UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA

Facoltà di Scienze Matematiche, Fisiche e Naturali

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XXIV ciclo

GENERATION AND CHARACTERISATION OF AN OUTER MEMBRANE PARTICLE-BASED VACCINE AGAINST NON-TYPHOIDAL *SALMONELLA*

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Coordinatore: Prof. Marco Vanoni

Tutor: Dr. Christiane Gerke

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Abstract

Non-typhoidal *Salmonella enterica* (NTS) serovars are a major cause of bacteraemia in Sub-Saharan Africa, affecting mainly children and immunocompromised adults, leading to case-fatality rates as high as 24%. Despite the high burden of disease and increasing emergence of multidrug resistant strains, there are currently no vaccines available against NTS disease. Vaccination is the most cost-effective way of disease prevention, so a broad-spectrum, effective and affordable vaccine against NTS would be highly desirable. NTS vaccine research efforts have focused both on the development of live attenuated strains and subunit vaccines. Whereas live attenuated strains might pose an important safety problem in populations with a high number of immunocompromised adults as found in an African setting, O antigen (Oag)-based vaccines are serovar specific and might elicit an immune response that generates inhibitory antibodies against the LPS and subsequently impair *Salmonella* killing in humans. Protein-based vaccines might be the most suitable approach in NTS vaccine development. Several protein antigens have shown protection against *Salmonella* infection in mouse models and it has been shown that antibodies against the outer membrane proteins effect *Salmonella* killing in human serum.

In this study, we investigated the use of Generalised Modules for Membrane Antigens, or GMMA, as a broad-spectrum protein-based vaccine against NTS. GMMA are outer membrane particles naturally released by *Salmonella* during growth and contain high amounts of periplasmic and outer membrane proteins. GMMA are usually known in literature as outer membrane vesicles (OMV), but are distinct from detergent-extracted OMV in their generation, content, and immunogenicity.

GMMA are released in small amounts during *Salmonella* growth, so we genetically engineered *tolR* deletion mutants of two NTS serovars, *S*. Typhimurium strain SL1344 and *S*. Enteritidis strain P12109, for increased GMMA release. These GMMA were optimised for protein antigen delivery by deleting the Oag, through disruption of the *wbaP* gene, thus overcoming the serospecificity of the Oag and unmasking protein antigens on the GMMA surface. A further genetic deletion in the *msbB* gene was done to overcome the GMMA reactogenicity associated with the lipid A portion of LPS. Overexpression of the highly conserved *Salmonella* iron-regulated outer membrane proteins (IROMPs) on the GMMA was achieved by growing the GMMA-overproducing *S*. Typhimurium and *S*. Enteritidis mutants under iron-limiting conditions.

The proteomic content of *S*. Typhimurium and *S*. Enteritidis GMMA was characterised and, as expected, found to contain a high number of outer membrane and periplasmic proteins. *Salmonella* GMMA were also tested for their ability to elicit an immune response in mice and were determined to be highly immunogenic by both subcutaneous and intranasal immunisation routes, generating high levels of GMMA-specific IgG. Immunoblots using GMMA immune sera showed that IgG antibodies were raised against many *S*. Typhimurium GMMA proteins and that these were cross-reactive in *S*. Enteritidis. In vitro, however, the presence of an Oag layer in live *Salmonella* was shown to impair the binding of protein antibodies to the bacterial surface, an effect that was abrogated in *Salmonella* strains lacking Oag.

In a murine challenge model of acute *Salmonella* infection, *S*. Typhimurium and *S*. Enteritidis GMMA lacking Oag impaired *S*. Typhimurium infection in the spleen, indicating that a protein-based response was able to impair homologous and heterologous challenge, respectively. *S*. Typhimurium

GMMA with Oag provided the same protective effect as the positive control, the live attenuated strain *S*. Typhimurium SL3261.

This is the first time that heterologous protection against *Salmonella* infection has been demonstrated for GMMA, indicating that they have the potential to be developed as a broad-spectrum protein-based vaccine against NTS.

