice immunised twice with the different OMVs.

(A) CD1 mice were immunised and boosted at days 0 and 35 with 1µg of OMV from the different mutants and infected at day 56 with STm D23580. Bacteria were counted one day after infection for the non-immunised and immunised group in the blood (B), spleen (C), and liver (D). Representative of 2 experiments where n=4 mice/group. ** = $p \leq 0.01$, *** = $p \leq 0.005$, and **** = $p \leq 0.001$.

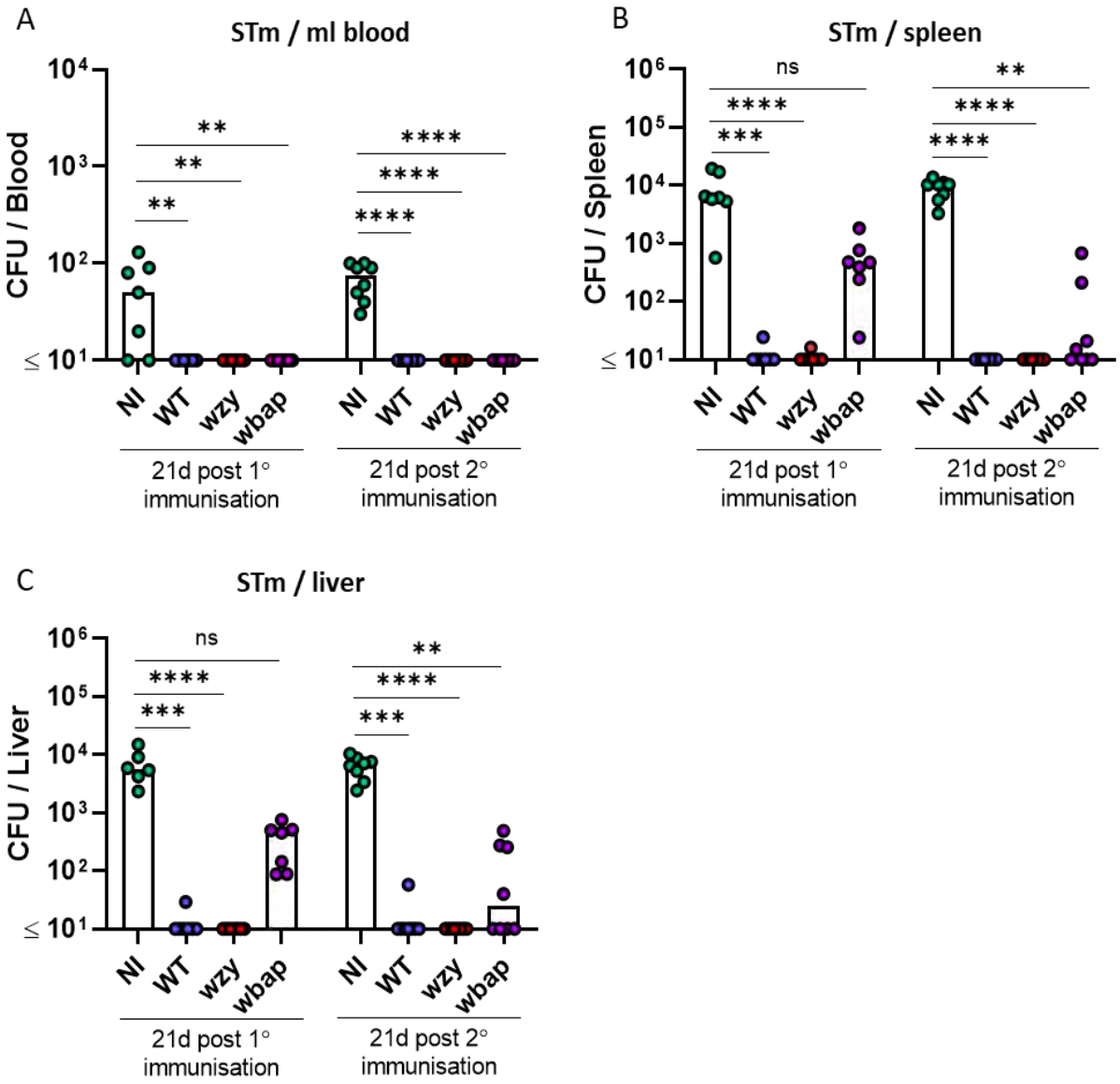


Figure 4.3 Bacterial burden after virulent STm challenge of mice immunised once or twice with the different OMVs.

Bacteria were counted one day after infection for the non-immunised and immunised group for both primary and secondary immunisations in the blood (A), spleen (B), and liver (C). Representative of 2 experiments where n=4 mice/group. ** = $p \leq 0.01$, *** = $p \leq 0.005$, and **** = $p \leq 0.001$.

We also assessed the ability of WT and wzy-OMV to protect against the laboratory strain of S. Typhimurium (*S*. Typhimurium SL 1344). Mice were immunised once with WT and wzy-OMV (wbaP-OMV not included) for 21 days and challenged *i.p.* with 1 x 10^4 CFU of *S*. Typhimurium SL 1344 (Figure 4.4 A). Bacterial loads were assessed in blood, spleen, and liver. Both WT and wzy-OMV resulted in >1000-fold lower bacterial numbers in both organs, and no bacteria were detected in the blood (Figure 4.4 B, C, and D). These results indicate that WT and wzy-OMVs are equally protected against *S*. Typhimurium infection in this model.

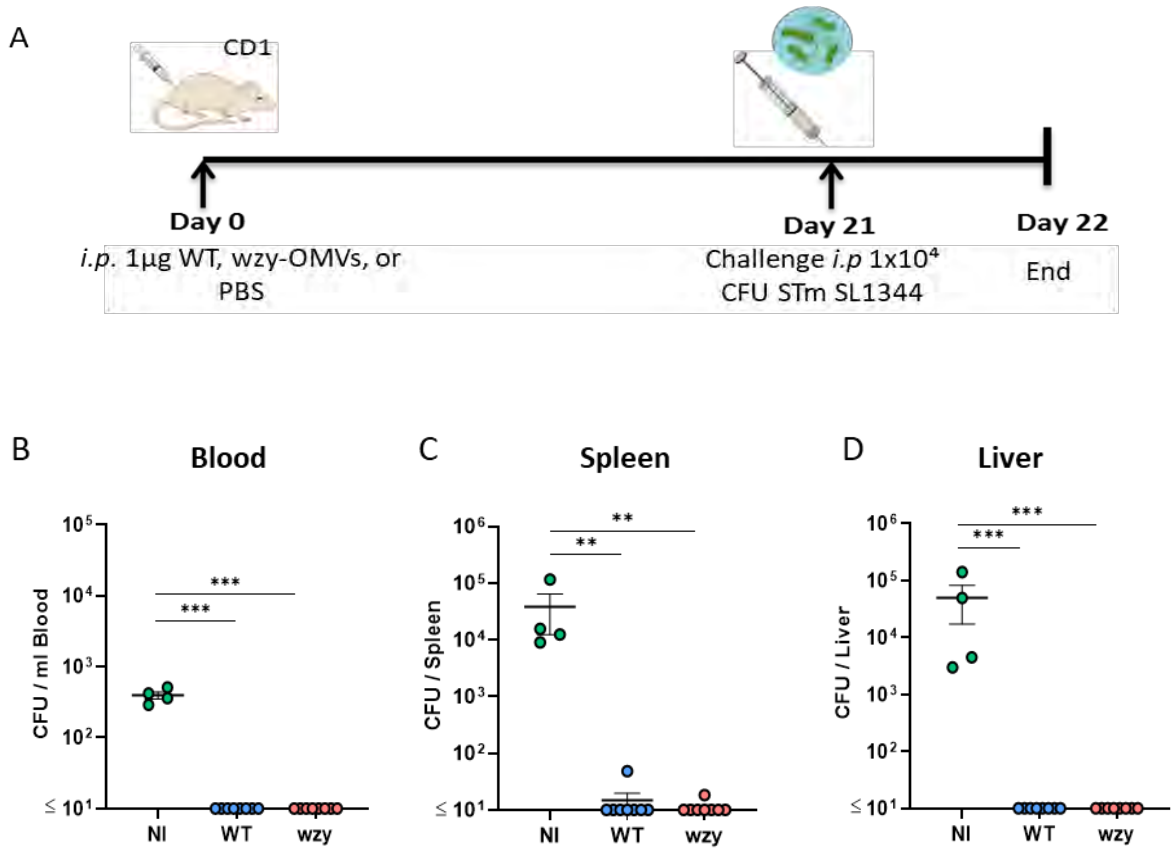


Figure 4.4 WT and wzy-OMV are equally protected against a laboratory strain of *S*. Typhimurium.

(A) CD1 mice were immunised at day 0 with 1µg of WT or wzy-OMV and infected at day 21 with STm SL1344 for 24 hours. Bacteria were counted one day after infection for the non-immunised and immunised group in the blood (B), spleen (C), and liver (D). Representative of 2 experiments where n=4 mice/group. ** = $p \leq 0.01$ and *** = $p \leq 0.005$.

To test if antibodies induced by WT or wzy-OMV immunisation could directly promote the killing of *S*. Typhimurium or *S*. Enteritidis, the bactericidal capacity of anti-OMV sera was assessed by SBA. An SBA was performed using sera from WT or wzy-OMVs- immunised and non-immunised mice (as a control) against invasive strains of *S*. Typhimurium (STm D23580) and *S*. Enteritidis (SEn D24954) (Figure 4.5). Antibodies from both sera promoted the killing of *S*. Typhimurium at a similar level (Figure 4.5 A). However, levels of antibody-mediated killing against *S*. Enteritidis were at higher levels in sera from wzy-OMV immunised mice than in WT-immunised mice (Figure 4.5 B). Therefore, OMV expressing a single O-antigen was able to induce bactericidal antibodies against both *S*. Typhimurium and *S*. Enteritidis.

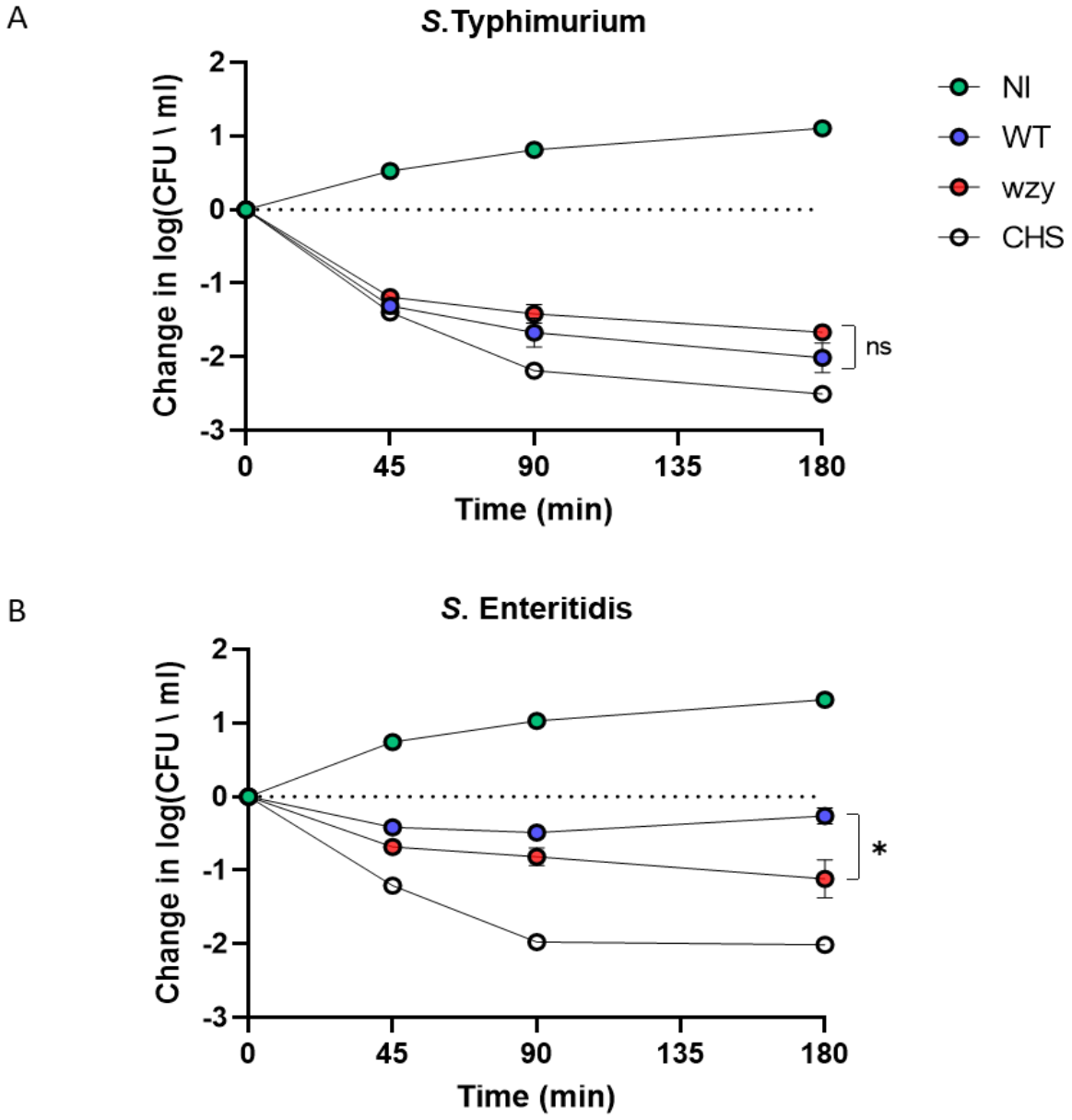


Figure 4.5 Serum bactericidal activity of WT and wzy-OMV immunised mice against *S.* Typhimurium and *S.* Enteritidis.

SBA was performed to measure the bactericidal activity of sera from immunised and non-immunised mice against *S.* Typhimurium (A) and *S.* Enteritidis (B). Data represented as mean change from the starting Log CFU/mL bacterial dose. Complete human serum (CHS) was used as a positive control. Error bars represent SEM. ns= non-significant and * = $p \leq 0.05$.

4.2.2 Boosting with OMV enhances control of bacterial infection after challenge with *S.* Enteritidis

Since immunisation with wzy- and wbap-OMV induced cross-reactive antibodies against *S*. Enteritidis (shown in Chapter 3) and were superior at killing in the SBA, the potential for cross-protection against *S*. Enteritidis *in vivo* was then examined through immunisation with *S*. Typhimurium -OMV. CD1 mice were immunised once with the different OMV and challenged *i.p*. with 1 x 10^4 CFU of the virulent strain *S*. Enteritidis D24954 for 24 hours (Figure 4.6 A), and bacterial load were assessed in blood, spleens, and livers. In this case, any antibody-mediated protection afforded is independent of antibody to LPS O4 as this is not expressed by SEn. After the challenge of once-immunised mice with SEn, bacteria were not detectable in the blood of most OMV-immunised mice despite relatively high bacterial burdens in the tissues (Figure 4.6 B). In addition, there was a median 5, 1.5 and 5-fold drop in bacterial numbers in the spleens of WT, wzy and wbaP-OMV immunised mice, respectively and an 8, 2 and 6-fold drop in bacterial numbers in the livers of WT, wzy and wbaP-OMV immunised mice respectively (Figure. 4.6 C and D).

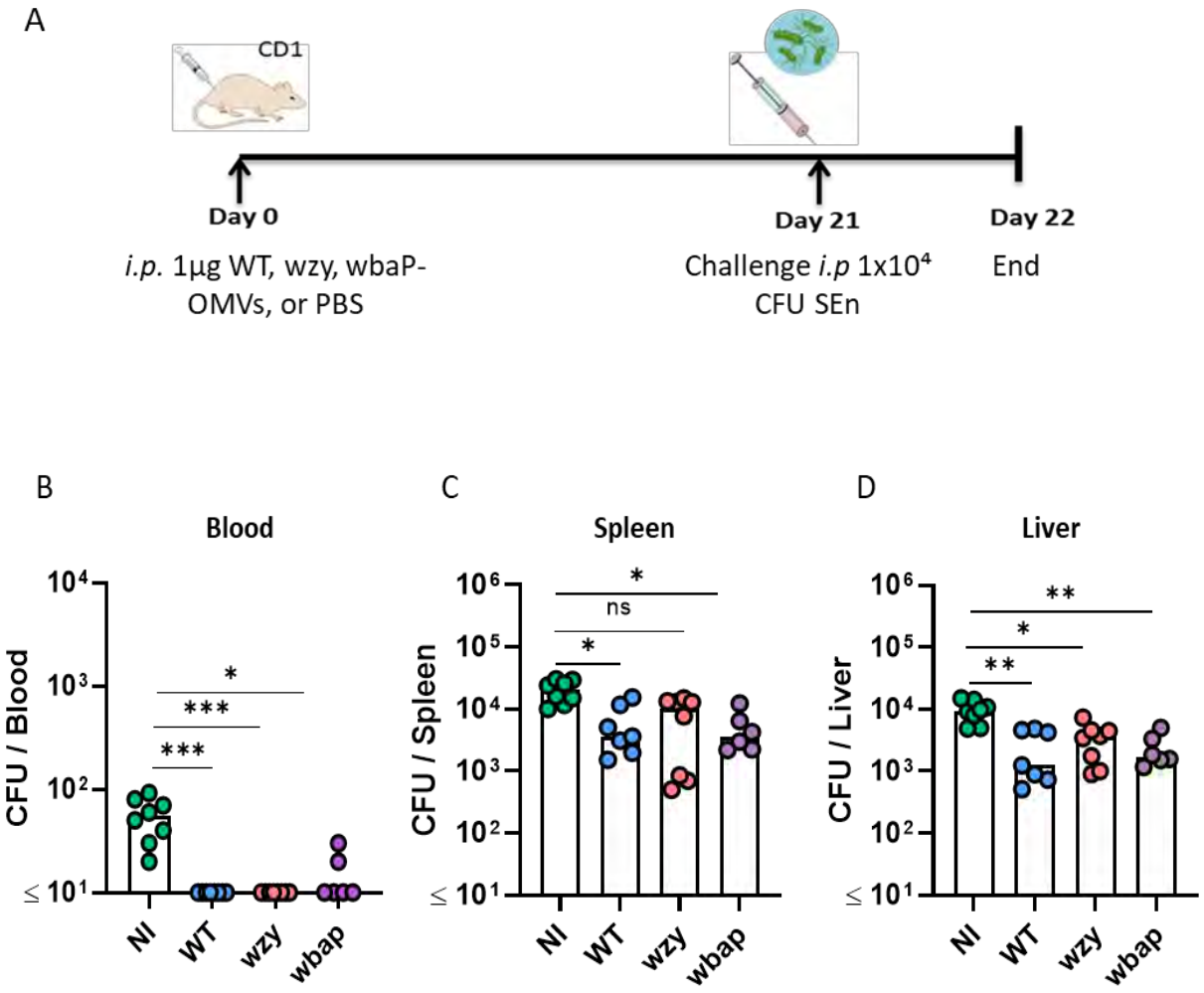


Figure 4.6 Primary immunisation with STm-OMVs provides limited protection against virulent S. Enteritidis.

(A) CD1 mice were immunised once with 1µg of the OMVs derived from different mutants for 21 days and infected with SEn D24954 for 24 hours. Bacteria were counted one day after infection for the non-immunised and immunised group in the blood (B), spleen (C), and liver (D). Representative of 2 experiments where n=4 mice/group. * = $p \leq 0.05$, ** = $p \leq 0.01$ and *** = $p \leq 0.005$.

To examine if the enhanced levels of cross-reactive antibodies induced after boosting improved protection, mice were immunised twice with the different OMV and challenged with the virulent strain *S*. Enteritidis for 24 hours (Figure 4.7 A). Unexpectedly, after challenge of boosted mice with *S*. Enteritidis there was an increase in the fold-reduction in nearly all instances. In the spleens of WT, wzy, and wbap-OMV boosted mice, the fold reduction was 44, 58 and 16-fold, respectively (Figure 4.7 C). In the livers of WT, wzy, and wbaP-OMV boosted mice, the fold reduction was 24, 18 and 7-fold, respectively (Figure 4.7 D). In addition, no bacteria were detected in the blood of any of the OMV-immunised mice (Figure 4.7 B). Thus, boosting can enhance cross-protection in an O4-antigen-independent manner.

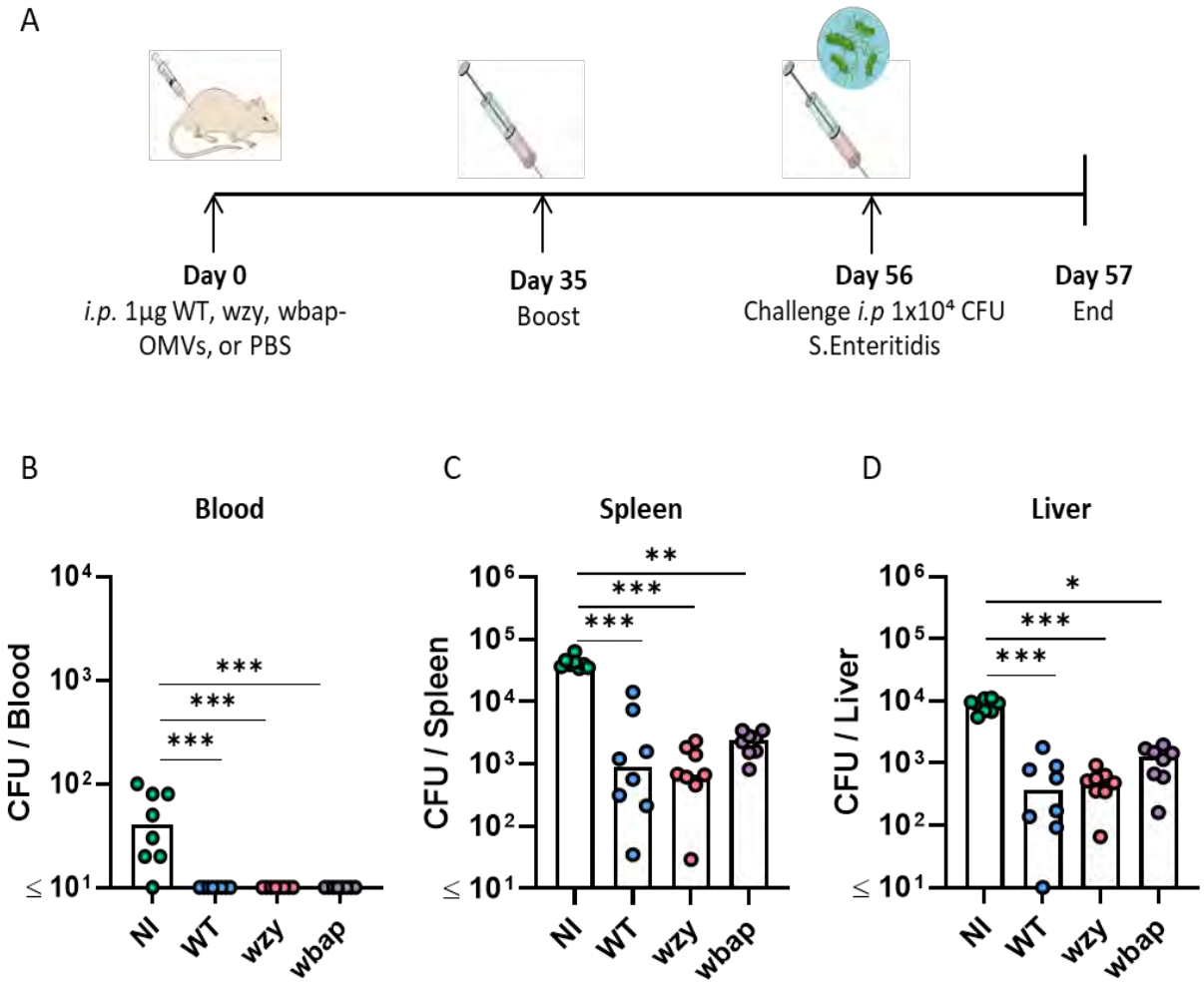


Figure 4.7 Enhanced cross-protection against virulent *S.* Enteritidis infection in mice after secondary immunisation with the different OMVs.

(A) CD1 mice were immunised and boosted at days 0 and 35 with 1µg of OMV from the different mutants and infected at day 56 with SEn D24954. Bacteria were counted after infection for the non-immunised and immunised group in the blood (B), spleen (C), and liver (D). Representative of 2 experiments where n=4 mice/group. * = $p \leq 0.05$, ** = $p \leq 0.01$ and *** = $p \leq 0.005$.

4.2.3 OMV vaccines are protective in the absence of IgM

Since immunisation with wzy-OMV induced low anti- *S*. Typhimurium IgM antibodies (shown in chapter 3, section 3.2.2.1.2) and still being able to protect against *S*. Typhimurium infection, it was hypothesised that IgM antibodies could be unnecessary for this protection. Mice lacking secretory IgM ($sIgM^{-/-}$) to test this hypothesis were used. $sIgM^{-/-}$ mice were immunised for 21 days with 1 µg of WT and wzy-OMV and then infected for 24 hours with 5 x 10^5 CFU of attenuated *S*. Typhimurium SL 3261 (Figure 4.8 A). ELISPOTs were performed to confirm phenotype (Figure 4.8 B). No IgM ASCs detected against LPS and porins in both the spleen and bone marrow; however, anti-LPS IgG ASCs were induced by immunisation with either OMV vaccine at comparable levels and higher anti-porins IgG ASCs were induced by immunisation with wzy-OMV than WT-OMV, in both the spleen and bone marrow (Figure 4.8 B and C). Bacterial burdens in blood, spleens, and livers were also assessed. No bacteria were detected in the blood of immunised mice (Figure 4.8 D). The two vaccinated groups resulted in significant decreases in the bacterial number in organs compared to mice controls (Figure 4.8 E and F). These results indicate that IgM is not necessary for the protection afforded against *S*. Typhimurium infection by OMV.