Riassunto

Salmonella enterica Non-Tifoidea (NTS) rappresenta una delle principali cause di batteriemia nell'Africa sub-sahariana che colpisce soprattutto bambini ed adulti immunocompromessi con tassi di mortalità maggiori del 24%. Nonostante l' elevata incidenza della malattia e l' emersione di ceppi multi resistenti agli antibiotici, non sono ad oggi disponibili vaccini contro NTS. La vaccinazione rappresenta il miglior metodo di prevenzione delle malattie per rapporto costi-efficacia pertanto un vaccino ad ampio spettro, efficace e conveniente contro NTS sarebbe auspicabile. Sforzi di ricerca su vaccini contro NTS si sono concentrati fino ad ora su due principali approcci: lo sviluppo di ceppi vivi attenuati o alternativamente lo sviluppo di vaccini a subunità. Da un lato un vaccino basato su ceppi vivi attenuati potrebbe rappresentare un importante problema di sicurezza nel contesto di popolazioni africane ad alto numero di adulti immunocompromessi. Dall' altro lato i vaccini a base dell'antigene polisaccaridico O (agO) del lipopolisaccaride (LPS) batterico sono sierotipo-specifici e potrebbero indurre una risposta immunitaria basata su anticorpi inibitori contro l' LPS non protettiva negli esseri umani. Vaccini a base proteica potrebbero rappresentare l' approccio più adatto nello sviluppo di vaccini contro NTS. Diversi antigeni proteici hanno dimostrato la capacità di indurre una risposta immunitaria protettiva contro l' infezione da *Salmonella* in diversi modelli murini ed è stato dimostrato che gli anticorpi contro le proteine di membrana batterica esterna nel siero umano sono responsabili della attività battericida contro *Salmonella* .

In questo studio, abbiamo valutato l'utilizzo di Moduli Generalizzati per Antigeni di Membrana (Generalised Modules for Membrane Antigens), o

GMMA, come vaccino proteico ad ampio spettro contro NTS. Le GMMA sono particelle di membrana esterna naturalmente rilasciate da *Salmonella* durante la crescita e contengono elevate quantità di proteine di membrana esterna e proteine periplasmatiche. Le GMMA sono comunemente conosciute in letteratura come vescicole di membrana esterna (OMV, per Outer Membrane Vesicles), ma si distinguono dalle OMV estratte con detergenti per la loro generazione, per i contenuti in termini proteici e per la loro immunogenicità.

Poichè le GMMA vengono rilasciate in piccole quantità durante la crescita di *Salmonella*, abbiamo modificato geneticamente due sierotipi di NTS (*S.* Typhimurium ceppo SL1344 e *S.* Enteritidis ceppo P12109) mediante delezione del gene *tolR*, al fine di indurre un maggiore rilascio di GMMA. Tali GMMA sono state ottimizzate per fungere da antigeni proteici abolendo l'espressione dell' agO nei ceppi produttori, mediante delezione del gene *wbaP*. In tal modo si evita l'immunodominanza serotipo-specifica dell' agO e si espongono maggiormente gli antigeni proteici sulla superficie delle GMMA. Una successiva delezione del gene msbB è stata effettuata per ridurre la reattogenicità delle GMMA associata alla regione del lipide A del LPS. La sovraespressione nelle GMMA delle proteine ferro-regolate della membrana esterna (IROMPs), altamente conservate in *Salmonella*, è stata raggiunta facendo crescere in condizioni restrittive di ferro i ceppi mutanti di S. Typhimurium e S. Enteritidis.

Il contenuto proteico delle GMMA di *S.* Typhimurium e *S.* Enteritidis è stato caratterizzato e, come previsto, è stata rilevata la presenza di un elevato numero di proteine di membrana esterna e di proteine periplasmatiche. Le GMMA di *Salmonella* sono state inoltre testate per la loro capacità di indurre una risposta immunitaria nel topo e sono risultate essere altamente immunogeniche, sia dopo immunizzazione per via sottocutanea che per via intranasale, generando alti livelli di immunoglobuline di tipo G (IgG)

GMMA-specifiche. Da analisi in Western blot, condotte utilizzando sieri immuni da immunizzazione con le GMMA, abbiamo dimostrato che le IgG sono dirette contro molte delle proteine delle GMMA di S. Typhimurium ed inoltrecross-regiscono con le proteine di S. Enteritidis. In vitro, tuttavia, la presenza di uno strato di agO sulla superficie di batteri vivi di *Salmonella* ostacola il legame degli anticorpi anti-proteina, un effetto abrogato nei ceppi di *Salmonella* senza agO.