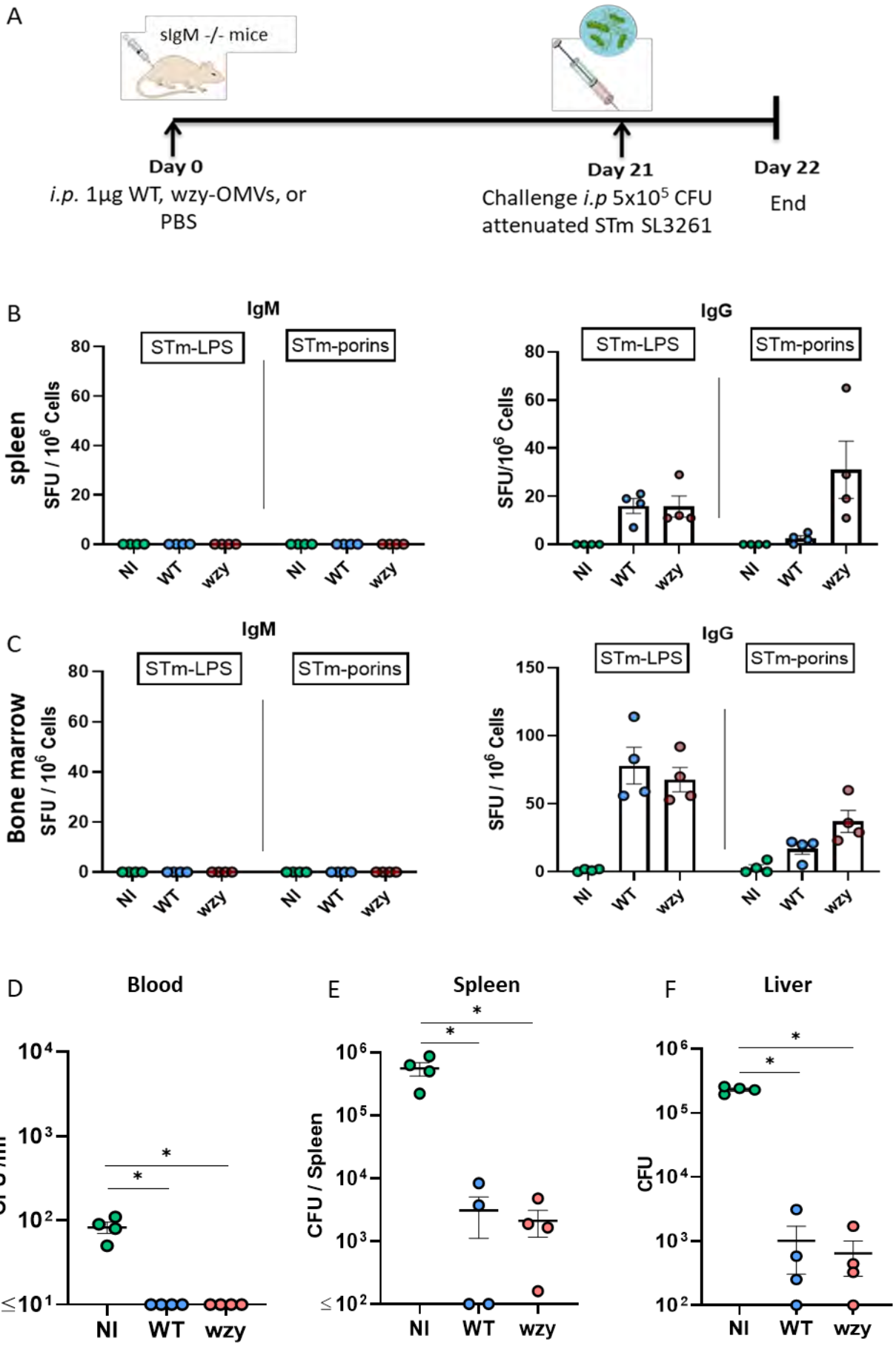

A
sIgM -/- mice
Day 0
i.p. 1µg WT, wzy-OMVs, or PBS
Day 21
Challenge i.p 5x10^5 CFU attenuated STm SL3261
Day 22
End
B
IgM
IgG
STm-LPS
STm-porins
spleen
SFU / 10^6 Cells
SFU/10^6 Cells
NI
WT
wzy
C
Bone marrow
SFU / 10^6 Cells
D
Blood
CFU /ml
E
Spleen
CFU / Spleen
F
Liver
CFU

Figure 4.8 OMV-immunised mice can impair STm infection in the absence of secretory IgM.

(A) sIgM$^{-/-}$ mice were immunised once with 1µg of WT or wzy-OMVs for 21 days and infected with attenuated STm SL3261 for 24 hours. Anti-LPS and anti-porins IgM+ and IgG+ antibody-secreting cells (ASCs) were quantified by ELISPOT in the spleen (B) and bone marrow (C). Bacteria were counted one day after infection for the non-immunised and immunised group in the blood (D), spleen (E), and liver (F). n=4 mice/group. * = $p \leq 0.05$.

4.2.4 Role of T-bet in protection against *S*. Typhimurium infection after immunisation with *S*. Typhimurium -OMV

In these models of *Salmonella* infection, antibodies need to be present at the time of infection to contribute to protection. In contrast, T cells, specifically T helper 1 (Th1) cells, contribute to clearance (269, 270). Polarisation to a Th1 phenotype is dependent upon the transcription factor T-bet and mice that lack T-bet cannot clear *Salmonella* infections and typically succumb to infection within the first month or so of challenge, even after infection with attenuated *Salmonella* strains (269). This allows us to assess whether mice that produce antibodies but fail to clear infection are protected by vaccination. This differs to INFγ -/- mice that cannot control *Salmonella* infections from the earliest times post-infection and the pivotal role of IFNγ in regulating *Salmonella* infection during the initial week has been demonstrated (271, 272). Therefore, whether CD4+ T cells that produce IFNγ are part of this process was assessed here. To investigate this further, cohorts of both wild-type (WT) and T-bet$^{-/-}$ mice were immunised *i.p*. either with PBS or 1 µg of WT-OMV. These mice were challenged at day 21 with *S*. Typhimurium SL3261 for 28 days except for the non-immunised T-bet$^{-/-}$ mice which were challenged for 14 days (as this was a reasonable humane end-point) (Figure 4.9 A). Bacterial burdens in blood, spleens, and livers were assessed by direct culturing from immunised and non- immuninsed WT and T-bet$^{-/-}$ mice. In all immunised groups a reduction in the bacterial numbers in the spleen and liver was observed compared to the non immunised groups (Figure 4.9 B and C). However, *S*. Typhimurium numbers were lower in WT immunised mice than T-bet$^{-/-}$ immunised group. Thus in the absence of T-bet and Th1-mediated control of infection, the antibody-mediated response to OMV is sufficient to prevent overwhelming infections from developing, at least in the timeframes of these experiments.

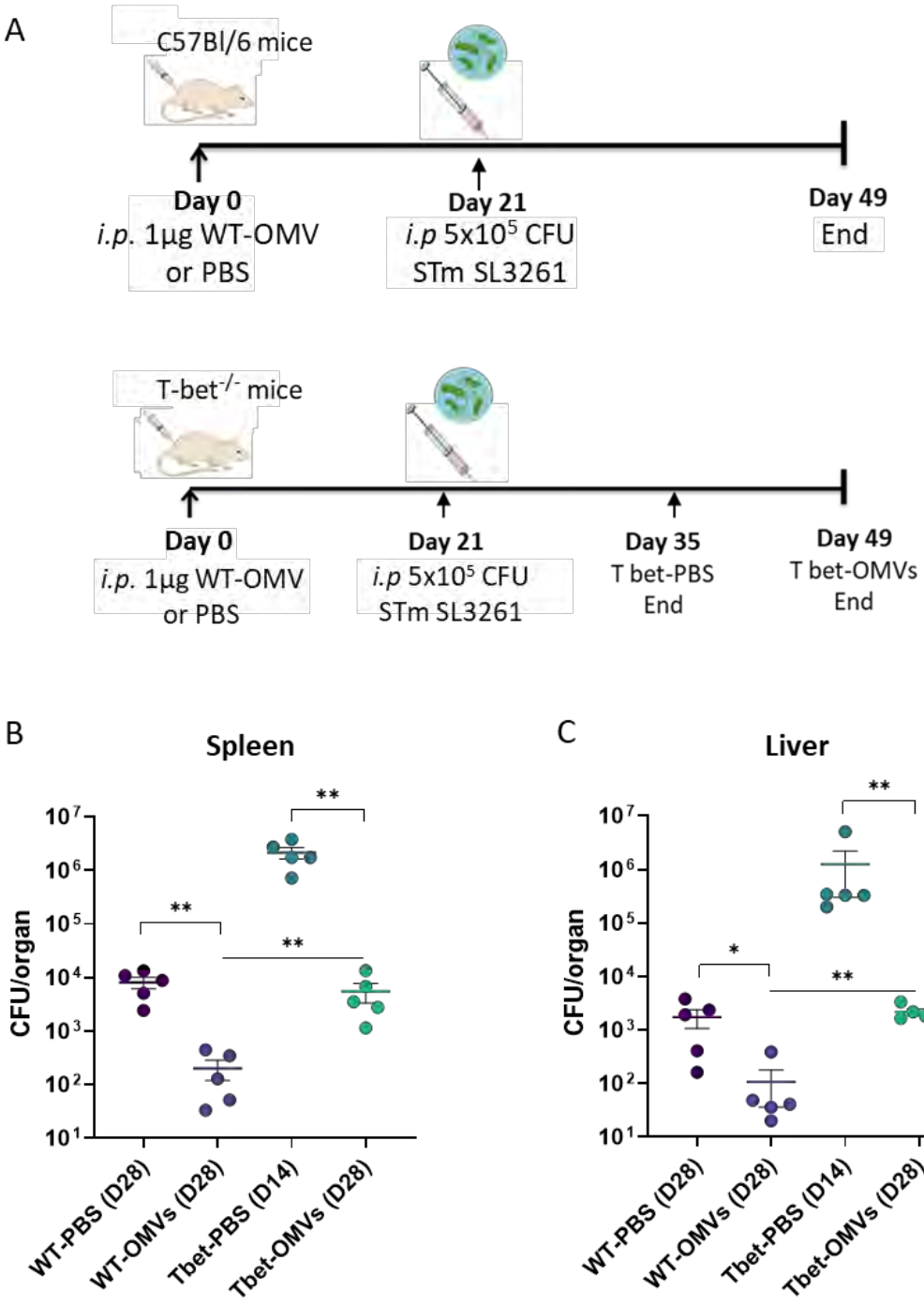


Figure 4.9 T-bet is not required for controlling *S.* Typhimurium infection after STm-OMV immunisation.

(A) WT and T-bet$^{-/-}$ mice were immunised at day 0 with 1µg of STm-OMV or PBS and infected at day 21 with attenuated STm SL3261 for 14 days (for non-immunised T-bet$^{-/-}$ mice) or 28 days (for other groups). Bacteria were counted after infection for the non-immunised and immunised group in the spleen (B) and liver (C). n=5 mice/group. * = $p \leq 0.05$ and ** = $p \leq 0.01$.

To examine factors associated with these results, serum IgM and IgG specific for LPS and porins were quantified by ELISA in all groups (Figure 4.10). Anti-LPS IgM and IgG antibody titres were significantly higher in OMV-immunised, infected groups than in non-immunised, infected mice (Figure 4.10 A). However, there was no difference in anti-LPS IgM and IgG antibody titres between WT and T-bet$^{-/-}$ mice that received *S*. Typhimurium -OMV. Anti-porins IgM and IgG antibodies were induced at a higher level in immunised and infected WT mice compared to non-immunised counterparts. At the same time, no differences were observed between the infected, non-immunised and immunised T-bet$^{-/-}$ groups (Figure 4.10 B).

The B cell responses were also examined by flow cytometry in these T-bet$^{-/-}$ mice (Figure 4.11). Splenic B cells were gated on CD19 and B220. While no significant differences in the proportion of B cells were detected between infected, non-immunised and immunised WT mice at day 28, a significant reduction was seen in the proportion of B cells in non-immunised, infected T-bet$^{-/-}$ mice compared to those immunised with OMV before infection (Figure 4.11 A). However, the B cell proportion was still lower in immunised T-bet$^{-/-}$ mice than in immunised WT mice.

Germinal centre B cell responses in the different groups of mice were then examined. The total number and frequency of GL7+ FAS+ GC B cells were assessed from spleens isolated from WT and T-bet$^{-/-}$ mice (Figure 4.11 B). Data showed that immunised T-bet$^{-/-}$ mice were able to induce equivalent GC B cell responses as WT mice (Figure 4.11 B). This indicates that T-bet induces similar Ab and GC responses after immunisation with *S*. Typhimurium -OMV and challenge.