In un modello murino di infezione acuta di *Salmonella*, abbiamo osservato un ridotto livello di infezione di *S.* Typhimurium nella milza dei topi precedentemente immunizzati con le GMMA senza agO di *S.* Typhimurium e di *S.* Enteritidis. Tali risultati indicano che la risposta immunitaria contro le proteine presenti nelle GMMA è in grado di compromettere l'abilità infettiva sia del ceppo omologo che del ceppo eterologo. L'immunizzazione con le GMMA contenenti l' agO di *S.* Typhimurium ha conferito lo stesso effetto protettivo dell' immunizzazione con il ceppo vivo attenuato di *S.* Typhimurium SL3261, utilizzato come controllo positivo..

Nel presente studio viene dimostrata per la prima volta la capacità delle GMMA di conferire una protezione eterologa contro le infezioni da *Salmonella*, indicando la potenzialità delle GMMA nello sviluppo di un vaccino proteico ad ampio spettro contro NTS.

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I would also like to thank Prof. Calman MacLennan for his supervision and review of the manuscript, as well as to the other members of my Ph.D. Committee – Dr. Laura Martin, Dr. Allan Saul, and Dr. Enzo Scarlato – for providing helpful feedback and guiding my project towards the right direction. Particularly I would like to thank Dr. Laura Martin for her supervision at the beginning of the project.

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Preface

The work presented in this thesis is the result of the work developed during my Ph.D. research project.

The experimental work was developed at the Novartis Vaccines Institute for Global Health (NVGH), in Siena Italy, and at the Wellcome Trust Sanger Institute (WTSI), in Cambridge UK. The work was co-supervised by Dr. Christiane Gerke and by Prof. Calman MacLennan at the NVGH, and by Dr. Robert Kingsley at WTSI.

Financial support was provided by NVGH, through a Ph.D. fellowship grant from the Università degli Studi di Milano-Bicocca.

This thesis is structured in 7 Chapters.

Chapter 1 is a review of the literature and comprises a general introduction to *Salmonella* epidemiology and pathogenesis, with a focus on invasive non-typhoidal *Salmonella* disease in Sub-Saharan Africa. A new vaccine approach based on outer membrane particles is explained in more detail. The aims and objectives of this thesis are included at the end of the chapter.

Chapter 2 includes the materials and methods used throughout chapters 3, 4, 5 and 6.

Chapter 3 describes the construction of different *Salmonella* mutants with increased GMMA production.

Chapter 4 characterises the proteomic content of *Salmonella* GMMA.

Chapter 5 highlights the immunological properties of *Salmonella* GMMA.

Chapter 6 analyses the ability of GMMA to protect against challenge using a murine model of acute *Salmonella* infection.

Each of these results chapters includes a short Introduction and Discussion.

Chapter 7 includes a general discussion of the results obtained, their relevance, and future perspectives.

Publications

As a result of the work described in this thesis we have submitted a patent application on “Hyperblebbing *Salmonella* strains”. Authors: **Sa Silva, S.**, Gerke, C., Martin, LB and Saul, A.

Table of Contents

Acronyms and Abbreviations

Ab – antibody

bp – base pairs

c.f.u. – colony forming units

cat – chloramphenicol resistance cassette

ELISA – Enzyme-linked immunosorption assay

GMMA – Generalised modules for membrane antigens

Ig - Immunoglobulins

iNTS – Invasive non-typhoidal *Salmonella*

IROMP – Iron Regulated Outer Membrane Proteins

kan – kanamycin resistance cassette

LC – Liquid chromatography

LPS – Lipopolysaccharide

MALDI-TOF – Matrix-Assisted Laser Desorption/Ionisation Time of Flight

MS – Mass spectrometry

NTS – Non-Typhoidal *Salmonella*

Oag – O antigen polysaccharide

OMV – Outer membrane vesicles

nOMV – Native outer membrane vesicles

SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis

TCA – Tricloroacetic acid

tet – tetracycline resistance cassette

Chapter 1

General Introduction

1.1 *The Salmonella enterica* species

Salmonellae are Gram-negative rod shaped bacilli that belong to the Enterobacteriaceae family (Agbaje, Begum, Oyekunle, Ojo, & Adenubi, 2011) and are an important bacterial pathogen who cause significant burden of disease worldwide. They are transmitted via the faecal-oral route and are responsible for a variety of diseases in humans and animals, ranging from mild gastroenteritis to severe systemic disease.