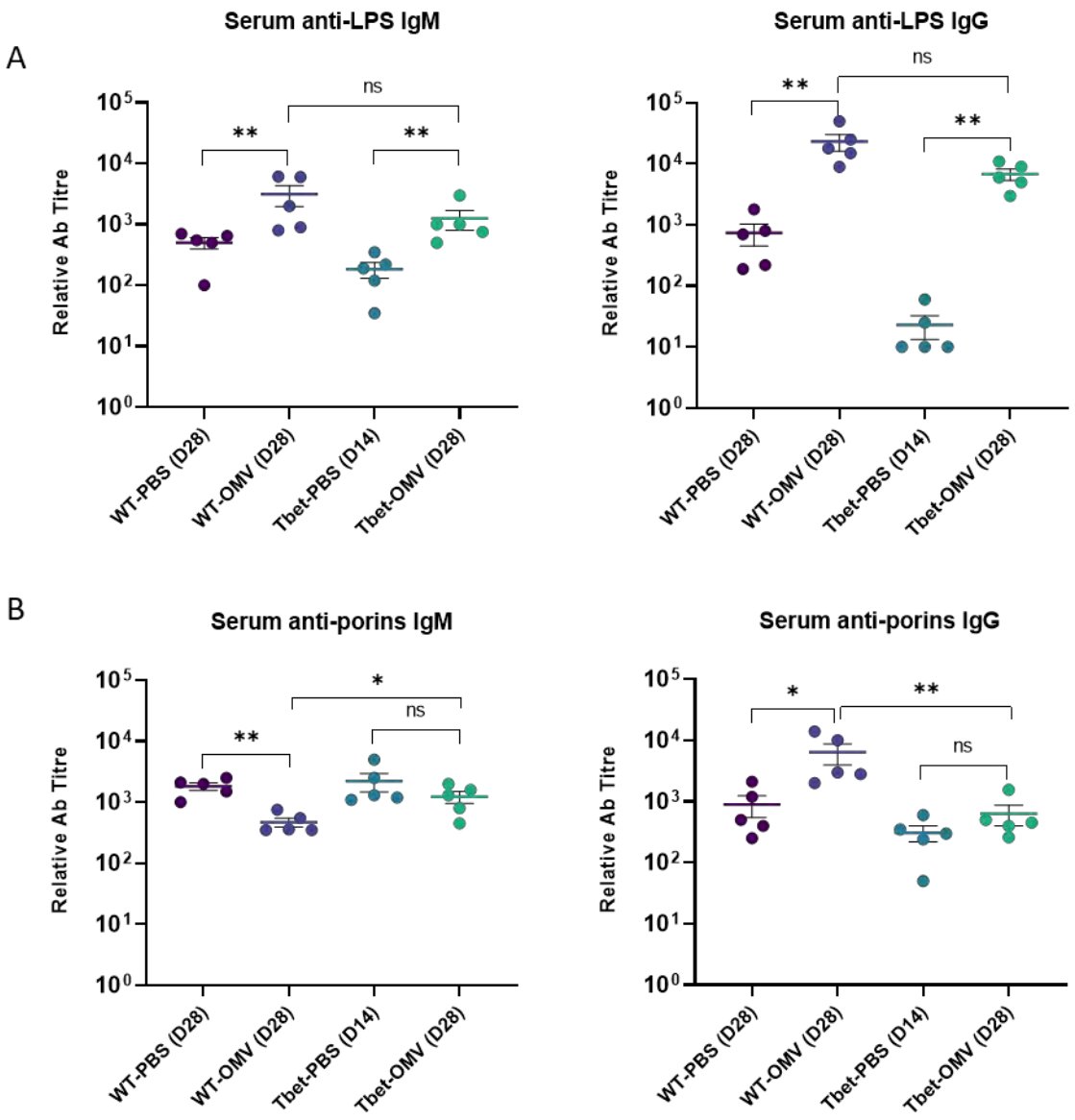


Figure 4.10 Serum IgM and IgG antibody titres in WT and T-bet-/- after immunisation and infection.

WT and T-bet-/- mice were immunised with 1 µg WT-OMV or PBS for 21 days and infected with the attenuated strain of STm. A) Relative IgM and IgG- antibody titres specific for LPS quantified by ELISA in sera from immunised and non-immunised groups. B) Relative IgM and IgG- antibody titres specific for porins quantified in sera from each group. n=5 mice/group. * = $p \leq 0.05$ and ** = $p \leq 0.01$.

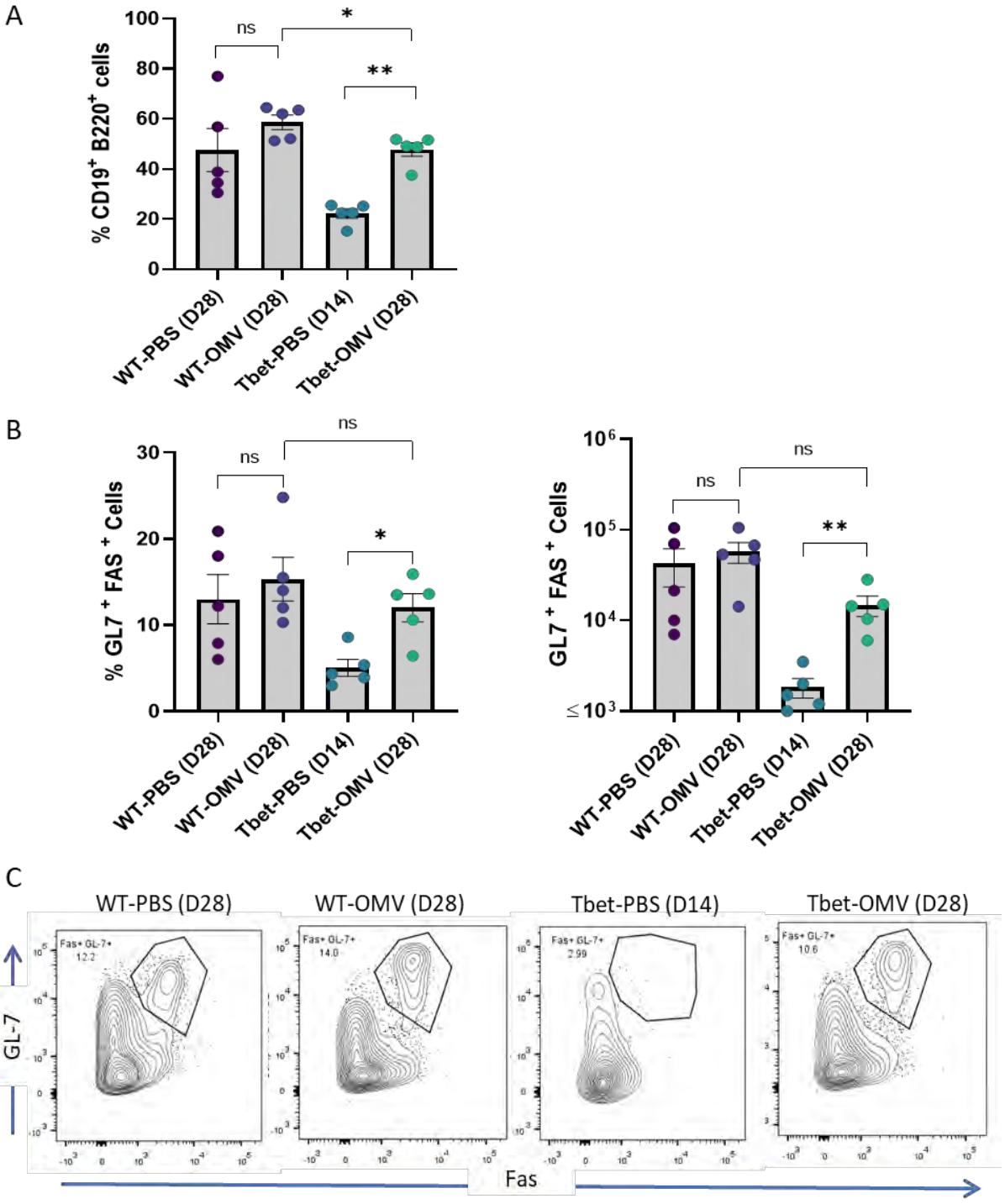


Figure 4.11 T-bet-/- mice were able to induce B cell responses and the formation of germinal centre (GC) B cells after immunisation with STm-OMV and challenge.

WT and T-bet-/- mice were immunised with 1 µg WT-OMV or PBS for 21 days and infected with the attenuated strain of STm. (A) Proportion of CD19+ B220+ B cells in the spleen post immunisation and infection for each group. (B) Proportion (left) and total number (right) of FAS+ GL7+ GC B cells for all groups. (C) Representative FACS plots for Fas+ GL7+ GC B cells in the spleen from immunised and non-immunised mice. n=5, * = $p \leq 0.05$, and ** = $p \leq 0.01$.

4.3 Discussion

Successful vaccines combine the ability to provoke long-lasting immune responses in combination with targeting one or more protective antigens. In the last chapter, the nature of the response induced by different OMV was examined, whereas in this chapter, the protective efficacy against the parent organism (*S*. Typhimurium) and the related organism *S*. Enteritidis was examined. Key to this is examining the level of protection afforded by OMVs that contain LPS with O-antigen of variable lengths, of one O-antigen repeat maximum and where O-antigen is absent. This combination allows us to dissect the contribution to protection of O-antigen and non-O-antigen molecules within the OMV. This is important because OMV contain many different antigens (Appendix C) and contrasts with viruses, which contain many fewer antigens to target than bacteria. This is demonstrated by licensed vaccines against SARS-CoV-2, where targeting a single antigen is sufficient to provide protection, even when multiple vaccine delivery platforms are used (273). In contrast, against bacteria, single antigen vaccines typically target exotoxins or capsular polysaccharides. Notably, after immunisation with the porin protein OmpD, the nature of the immune response induced correlates strongly with the level of protection afforded since mice lacking IgG1 are not as protected as wild-type mice, whereas mice lacking IgG2a were similarly protected (36). The key factor that is likely to be important between OmpD and OMV is the presence of O-antigen within OMV. This is because LPS O-antigen on the bacterial surface provides not just a physical b. arrier but that the chemical structure of O-antigen itself affects antibody access to this porin (12). A potential effect of this is that there is less immune-driven selective pressure on OmpD, and there is only a single amino acid difference between OmpD from *S*. Typhimurium and *S*. Enteritidis. This means that the physical and chemical relationship between surface antigens is an important

determinant of whether a given antigen is a good target of protective antibodies. It was these findings that led us to examine the response to LPS itself and the protection afforded after immunisation with OMVs where O-antigen expression was not controlled, or where it was restricted to a maximum length of one O-antigen repeat or where there was no O-antigen present at all.

As expected, the targeting of O-antigen enhanced the protection afforded to the homologous serovar. Nevertheless, surprisingly similar levels of IgG induction to LPS and protection against infection with *S*. Typhimurium were observed when O-antigen expression was restricted to a single O-antigen unit per LPS molecule. This suggests that the generation of O-antigen chains is not essential for this protection. As stated in the last chapter, this suggests that a single O-antigen unit can constitute a single epitope and that such single O-antigen-containing epitopes are protective. Reduced bacterial burdens were observed after challenge of mice immunised with wbaP-OMV, indicating that non-O-antigen components of OMV induce protective antibodies. Whilst it cannot be fully ruled out that these effects are because of antibodies targeting core oligosaccharide, and core oligosaccharide antibodies can be protective (12), the greater reduction in bacterial numbers after challenge with *S*. Typhimurium compared to *S*. Enteritidis makes this a less likely option in the current context. Instead, this is hypothesised that proteinaceous antigens within the OMV contribute to this. Some proteins that are known targets of such antibodies, such as OmpD (34), are present within the OMV so may contribute to these effects, but other antigens are likely to contribute. Nevertheless, more work is needed to identify the antigens in wbaP-OMV that are protective and whether enriching for these antigens within OMV enhances protection to wbaP-OMV.

Previous reports are not consistent in ascribing a protective value to levels of cross-reactive antibodies induced after immunisation with truncated LPS mutant bacteria, OMVs and OMPs (235, 256, 257, 258). It was found that boosting had a dramatic impact on levels of cross-protection, despite there being only modest increases in anti- *S*. Typhimurium, LPS or porin-specific antibodies between day 35 after priming and day 21 after boosting. Moreover and counterintuitively, the greatest fold reduction in bacterial numbers was observed for the WT and wzy-OMV groups, rather than for the wbaP-OMV groups. This would suggest that the improved efficacy is not due to changes in titres against core oligosaccharide or lipid A. Multiple reasons could explain these observations, none of which are mutually exclusive. One possibility is that boosting with the two O-antigen containing OMVs increases antibodies against other O-antigen species common to *S*. Typhimurium and *S*. Enteritidis (O1 and O12). The literature is limited on the value of such antibodies, but most studies suggest that antibodies to O12 for instance are generally less protective than antibodies to the O4 and O9 moieties of *S*. Typhimurium and *S*. Enteritidis respectively (206, 240, 274). Additionally, after natural infection there is little cross-protection observed (228) suggesting that anti-O1 or O12 antibodies only make a minor difference. The working hypothesis on these findings is that boosting increases germinal centre-mediated selection of cross-reactive antibodies that do not target any component of LPS. It is proposed that this process is enhanced through the acquisition of mutations in the antibody sequence in both the complementarity determining regions of the antibody but also in the mainframe resulting in increased access to conserved, but normally inaccessible epitopes. If so, then the consequences are this are that not only would the target antigen be bound with greater affinity, but that access through the LPS barrier to the antigen is enhanced. This could broaden the range of antigens targeted within the proteinaceous component of the OMV, such as conserved epitopes within porins, which

are made less accessible by the presence of O-antigen (12). Nevertheless, it is important to note that the final formulation of a vaccine against non-typhoidal *Salmonella* will include OMVs from multiple serovars and it is proposed that boosting with such an OMV-based vaccine would enhance cross-protection against a range of different *Salmonella* serovars and potentially other Gram-negative bacteria not included in that vaccine. Encouragingly, there is precedent to this concept since epidemiological evidence suggests that vaccination with OMV derived from meningococococcus group B may provide some cross-protection against gonococcal infections (112, 244). Thus, OMV-based vaccines have great potential to be tailored to regulate the extent of antibody responses induced to particular antigens and induce robust protection against disease caused by Gram-negative bacteria.