The genus name *Salmonella* was adopted in honour of Dr. D.E. Salmon, who first isolated *Salmonella choleraesuis*, at the time thought to be responsible for swine plague, from a pig in 1885 (Salmon, 1885). *Sallmonella* contains several species (eg. *Salmonella enterica* or *Salmonella bongori*) and subspecies (eg. *enterica*, *salamae*, or *arizona*). *Salmonella* can be further divided into serovars is based on the expression of three surface antigens, according to the Kauffman-White-Le Minor Scheme (Le, Popoff, & Bockemuhl, 1990; Guibourdenche et al., 2010): the somatic O antigen, the flagellar H1 and H2 antigens, and the capsular Vi antigen.

Salmonella enterica (*S. enterica*) alone is composed of more than 2,000 serovars, and even though they are closely related, they have an extended host range and cause different disease symptoms (Baumler, 1997). Human disease-relevant *S. enterica* subspecies *enterica* serovars are usually broadly divided into two types, based on epidemiology and clinical presentation: typhoidal or enteric *Salmonella*, which is endemic in South and South East

Asia and causes systemic disease, and comprises mainly serovars Typhi and Paratyphi A; and non-typhoidal *Salmonella* (NTS), which presents as either gastroenteritis in most settings or as bacteraemia in sub-Saharan Africa, and includes predominantly serovars Typhimurium and Enteritidis (Figure 1).

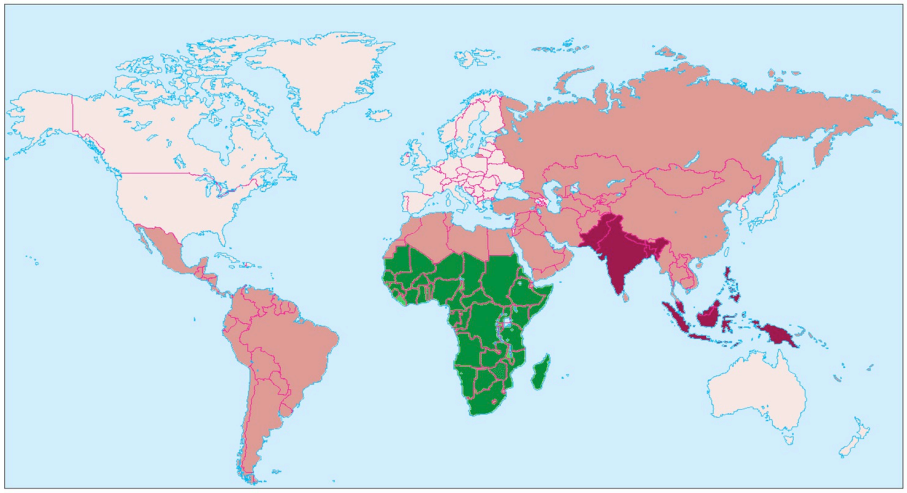

Figure 1. Global distribution of *Salmonella* in humans. Non-typhoidal *Salmonella* is endemic in sub-Sarahan Africa (highlighted in green) and Typhoidal and Paratyphoidal *Salmonella* are present in Central and South America, Africa, and Asia (highlighted in pink), with very of high incidence in South and Southeast Asia (highlighted in burgundy) (adapted from Crump et al., 2004).

Table 1. Hosts and diseases caused by *S. enterica* serovars. (adapted from (Mastroeni & Grant, 2011)

Syndrome	Serovar	Host specificity	Disease
Typhoid	Typhi Paratyphi A	Human only	Enteric fever: fever, headache, headache, abdominal discomfort, dry cough, myalgia (Bhan et al. 2005; Parry et al, 2002; WHO, 2003)
Non-typhoidal (non invasive)	Typhimurium Enteritidis	Broad host range	Gastroenteritis: abdominal pain, vomiting and inflammatory diarrhoea
Non-typhoidal (invasive)	Typhimurium Enteritidis	Human only	Bacteraemia

1.2 Invasive Non-Typhoidal *Salmonella* in Africa

1.2.1 Epidemiology and transmission

Non-typhoidal *Salmonella* (NTS) are an important cause of disease worldwide. Whereas in developed countries they are associated with food-borne infections and acute gastroenteritis (Hohmann, 2001), in developing countries, and in Sub-Saharan Africa in particular, they are an important cause of invasive disease.

Invasive NTS (iNTS) disease in Africa has been reported since the 1980s (Mabey, Brown, & Greenwood, 1987; Lepage et al., 1987; Nesbitt & Mirza, 1989; Green & Cheesbrough, 1993), but it was only recently that it became acknowledged as one of the major causes of invasive disease in children (Graham & English, 2009; Reddy, Shaw, & Crump, 2010; Sigauque et al., 2009) and HIV-infected adults (Graham et al., 2000; Gordon, 2008; Morpeth, Ramadhani, & Crump, 2009; Reddy et al., 2010). There is a clear bimodal age distribution, with iNTS peaks occurring in children aged between 6 months and 3 years of age (MacLennan et al., 2008; Feasey et al., 2010) and in adults aged 25-40 years of age (Feasey et al., 2010). Annual incidence of iNTS disease in Africa is estimated to be 175-388 per 100,000 in children under 5 (Gilks, 1998; Berkley et al., 2005) and 1,800-9,000 of 100,000 per person years of observation among HIV+ cohorts (non-antiretroviral therapy-treated) (Gilks, 1998; van Oosterhout et al., 2005; Watera et al., 2004). In both cases, case-fatality rate is high and ranges 22-25% (Vandenberg et al., 2010; Gordon et al., 2008).

A recent systematic review of bloodstreams infections in Africa (Reddy et al., 2010), which covered 22 prospective studies in 34 locations, showed that *S. enterica* was the most commonly identified pathogen overall (29.1% of isolates) and among adults (42.3%), and the second most common in children (21.4%). These isolates, however, also included *S.* Typhi and *S.*

Paratyphi, which were found mainly in North Africa. In other African regions, *S.* Typhimurium is the predominant serotype isolated from patients with iNTS disease, followed by *S.* Enteritidis (Gordon et al., 2008; Kariuki et al., 2006a; Mandomando et al., 2009; Reddy et al., 2010; Vandenberg et al., 2010).

Despite increased recognition of iNTS in Asia, its burden is still limited in the region, with only six cases identified amongst 20,537 febrile events reported in a five site population-based study (Khan et al., 2010). Nonetheless, and according to a recent report from Vietnam (Nga et al., 2012), there has been an increase in the number of cases of iNTS, concurrent with an increase in HIV prevalence. There has also been a steady decrease in *S. Typhi* since 2002, indicating a possible shift in the epidemiology of invasive *Salmonella* disease in this region. Recent studies of iNTS disease in two industrialised countries have shown that incidence is very low in this setting (1.02 per 100,000 population) (Laupland et al., 2010).

The relationship between invasive and diarrhoeal NTS disease in Africa is still under investigation. In recent studies (Musiime, Kalyesubula, Kaddu-Mulindwa, & Byarugaba, 2009; Simpore et al., 2009), NTS was isolated from a small proportion of children with acute diarrhoea, which is not a feature of iNTS, but overall prevalence of diarrhoeal NTS disease in Africa is unknown. However, it is now known that some *S.* Typhimurium isolates causing invasive disease in Sub-Saharan Africa are phylogenetically distinct, as revealed by multilocus sequence typing, from *S.* Typhimurium responsible for acute gastroenteritis in other regions of the world (Kingsley et al., 2009). These isolates belong to a recently evolved pathovar – ST313 – which has become host-adapted to human invasive disease as a result of genome degradation (Kingsley et al., 2009).

A study in Kenya showed that transmission of iNTS is primarily human-to-human rather not zoonotic, with a possible important role played by asymptomatic carriers (Kariuki et al., 2006b). Similar results were observed in a study in the Gambia (Dione et al., 2011). There have also been reports of human-to-human transmission in two hospital outbreaks of NTS (Keddy et al., 2009; Ktari et al., 2009), with the potential of transmission back to the community. Water is an important vehicle of transmission, with NTS having been isolated from a number of different water sources (Akoachere, Tanih, Ndip, & Ndip, 2009; Ktari et al., 2009; Oluyege, Dada, & Odeyemi, 2009).