We recently examined the relative efficacy of different conjugated and non-conjugated Vi-based vaccines against *Salmonella* expressing Vi in a murine challenge model. Strikingly, vaccine-specific IgM or IgG alone were sufficient to provide protection (275). Here, this concept were examined by immunising mice that do not secrete serum IgM with different OMV and challenging with attenuated *S*. Typhimurium. Immunised mice had lower bacterial burdens that non-immunised mice and this demonstrates that IgM is not essential for protection to OMV either.

Finally, it was showed that after immunisation, and in the timeframes assessed, T-bet was not required to suppress clinical signs. Mice had low bacterial burdens and were well and active. Thus, if bacterial burdens are maintained at a low enough level, then it suggests Th1 cells are not needed to maintain control. Nevertheless, the bacterial burdens in T-bet$^{-/-}$ mice at day 28 were higher than in their WT mice counterparts. This may mean that over a sufficiently protracted period then bacterial outgrowth occurs and that they eventually succumb to

infection. Alternatively, it may mean that chronic, non-resolving, infections are maintained in these mice but that they never get sick. Lastly, alternative mechanisms may control such chronic bacterial infections and that they finally resolve. More studies are needed to interrogate this further.

Chapter 5. FUCTIONAL CHARACTERISATION OF MONOCLONAL ANTIBODIES DIRECTED AGAINST OUTER MEMBRANE PROTEIN D (OmpD) OF *S*. TYPHIMURIUM

5.1 Introduction

Salmonella outer membrane antigens play a crucial role in the pathogenesis and immune response against the infection (34, 226). It was reported in the previous chapters that antibodies induced in responses to OMV can potentially protect against *Salmonella*. It was also showed how these antibodies interacted with different *S*. Typhimurium surface antigens and how the O-Antigen in LPS influences access to other epitopes such as porins. Therefore, the generation of monoclonal antibodies (mAbs) that specifically target epitopes such as outer membrane protein D (OmpD), coupled with the creation of structural models to simulate the interactions between LPS and OmpD, would provide a better understanding of the impact of substitutions on the loss of binding and the resulting alterations in the architecture of this protective epitope. Additionally, understanding how antibodies specific to certain *S*. Typhimurium antigen responses to *Salmonella* and other *Salmonella* outer membrane antigens are regulated would help in vaccine development. This chapter aimed to explore the functional characteristics of monoclonal antibodies specific to *S*. Typhimurium -OmpD and identify the level of binding to *S*. Typhimurium antigens and different *Salmonella* strains.

Specific monoclonal antibodies to *Salmonella* antigens have emerged as valuable tools for studying and combating infectious diseases. The presence of antibodies targeting the LPS O-antigen, outer membrane proteins, and flagellar protein in response to *Salmonella* infection has been extensively studied (32, 276). Specific antibodies against the O-antigen have been

shown to play a crucial role in protection, as demonstrated by monoclonal antibody studies and experiments using heat-killed invasive *Salmonella* in mice (204, 207). Monoclonal antibodies against the O-antigen have been found to impair the serum killing of *Salmonella* and can directly inhibit bacterial killing by serum from healthy individuals (206). Furthermore, mice receiving a passive transfer of monoclonal antibodies generated against smooth LPS- but not rough LPS lacking O-antigen- provided protection against infection. mAbs that likely targeted epitopes found in porin-LPS complexes demonstrated protection against both endotoxemia and mouse typhoid (204). The findings provide evidence for the involvement of antibodies against LPS O-chains and porin-LPS complexes in the induction of acquired resistance against *S*. Typhimurium infection.

In this chapter, mAbs using a preparation of OMPs enriched for S. Typhimurium OmpD were generated. The specificity of each antibody clone was determined against *S*. Typhimurium OMPs, *S*. Typhimurium WT, or *S*. Typhimurium ΔwbaP. Five monoclonal antibodies were characterised by Western immunoblots, and their binding levels to *S*. Typhimurium antigens and different *S*. Typhimurium mutants were assessed by ELISA. Additionally, the ability of the monoclonal antibodies to kill *Salmonella* in vitro was examined. Finally, an attempt was made to raise mAbs against a mixture of OMV from the different *S*. Typhimurium mutants and *S*. Typhimurium -OpmD. Intriguingly, despite inducing very high levels of serum antibodies, no mAbs were generated. These studies are not discussed further.

5.2 Results

5.2.1 Generation of monoclonal antibodies specific to *S*. Typhimurium -OmpD

To generate anti-OmpD mAbs, female BALB/c mice were immunised and boosted *i.p.* with 5 µg of OmpD from *S.* Typhimurium on day 1 and day 14. Each mouse was given a third dose, and spleens were collected on the third day after the last immunisation. Fusions were carried out as described previously (223), and this work was performed in collaboration with Dr. M. Goodall, in Monoclonal Antibody Production Unit, University of Birmingham. Hybridoma supernatants were initially assayed by ELISA for IgG production against OMP and whole cells of WT *S*. Typhimurium and *S*. Typhimurium ΔwbaP, which expressing wild-type length O-antigens or no O-antigen, respectively. Five Hybridomas were selected for clone expansion.

5.2.2 Characterisation of monoclonal antibodies raised against *S*. Typhimurium-OmpD

We characterised a panel of five mAbs to the OmpD of *S*. Typhimurium. These mAbs were tested for reactivity with different *S*. Typhimurium antigens, including LPS, OmpD, and OMVs, from the three strains used in the previous chapters (Figure 5.1). Data showed that three mAbs, 1.326, 1.36, and 1.105, reacted with OMVs and did not target the O-antigen (Figure 5.1 A, B, and C). However, mAb 1.143 showed an increase in the reactivity with OMV as the O-antigen chain decreased. Furthermore, it was the only monoclonal with a high binding with *S*. Typhimurium -OmpD (Figure 5.1 D). This may indicate that mAb 1.134 was a monoclonal antibody specific to OmpD. A weak reactivity of mAb 1.112 was observed with *S*. Typhimurium -LPS (Figure 5.1 E).

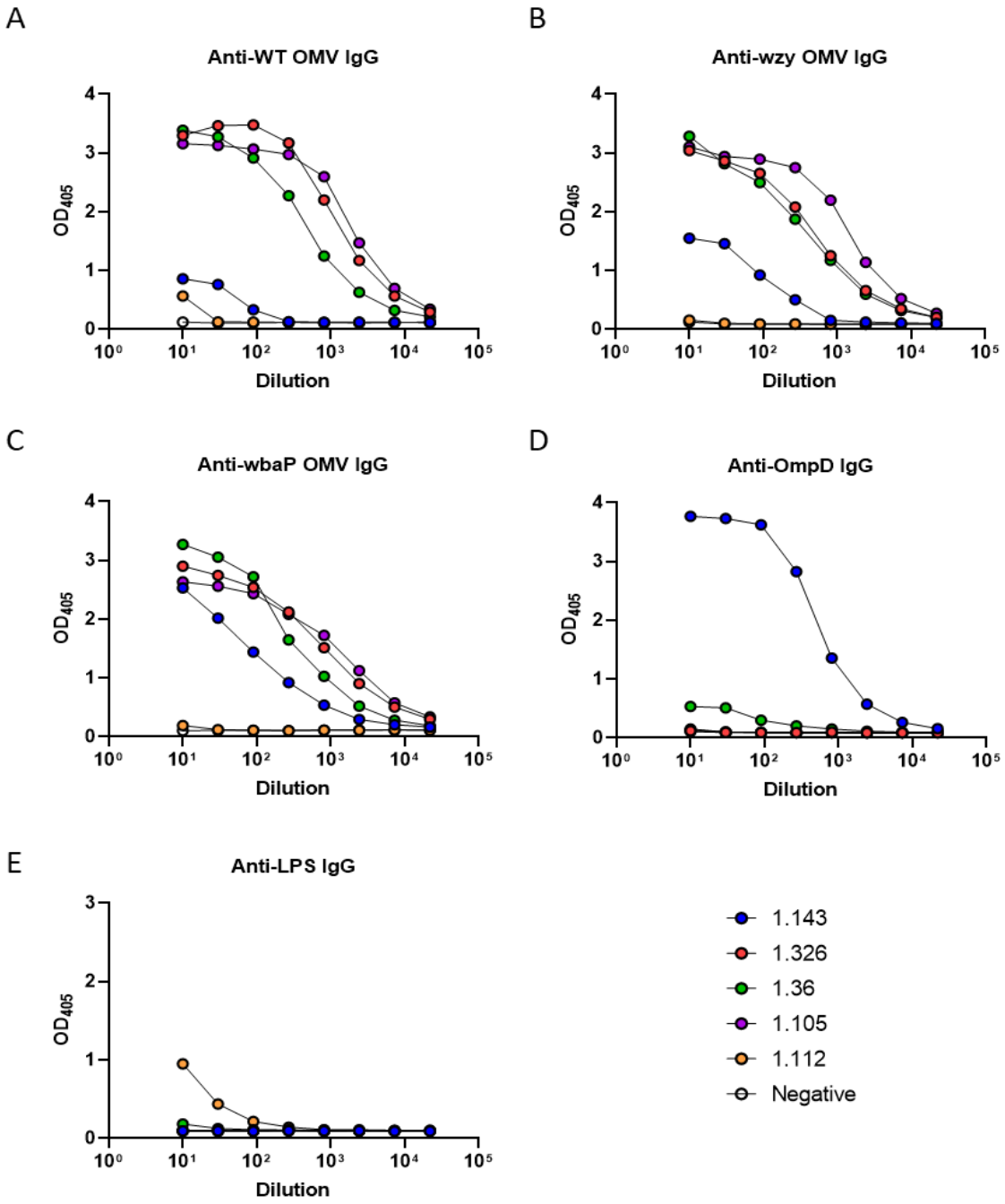


Figure 5.1 Binding levels of monoclonal antibodies raised against STm-ompD to different STm antigens.

ELISA was done to measure the reactivity of monoclonal IgG with (A) WT-OMV, (B) wzy-OMV, (C) wbaP-OMV, (D) STm-OmpD, and (E) STm-LPS.

These mAbs were further tested for binding to the whole bacteria of the virulent *S*. Typhimurium (D23580) and *S*. Enteritidis (D24954), as well as mutant *S*. Typhimurium and *S*. Enteritidis, which lack O-antigen chain (Figure 5.2). A very weak reactivity of mAb 1.143, 1.326, 1.36, and 1.105 was observed with the intact bacteria of *S*. Typhimurium (Figure 5.2 A), while this reactivity increased when the O-antigen chain was missing (Figure 5.2 B). This phenomenon was also observed with *S*. Enteritidis and *S*. Enteritidis lacking O-antigen for mAbs 1.143 and 1.36 (Figure 5.2 C and D). This supports the fact that the O-antigens might impede access to other OM antigens. Reactivity of mAbs were summarised in table 5.1.