1.2.2 Risk factors

In children, risk factors for iNTS disease include anaemia (Mandomando et al., 2009), malnutrition, sickle cell disease (Williams et al., 2009), and, more importantly, malaria (Brent et al., 2006). In Kenyan children with severe malaria, NTS was the most common cause of invasive bacterial disease (Berkley et al., 2009), whereas *Streptococcus pneumonia* and *Haemophilus influenzae* type B were most commonly found in children without malaria parasitaemia. The relationship between iNTS and malaria has been demonstrated in a number of studies (Berkley et al., 2009; Calis et al., 2008; Mackenzie et al., 2010; Mandomando et al., 2009; Mtove et al., 2010).

In adults, invasive NTS disease is associated with HIV infection (Gordon, 2008; Gordon et al., 2008; Gordon et al., 2010; Reddy et al., 2010). Even at low levels, immunosuppression is associated with increased risk of invasive bacterial disease (Danel et al., 2006). Increased susceptibility to iNTS in HIV-infected individuals is likely a result of impaired cell-mediated immunity in the gut mucosa (Raffatellu et al., 2008), as well as iNTS intracellular persistence and recrudescence , which happens in 30-40% of cases (Gordon et al., 2002; Gordon et al., 2003; Gordon et al., 2008; Gordon

et al., 2010). Anti-retroviral therapy in HIV-infected adults has been shown to reduce invasive NTS disease (Gordon, 2008; Hung, Hsieh, Hsiao, Chen, & Sheng, 2001).

Interestingly, there is an inverse association between *S.* Typhi bacteraemia and HIV (Reddy et al., 2010), which indicates that HIV has a protective effect against typhoid fever.

1.2.3 Clinical diagnosis and treatment

The clinical presentation of iNTS is characterised by fever due to bacteraemia, meningitis and/or septic arthritis. Diarrhoea is usually not one of the symptoms of iNTS (Graham et al., 2000; Mandomando et al., 2009; Mtove et al., 2010; Vandenberg et al., 2010). In cases where both iNTS and *S.* Typhi were identified in bacteraemic children (Mtove et al., 2010), these two forms of invasive *Salmonella* disease could nonetheless be distinguished by their clinical features: children suffering from *S.* Typhi infection were older and fever lasted for a longer period; in the case of iNTS disease there was association with previous malaria treatment, complications from malaria, and a higher risk of death.

Symptoms of iNTS are however very similar to those of other common diseases in Sub-Saharan Africa, such as malaria and pneumonia, which presents a problem at the time of diagnosis and treatment (Graham & English, 2009; Nadjm et al, 2010). Misdiagnosis of iNTS for other diseases is frequent (Berkley, Mwarumba, Bramham, Lowe, & Marsh, 1999; Dougle, Hendriks, Sanders, & Dorigo-Zetsma, 1997). Despite the existence of PCR-based tools for quick diagnosis of NTS infection in stool (Cunningham et al., 2010; Lin, Tsai, Hung, Fang, & Ling, 2011) or iNTS disease (Tennant et al.,

2010), these are not yet sensitive enough to detect iNTS in the blood, as it can be present at levels as low as 1 c.f.u/mL of blood (Gordon et al., 2010).

A recent study assessing the performance of WHO guidelines for detecting invasive bacterial disease and treatment initiation showed that almost one third of children were misdiagnosed (Nadjm et al., 2010). The WHO treatment guidelines for acute illness in children are based around malarial disease and recommend the use of antimalarial drugs before follow-up (Reddy et al., 2010). However, as mentioned in section 1.2.2, malaria parasitaemia and bacteraemia can coexist, and treatment of malaria alone will not adequately address the needs of patients with invasive bacterial infections such as iNTS (Reddy et al., 2010).