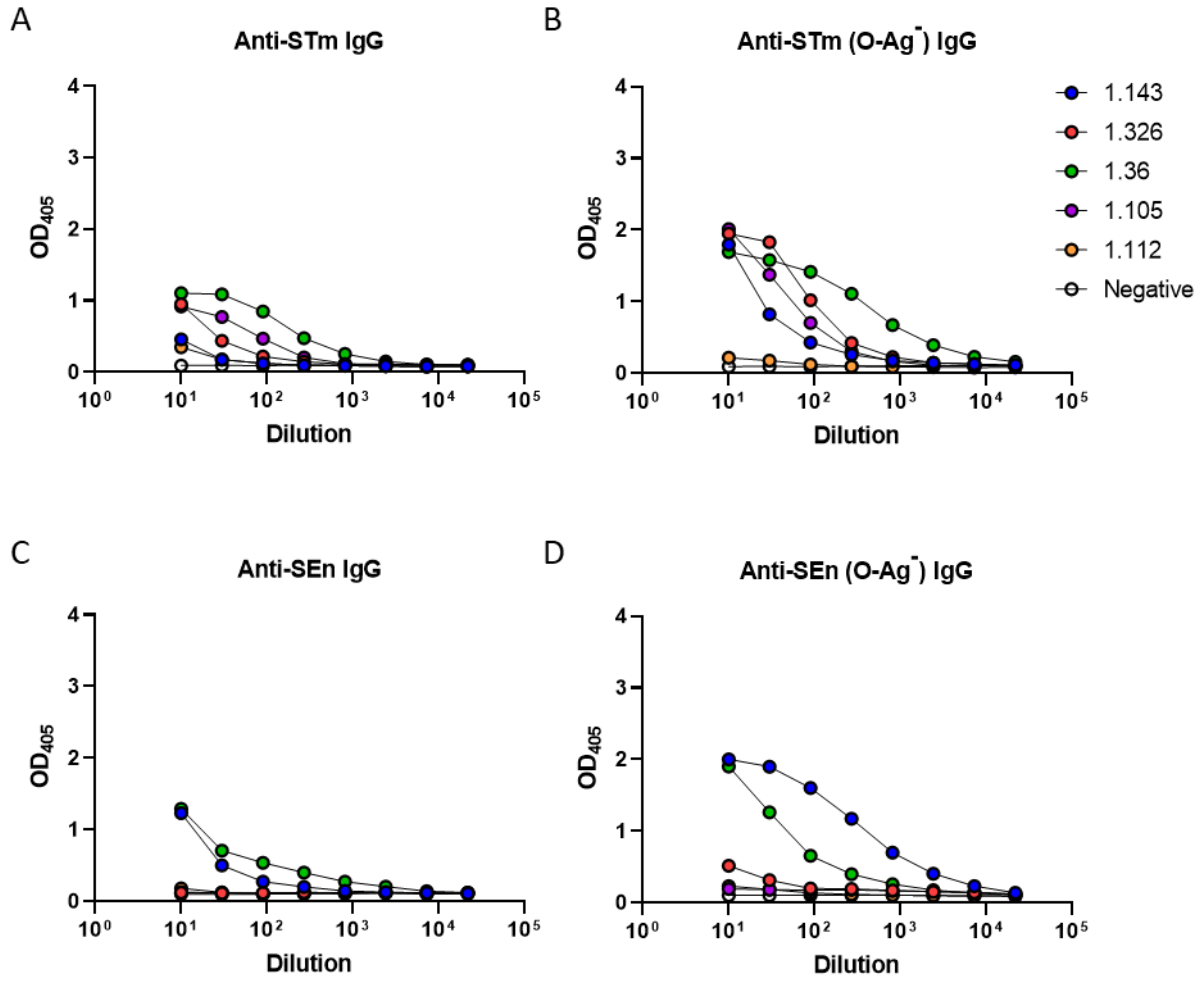


Figure 5.2 Binding levels of monoclonal antibodies to whole cells of different *S*. Typhimurium and *S*. Enteritidis mutants.

The reactivity of monoclonal IgG antibodies raised to Stm-OmpD was assessed by ELISA with (A) the virulent strain of STm (STm D23580), (B) the virulent strain of STm lacking O-antigen (STm D23580 ΔwbaP), (C) the virulent strain of SEn (SEn D24954), (D) the virulent strain of SEn lacking O-antigen (SEn D24954 ΔwbaP)

Table 5.1. Reactivity of mAbs with STm- LPS, OmpD, OMVs, and whole cells of *S*. Typhimurium and *S*. Enteritidis measured by ELISA*

	LPS	OmpD	WT-OMV	Wzy-OMV	wbaP-OMV	STm	STm ΔwbaP (O-Ag-)	SEn	SEn ΔwbaP (O-Ag-)
1.143	-	+	-	+	+	-	+	+	+
1.326	-	-	+	+	+	+	+	-	-
1.36	-	-	+	+	+	+	+	+	+
1.105	-	-	+	+	+	+	+	-	-
1.112	+	-	-	-	-	-	-	-	-

* Plates were coated with 100 µl of intact cells (10 µg/ml), porins (5 µg/ml), and LPS (5 µg/ml). Antibodies were used with an initial dilution at 1:10 for a serial dilution of 3-fold. Positive reactions in ELISA were scored weakly positive (+) or strongly positive (**+**) if the absorbances were greater than two times (but less than three times) the background or greater than three times the background, respectively. Each ELISA was repeated twice.

Isotype testing was performed for these mAbs against wbaP-OMV (Figure 5.3). Two were identified as IgG1 (mAb 1.326 and 1.36) (Figure 5.3 A), and two were identified as IgG2a (mAb 1.143 and 1.105) (Figure 5.3 B).

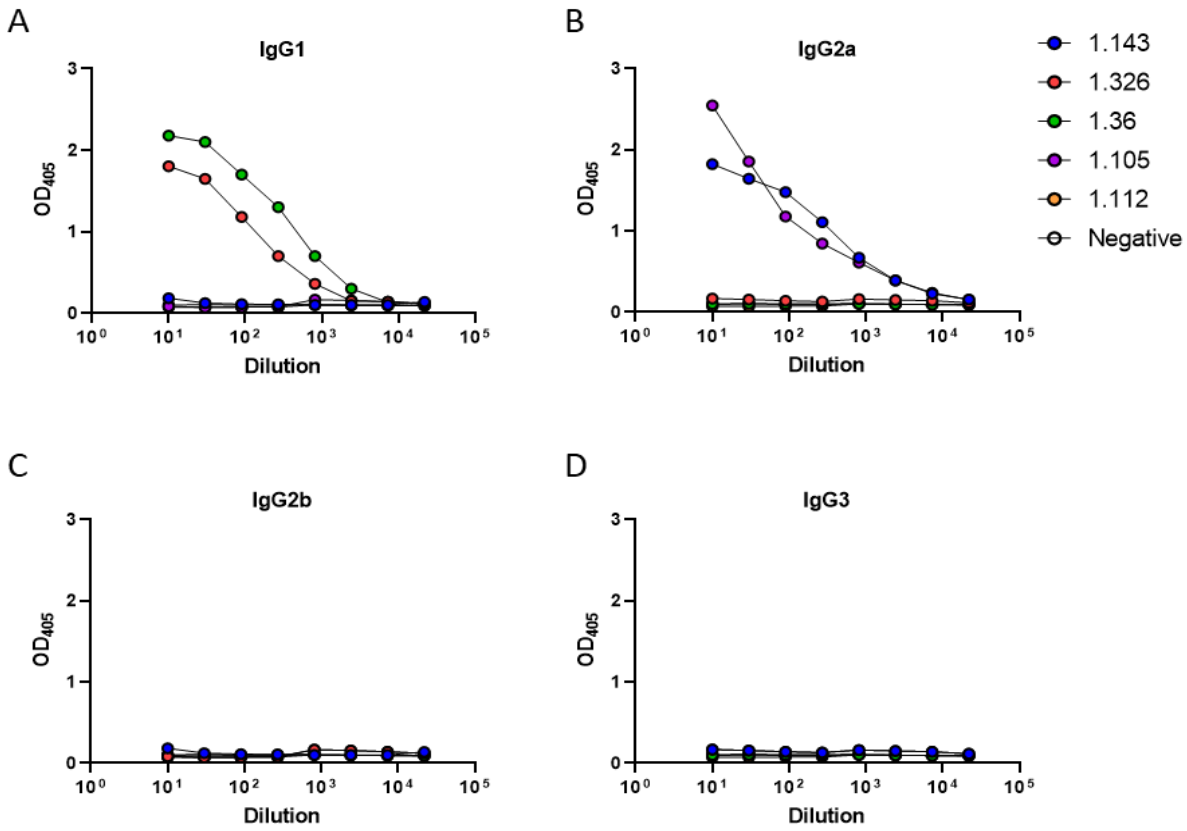


Figure 5.3 Isotype testing of monoclonal IgG antibodies.

ELISA was performed against wbaP-OMV to identify the isotype of each monoclonal antibody. Among these, two monoclonals were identified as IgG1 (1.36 and 1.326) (A), and two were identified as IgG2a (1.143 and 1.105) (B). No monoclonals were identified as IgG2b or IgG3 (C) and (D)

The specificities of the mAbs raised in this study were also determined by Western blot against wzy-OMV (Figure 5.4). Wzy-OMV was subjected to SDS-PAGE and stained with Coomassie blue (Figure 5.4 A). A serum from a mouse immunised with wzy-OMV was used as a positive control (Figure 5.4 B). MAb 1.143 reacted strongly with the OmpD in both ELISA and Western blot (Figure 5.4 C). Other mAbs, 1.326, 1.36, and 1.105, are likely to recognis the same antigen (Figure 5.4 D, E, and F). This epitope recognized by these mAbs are probably buried in the OMV, but not in the LPS.

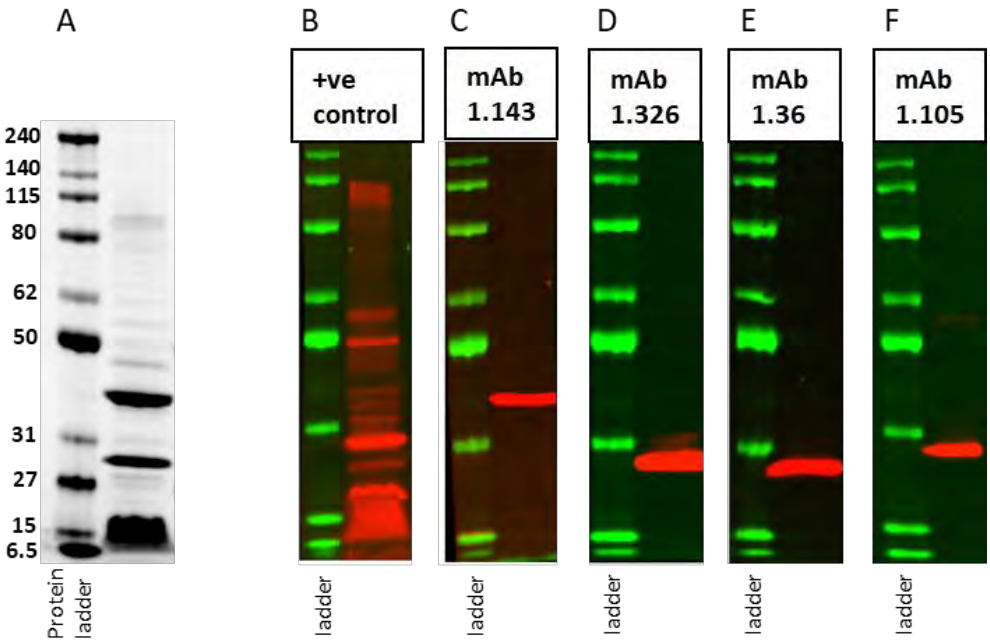


Figure 5.4 Reactivity testing of mAbs with wzy-OMV by Western blot.

(A) Wzy-OMV was subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was cut into strips and incubated with the following: Serum from a mouse immunised with wzy-OMV as a positive control (B), mAbs 1.143 (C), mAbs 1.326 (D), mAbs 1.36 (E), and mAbs 1.105 (F).

Bactericidal potential of these mAbs against the virulent strain of *S*. Typhimurium D23580 was assessed in vitro by SBA, using human complement as an exogenous complement source. No mAbs showed bactericidal activity against *S*. Typhimurium D23580.

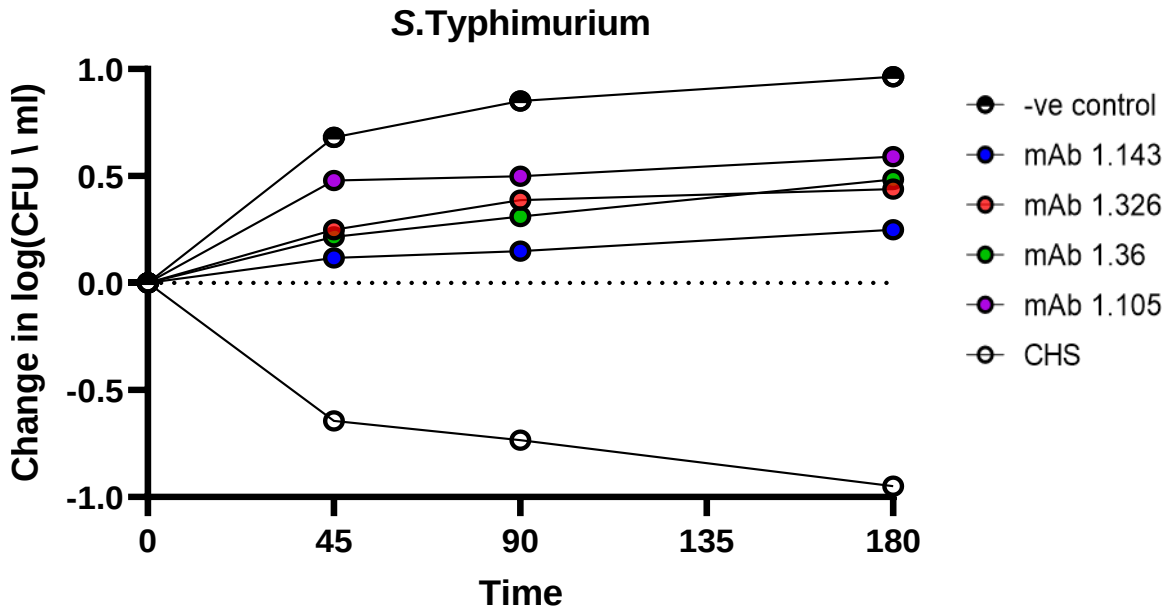


Figure 5.5 Bactericidal activity the mAbs against the virulent strain of S. Typhimurium.