In cases where iNTS is correctly diagnosed, treatment is complicated by the emergence of multi-drug resistant strains (Brent et al., 2006; Gordon et al., 2008; Kariuki et al., 2006c; Kariuki et al., 2005). A recent systematic review of bloodstreams infections in Africa (Reddy et al., 2010) showed that less than 30% of NTS isolates were ampicillin-susceptible, with high levels of resistance to cotrimoxazole in *S.* Typhimurium and to chloramphenicol in *S.* Enteritidis. Most isolates were susceptible to third-generation cephalosporins and fluoroquinolones, but an increase in widespread use might further increase antibiotic resistance (Reddy et al., 2010; Keddy et al., 2009).

1.2.4 Prevention

Clean water, safe food, and improved sanitation are among the best prevention methods currently available. However, providing adequate sanitation and water are expensive long-term strategies that are linked to economic development. Vaccination would be the most useful and cost-effective measure (Rappuoli, Miller, & Falkow, 2002) for prevention of

iNTS disease in Sub-Saharan Africa. At present there are no commercially available vaccines against NTS for use in humans.

1.3 GMMA as a protein-based vaccine against NTS

1.3.1 What are GMMA?

Salmonellae, like most Gram-negative bacteria, naturally secrete outer membrane particles during all stages of growth (Yoon, Ansong, Adkins, & Heffron, 2011; Kitagawa et al., 2010). These outer membrane particles are generally 50-100 nm in size and are composed of outer membrane (OM) and periplasmic components – LPS, outer membrane proteins and periplasmic proteins (Figure 2) (Kuehn & Kesty, 2005; Beveridge, 1999). They are usually known in the literature as outer membrane vesicles (OMV) (Alaniz, Deatherage, Lara, & Cookson, 2007; Berlanda et al., 2008) or native OMV (Koeberling et al., 2011), but as they are distinct from detergent-extracted OMV in the way they are generated (Giuliani et al., 2006; Toneatto et al., 2011), we have named them GMMA, or Generalised Modules for Membrane Antigens.

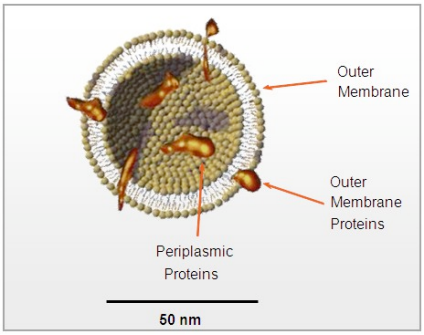

Figure 2. Schematic representation of GMMA. GMMA, which are composed of outer membrane and periplasmic components, exhibit outer membrane proteins on the surface in their natural conformation as they would be on the *Salmonella* surface during infection. Adapted from (Berlanda et al., 2008).

1.3.2 GMMA as a protein-based vaccine

The efficacy of detergent-extracted OMV as a vaccine against *Neisseria meningitidis* (*N. meningitidis*) was well-established during disease outbreaks in Norway, Cuba and New Zealand (Holst et al., 2009; Oster et al., 2007), but it has been demonstrated that naturally-released *N. meningitidis* GMMA have increased immunogenicity, likely due to differences in antigen presentation on the OMV versus GMMA surface. The potential role of GMMA as vaccines has also been demonstrated for a diverse range of organisms, such as *Salmonella*, using animal models of disease (Alaniz et al., 2007).

Although Gram-negative bacteria produce GMMA through natural processes, this production is generally low, which hinders their isolation and characterisation for vaccine development. Several methods have been used to obtain GMMA on a larger scale, such as the addition of gentamicin to the growth media (Martin & Beveridge, 1986; Kadurugamuwa & Beveridge, 1997). The release of GMMA can also be increased through the knock-out of proteins associated with the outer membrane – such as Gna33 in *N. meningitidis*, Dam in *S.* Typhimurium (Pucciarelli, Prieto, Casadesus, & Garcia-del, 2002), or Tol-Pal proteins in *E.* coli (Adu-Bobie et al., 2004; Bernadac, Gavioli, Lazzaroni, Raina, & Lloubes, 1998; Berlanda et al., 2008). In *E. coli*, the Tol-Pal proteins form two complexes in the cell envelope (Bernadac et al., 1998). These are involved in outer membrane integrity (Lazzaroni & Portalier, 1992; Muller, Vianney, Lazzaroni, Webster, & Portalier, 1993; Vianney et al., 1994), and their disruption of these complexes leads to increased GMMA production. The Tol-Pal system is also present in *Salmonella enterica*, and even though it has been shown that overexpression of Tol components increases GMMA release (Henry et al.,

2004) or that *tolA* mutants are attenuated *in vivo* (Paterson et al., 2009), it has not yet been explored whether *Salmonella* Tol-Pal mutants have increased GMMA production in a manner similar to *E. coli*.