SBA was performed to measure the bactericidal activity of each mAb against the virulent STm (STm D23580) with Ab-depleted human serum as a complement source. Data represented changes from the starting Log CFU/mL bacterial dose over time. Complete human serum (CHS) was used as a positive control.

5.3 Discussion

Our recent publication and the work within the other results chapters showed the importance of targeting specific epitopes for protection and the "shield" effects of LPS O-antigen (12). To study this further, the focus was Initially placed on generating monoclonal antibodies to a preparation of OMPs highly enriched for OmpD. Only one mAb showed unequivocal binding to OmpD (1.143). Consistent with our previous work (12) showing the importance of shielding by O-antigen, this bound relatively poorly to the surface of *S*. Typhimurium or *S*. Enteritidis, unless O-antigen was absent in which case binding was enhanced. This suggests that the epitope target by this mAb is one that is conserved between *S*. Typhimurium and *S*. Enteritidis. Furthermore, this mAb was not bactericidal in an SBA despite being of an IgG2a isotype, the murine isotype most associated with complement activation. This may mean that this epitope is not one targeted by protective antibodies or that multiple epitopes need to be targeted for protection. Indeed, a major conclusion from this chapter is that most of the mAbs generated showed enhanced binding to the bacterial surface in the absence of O-antigen. Moreover, most mAb bound OMV strongly, and even WT-OMV contain shorter O-antigen than WT bacteria counterparts (116). This could be because these antigens are more readily accessible or enriched in OMV than on the bacterial surface or because the shorter LPS provides steric hinderance that restricts binding. Nevertheless, despite us not identifying the target for all of these mAbs due to time constraints, it is clear these antibodies provide valuable tools to investigate the role of LPS and O-antigen in occluding access to the bacterial surface.

An intriguing mAb is 1.112 which showed consistent, but weak, binding to LPS but no binding to *S*. Typhimurium or *S*. Enteritidis bacteria. Indeed, this mAb did not bind O-Ag-deficient mutants of *S*. Typhimurium or the different OMV. This suggests weak binding or an unstable

epitope, or possibly one that is generated through the juxtaposition of two distinct antigens. In contrast to the weak binding of 1.112, the mAb 1.326, 1.36, 1.105 all bound *S*. Typhimurium and OMV strongly. Based on the western blot results, it is probable that these mAb target the same antigen. Interestingly, all three mAb bound *S*. Typhimurium, but only two bound *S*. Enteritidis, suggesting some antigen variability between the target antigen in *S*. Typhimurium and *S*. Enteritidis. Notably, loss of O-antigen on the bacteria is again associated with enhanced binding to the bacterial surface.

None of the mAb individually or collectively (not shown) induced killing in the SBA. This demonstrates the difficulty in generating bactericidal antibodies and collectively is consistent with the data presented throughout this thesis that generating protective antibodies to non-O-antigen targets, although possible, is difficult. Within this statement, however, is the caveat that the isotype of the antibody matters. This is potentially of most importance to non-O-Ag antigens, as has been shown previously (12), where the structural characteristics of the antibody, i.e. its ability to "reach" and bind its target, in conjunction with its effector function is important. Thus, these antibodies may be more active and beneficial *in vivo,* where the mechanisms of action are less well-known.

Chapter 6. FINAL DISCUSSION

The aim of this chapter is to bring some key concepts together that overlap the different results chapters, discuss the limitations of the work, also identify future studies that could be valuable or of interest to perform. Most of the work has been discussed and contextualised in detail in each Results chapter.

In this thesis, the work detailing how immune responses to OMV have used to understand the immune response to *Salmonella* antigens and the protection afforded to these antigens after immunisation. To do this, OMVs wrer generated, which have truncated or lack O-antigen chains. All OMV types contain similar protein antigens within and induce similar “high-level” responses in terms of plasmablast(s) and germinal centres. Nevertheless, the different O-antigen content clearly impacted the level of responses to the individual protein antigens, as the anti-porin data demonstrates. The protection data suggests that these changes are not sufficient for the wbaP-OMV to drive equivalent protection as the O-antigen+ OMV to the parent serovar. This demonstrates how important O-antigen can be against the homologous serovar. However, since there was the least cross-protection against *S*. Enteritidis afforded by immunisation with a booster dose of wbaP-OMV, it also suggests other factors may also be important. It is hypothesised that the O-antigen itself is interacted with directly by non-LPS antigens to alter epitope stability, and it is proposed that the lack of "rigidity" afforded by O-antigen interactions could result in a diminished antibody response to "protective" epitopes, while the overall antibody responses to protein antigens remain equivalent. This is further supported by the relatively greater protection seen to *S*. Enteritidis after immunisation with wzy-OMV. In ways not yet known, this may indicate that OMV gets processed intracellularly

for presentation in GC in a more molecularly constrained way, increasing the likelihood of selecting protective epitopes.

The combined studies on protection and immunogenicity and examination of the immune response to the OMV in terms of the antibody response to component antigens within OMV allowed me to interrogate more deeply the contribution of non-O-Ag to protection. Although there were elevated responses to protective antigens like porins, these were insufficient to compensate for the loss of O-antigen. This is likely to be that although detectable within OMV, they make up an insufficient proportion of the OMV protein mass to induce enough protective epitopes, despite the overall anti-porin response being substantial. There has been little effort to optimise protein content within OMV, with the exception of 4CMenB, where the response to PorA is probably the key contribution anyhow and where MenB expresses a LOS rather than an LPS. Additionally, OmpA in all of the OMVs was readily detected and has been in other OMVs too, yet antibody to this protein is not strongly associated with protection. Therefore, optimisation of OMV to enrich selectively the protein content is important.

The finding that O-antigen chains were not necessary to induce an anti-O-antigen response equivalent to that of WT-OMVs was a key finding in this study. Moreover, the level of protection afforded by these O-antigen+ OMVs was equivalent. This is likely to be because a single O-antigen is sufficient size to generate a single epitope (personal communication Prof. Vass Bavro, School of Biological Sciences, University of Essex). These studies do not inform on the diversity of the response, i.e., does having longer O-antigens result in a more diverse B cell response and if it is more monoclonal. They do, however, demonstrate the importance of using structural studies to contextualise surprising findings. This has been done previously

with our original studies examining immune responses to OmpD and has also been widened here to include how O-antigen may relate to B cell epitopes and immunoglobulin structure.

Although the immune response to OMV was examined in detail, the immune response at different times post-immunisation was not looked at in great detail. This is likely to be important as total antibody responses may wane over time, yet protective antibody responses be maintained. Assessing this further would require a combination of studies again examining immune responses in the germinal centre alongside the plasma cell output over time. The SBA provides a convenient way to test these whilst reducing the number of animal studies. In particular, immunisation with OMV from *S*. Typhimurium and examining the killing of heterologous serovars such as *S*. Enteritidis would be a valuable tool here as it would be possible to standardise for total antibody levels and numbers of immunisations. However, a complication in such studies is that the responses to multiple antigens would need to be incorporated and so interpreting results may be complicated by this. These studies generating and testing the monoclonal antibodies were revealing as it suggests that identifying single epitopes that are protective may be difficult. For vaccines, this emphasises the importance of polyclonal responses, and for immunotherapy indicates that when not targeting O-antigen, there may be significant challenges in identifying target molecules. In addition, the work with monoclonals showed how limited the value of examining responses to purified proteins is and how examining binding to the whole organism is important. It also highlights how O-antigen provides a powerful barrier that needs to be overcome for antibodies to bind. Lastly, and possibly crucially, the binding of these antibodies was clearly demonstrated in WT bacteria, yet these mAbs were not bactericidal in the presence of human complement. This is an important finding as it suggests that binding of the bacterial surface is not sufficient on its own

to promote killing, at least not in the context of this SBA. Potential future experiments could test this using adoptive transfer or opsonisation of monoclonal antibodies followed by a challenge.

When developing a candidate vaccine, it is crucial to achieve a balance between stimulating an optimal protective immune response and minimising undesirable inflammatory reactions such as systemic reactogenicity. While LPS present in *S*. Typhimurium -OMV has the ability to stimulate the production of protective antibodies, it also exhibits the disadvantage of being pyrogenic and potentially inducing reactogenicity. Modification of OMV LPS lipid A resulted in a reduction of inflammatory cytokines in response to OMV compared to the unmodified version (107). In this project, reactogenic effects following OMV vaccination in mice were consistently observed, primarily characterised by weight loss *in vivo*. However, human studies on *Shigella* OMV with penta-acetylated lipid A demonstrated a correlation between dose and local reactions. It also showed that even at a high dose (5.9/100 μg), the vaccine exhibited an acceptable reactogenicity profile, which was lower than that reported for the licensed OMV-based vaccine (4CMenB)(277).

6.1 Future studies

The inherent qualities of OMV suggest their potential to trigger robust immune responses. However, our understanding of OMV's mode of action remains limited, especially in humans. Further research is needed to elucidate the mechanisms by which OMV operate, which will enhance the development of OMV-based vaccines. Clinical studies are essential to validate preclinical findings. Despite the broad application of the OMV platform in preclinical studies for various pathogens, only a select few OMV vaccine candidates have progressed to clinical trials. This discrepancy likely stems from the relative unfamiliarity with the OMV platform

compared to traditional vaccine technologies, regulatory hurdles in novel vaccine development and production, and the analytical complexity of OMV-based vaccines compared to more straightforward subunit vaccines (99).

OMV has demonstrated its versatility as a vaccine platform, with numerous instances of displaying both protein and polysaccharide antigens. Utilising OMV decorated with antigens from a different pathogen offers the potential for developing vaccines effective against multiple pathogens. However, achieving this requires expertise and time for genetic manipulation not only to express antigens but also to control blebbing and minimise reactogenicity without compromising other essential immunogenic features of OMV. For instance, mutations aimed at enhancing blebbing or altering lipid A structure may inadvertently reduce levels of LPS O-antigen, which is a crucial target for protective immunity, or lead to compensatory mutations maintaining lipid A acyl chain numbers and thus endotoxicity (106, 116).

Recently, OMV have been investigated for various applications such as cancer immunotherapy, drug delivery, and carrying viral antigens, thereby broadening their utility. Several instances have demonstrated that modified OMV can target tumour tissues, trigger an immune response against tumours, and ultimately eliminate them following intravenous injection in mice (278). An interesting observation was reached by a previous researcher within our group; it was that the reactogenicity of fragmented Vi conjugated to lipid A detoxified GMMA (dGMMA-fVi) was significantly reduced compared to standard GMMA. It has been noted that the deletion or overexpression of specific genes involved in the LPS biosynthesis pathway could provide a better solution for reducing endotoxin activity (256, 279). The purified OMVs described in this project were all generated from Δ*tolR* mutants of *S*.

Typhimurium. Further engineering these OMVs by deleting endogenous acylating enzymes might help produce vaccine candidates that are potentially less reactogenic.

The nature of the immune response to polysaccharides antigen can be influenced by the balance between Th1 and Th2 responses. Th1 responses typically involve the activation of macrophages and cytotoxic T cells, which are important for clearing intracellular pathogens. Th2 responses, on the other hand, produce IL-4, IL-5, and IL-13 and induce B cell activation and differentiation, and the production of antibodies. In this project, the primary focus has been on examining the B-cell response, specifically the antibody response, although there are other important immunological aspects that deserve further investigation. For instance, it is essential to characterise the involvement of some cytokines such as IFNγ and TNFα in response to *S*. Typhimurium -OMV immunisation as they play significant roles in the protection against live *S*. Typhimurium (280). Additionally, the investigation only delved into the role of T-bet in protection, whereas exploring T-cell effector subsets and T-cell memory would provide valuable insights into different facets of how *S*. Typhimurium -OMV confers protection. Furthermore, AID KO mice could also be utilised to understand whether IgM and IgG induced by OMVs are independently protective.

6.2 Final statement

In conclusion, in this work, the immunological response to *S*. Typhimurium -OMV and the effect of the reduction of O-antigen expression of the OMV on protection against *Salmonella* infection were investigated. The relevance of how antigens are presented were also highlighted. The extensive immune response to *S*. Typhimurium -OMV is due to its ability to mimic the surface of live bacteria, presenting antigens in a way that efficiently triggers the immune system. One significant finding in this project was that all OMV induced similar plasma

cell and germinal centre responses, while antibodies are not induced with similar kinetics to different antigens. In addition to this, more significant responses to non-O-antigens developed when O-antigen expression is limited. Furthermore, immunisation with OMV containing single O-antigen repeats induced protection against *S*. Typhimurium comparable to that induced by OMV with variable O-antigen length. Boosting can enhance the level of cross-protection against related *Salmonella* serovars.

In addition, the functional characteristics of mAbs raised against *S*. Typhimurium -OmpD were explored and the level of binding to *S*. Typhimurium antigens and different *Salmonella* strains were identified. Among the mAbs tested, only one exhibited clear binding to OmpD. However, the binding to the surface of *S*. Typhimurium or *S*. Enteritidis was relatively weak. In the absence of the O-antigen, the binding was enhanced, demonstrating the shielding effect of the O-antigen on outer membrane antigens. Furthermore, most mAbs bound OMV stronger than their binding to the intact bacteria. None of the mAb induced killing against *S*. Typhimurium in an in-vitro assay.

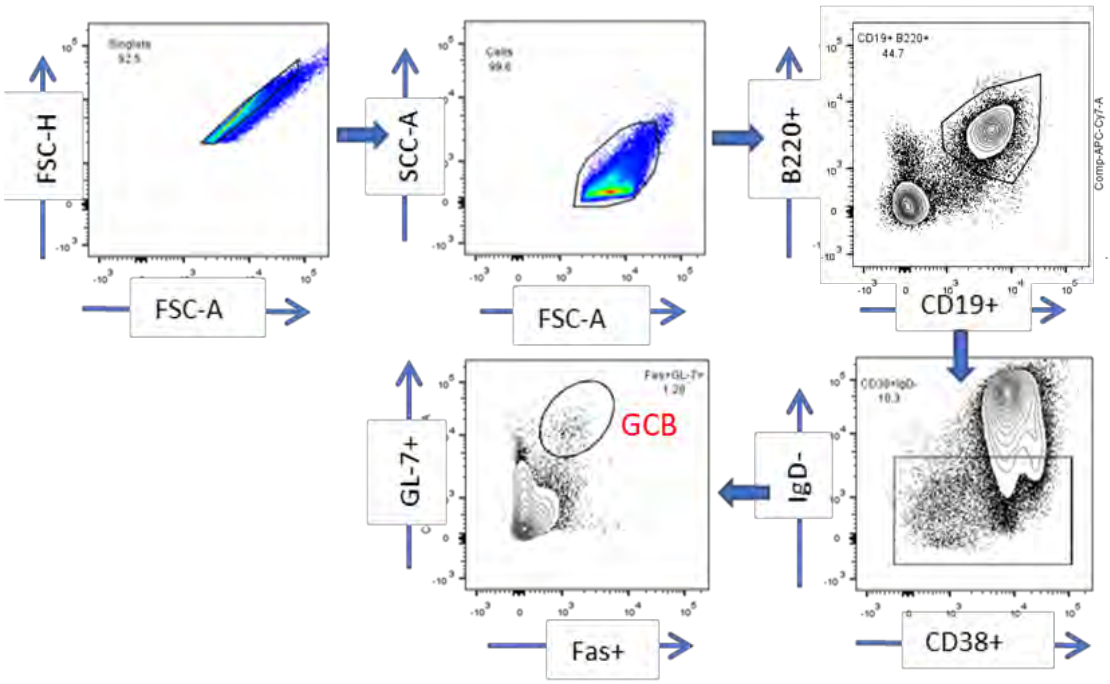
APPENDIX A - ABBREVIATIONS

Ab	Antibody
AF	Alexafluor
Ag	Antigen
AID	Activation-induced deaminase
Amp	Ampicillin
AP	Alkaline phosphatase
APC	Allophycocyanin
APCs	Antigen presenting cells
ASC	Antibody secreting cell
BCA	bicinichonic acid assay
BCIP/NBT	Bromo-4-chloro-3-indolyl phosphate / Nitro blue tetrazolium
BCR	B cell receptor
BSA	Bovine serum albumin
BV	Brilliant Violet
CD	Cluster of differentiation
CFU	Colony forming unit
DC	Dendritic cell
EF	Extrafollicular
eF	eFluor
ELISA	Enzyme-linked Immunosorbent assay
ELISPOT	Enzyme-linked ImmunoSpot
FACS	Fluorescence-activated cell sorting
FBS	fetal bovine sera
Fc	Antibody fragment crystallisable
FcγR	Antibody fragment crystallisable gamma receptor
FDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate
FO B	Follicular B cells
GC	Germinal centre
GCB	Germinal centre B-cell
GMMA	Generalized modules for membrane antigens
HI	Heat inactivated
HIV	Human immunodeficiency virus
i.p.	Intraperitoneal
i.v.	Intravenous
iCOS	Inducible nitric oxide synthase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
iNTS	invasive Non-Typhoidal *Salmonella*

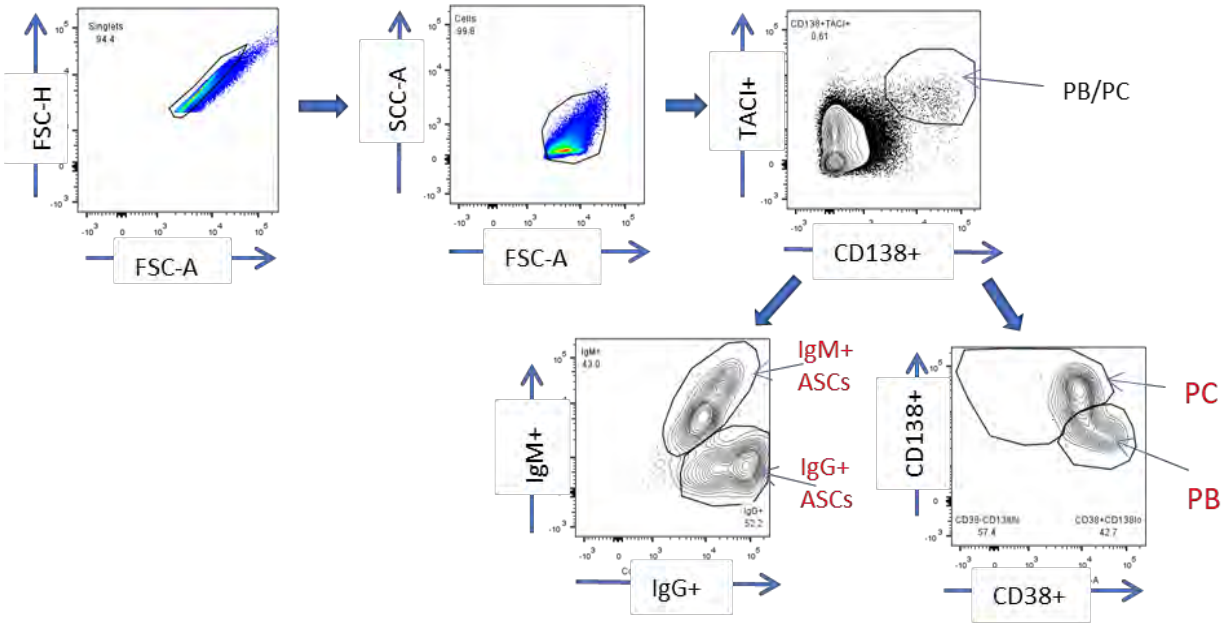
kan	Kanamycin
kDa	Kilodalton
KO	Knock out
LB	Luria Bertani
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation primary response gene
MZ	Marginal zone
MZB	Marginal Zone B cell
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium Bicarbonate
NK	Natural Killer
NLR	NOD-like receptor
Nramp-1	Natural resistance associated macrophage protein 1
NTS	Non-typhoidal *Salmonella*
O-Ag	O-polysaccharide antigen
OD	Optical density
OMP	Outer membrane porin
OMV	Outer membrane vesicle
OVA	Ovalbumin
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PC	Plasma cell
PCR	Polymerase chain reaction
PeCy7	Phycoerythrin-Cyanine7
pNPP	p-Nitrophenol phosphate
PRR	Pathogen recognition receptor
R-10	RPMI 10% FBS
RPMI	Roswell Park Memorial Institute 1640 medium
SBA	Serum bactericidal assay
SCV	*Salmonella* containing vacuole
SEn	*Salmonella enterica* subsp. *enterica* serovar Enteritidis
SFU	Spot forming unit
SPI	*Salmonella* pathogenicity island
STm	*Salmonella enterica* subsp. *enterica* serovar Typhimurium
STy	*Salmonella* enterica subsp. enterica serovar Typhi
T3SS	Type 3 secretion system
TACI	Transmembrane activator and CAML interactor
TBS	Tris buffered saline
TCR	T cell receptor
TD	T-cell dependent
Tfh	T follicular helper
Th1	T helper 1
TH17	T Helper 17
Th2	T helper 2

TI	T-cell independent
TI-1	T cell independent type 1
TI-2	T cell independent type 2
TIR	Toll-interlekuin-1 receptor
TLR	Toll like receptor
TNF	Tumour necrosis factor
T reg	Regulatory T-cells
TRIF	TIR domain-containing adapter-inducing interferon β
Vi	Vi-capsule antigen polysaccharide
WT	Wild type

APPENDIX B - Flow cytometry gating strategy for B cell subsets

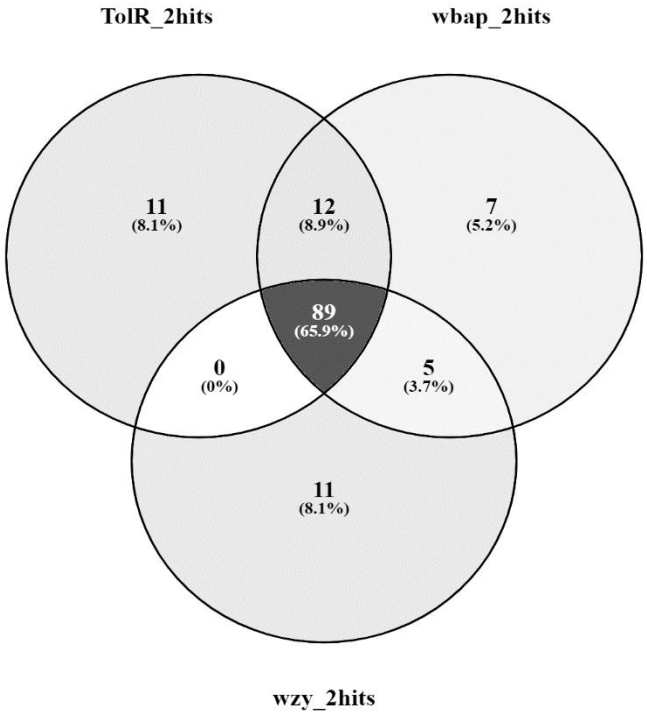


Singlets and then cells were isolated using forward and side scatter area and height parameters. B220 and CD19 were used to identify B cells. Germinal B cells (GCB) were identified as IgD- CD38+ GL-7+ Fas+ B cells.



Antibody secreting cells (ASC) were identified as TACI+ CD138+ single cells and separated into as either plasma cells (PC) and plasma blasts (PB) based on CD38 expression. IgG and IgM were used to identify the class of ASCs.

APPENDIX C - mass spectrometry



Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams.

https://bioinfogp.cnb.csic.es/tools/venny/index.html

TolR only	wbap only	wzy only	TolR + wbap	TolR + wzy	wbap + wzy
P52616	Q56111	Q8ZP20	P06179		A9N876
P52615	A9N8B8	B5FTN7	A9MVX4		Q5PK07
A9MFR9	A9MPV4	Q7CPK0	Q8ZNQ9		A9N240
P0A1J1	Q8Z740	Q8ZPD6	A9MRX7		Q8ZMB5
A9N0I9	A9MN51	P06175	A9MZ51		A9MH07
P35672	A9MFK9	Q8Z289	A9N0R7		
P0A283	P0A2A5	D0ZSY7	A9MN53		
Q7CQY4		Q7CR85	A9MNY0		
A9MN68		Q8ZQC4	A9MN70		
P0A297		A9N7A9	Q5PJK8		
Q8Z418		P67714	O54297		
			P40810		

TolR + wbap + wzy							
P02936	Q5PDE6	Q8ZP50	A9N4R0	Q06399	P0A1I1	P66950	P0CL43
Q8ZRP0	A0A0H3NBQ0	Q7CQN4	P39434	P06196	P58683	P40680	Q8ZRS2
Q8Z9A3	P37600	P40827	Q57JI4	Q8ZQZ7	P23988	O68901	A9MGK5
B5BC62	P26466	Q8ZR01	P50335	P23905	P64437	P63727	A9MN54
Q8ZRW0	A9N5G2	B5R535	A0A0H3N9T8	P55890	P0CW86	Q57I62	P66643
H9L451	P0A1X0	P26478	A9MZG4	O33921	P0A1Z2	Q8ZML1	
P37409	P26265	P0A2H9	A9MVX1	Q8ZQ08	B5BBD9	A9MR48	
P26982	P43669	P06202	E1WAU4	A9MP99	P26976	A9MRN3	
Q54001	Q56078	Q8ZR06	Q56030	P20753	A9MUZ0	Q8ZQX4	
Q8ZLB8	P19576	Q7CPZ3	P37723	A9N7X9	P0A1C5	Q8ZQM3	
P0A261	P0A2C7	P0A231	P0A2C5	Q8ZRJ9	P0A1T6	P26366	
A0A0H3NJI9	Q8ZNA5	P02910	P22107	B5R236	A9MHG0	Q9ZF60	

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