Given their rich outer membrane protein (OMP) composition, GMMA are an attractive method for delivery of these protein antigens. There are two main advantages over recombinantly-expressed protein-based vaccines: firstly, OMP, which are usually difficult to express recombinantly due to their highly hydrophobic trans-membrane domains, are delivered on the GMMA surface and correctly exposed in their natural conformation (Bernadac et al., 1998); secondly, a large number of protein antigens are delivered as a single dose to generate a more robust immune response (Ellis, Leiman, & Kuehn, 2010; Blander & Medzhitov, 2006).

The antigenic composition of the GMMA can also be altered and optimised through genetic modification. Variable and highly immunogenic components can be downregulated, conserved antigens can be upregulated, and detoxification of the LPS can be achived. For a detailed description of these modifications, please refer to Chapter 3.

1.4 Aims and Objectives of Project

The overall aim of this project was to generate and characterise a broad-spectrum vaccine against non-typhoidal *Salmonella* (NTS) based on outer membrane particles, or GMMA. This aim was divided into different objectives:

1. Generation of GMMA from different NTS serovars and their optimisation for protein antigen delivery, by overcoming the serospecifity inherent to the O antigen and the reactogenicity associated with LPS endotoxin, the details of which are discussed in Chapter 3.

2. Characterisation of the proteomic content of NTS GMMA in order to identify the protein antigens present in GMMA, which is discussed in Chapter 4.

3. Assessment of the ability of NTS GMMA to elicit an immune response in mice, both humoral and cell-mediated, and determination of whether this response is reactive across NTS, the results of which are described in Chapter 5.

4. Evaluation of the ability of NTS GMMA to generate a cross-protective immune response using an acute model of *Salmonella* infection in mice, which is described in Chapter 6.

So far no other broad-spectrum GMMA-based vaccines have been described in the literature, so this represents a novel approach in *Salmonella* vaccine development.

Chapter 2

Materials and Methods

Materials

Unless otherwise stated, chemicals and reagents were obtained from Sigma-Aldrich.

Bacterial strains

The bacterial strains used and mutants created are described in Table 1.

Plasmids

The plasmids used are described in Table 2.

Inactivation of gene *tolR* with long PCR product

The upstream and downstream regions of *tolR* were amplified by PCR with primers tolR1 and tolR2 for the upstream region and tolR3 and tolR4 for the downstream region (see Table 3 for list of primers), which contained restriction sites at the 5' and 3' end (XbaI and EcoRV for the upstream and EcoRV and KpnI for the downstream region), to generate ~500-base pair (bp) long DNA fragments. These fragments were digested with the respective restriction enzyme (RE) and ligated to a previously XbaI and KpnI-digested pBlueScript18 (pBS) (Qiagen) to generate a pBS containing the 500 bp upstream and downstream regions of *tolR* with a restriction site for EcoRV in the middle (pBStolR5'tolR3'). This plasmid was digested with EcoRV and ligated to a kanamycin resistance cassette (*kan*^{r}) previously amplified from pUC4K (Taylor & Rose, 1988) with primers tolR5 and tolR6 (Table 3), corresponding to the same EcoRV sites at both the 5' and 3' end,

and digested with RE, to generate a plasmid containing the 500 bp *tolR* upstream region, the *kan*^{r} cassette, and the 500 bp *tolR* downstream region (pBS tolR5' kan^r tolR3'). This plasmid was linearised with an RE and a linear DNA fragment was amplified by PCR with primers tolR1 and tolR4 to yield the DNA fragment tolR5'*kan*^{r}tolR3'. 1µg of this fragment was electroporated into *S.* Typhimurium LT2, *S.* Typhimurium SL1344 or *S.* Enteritidis P125109 expressing the phage λ red homologous recombination system encoded by plasmid pAJD434, and mutants where the *tolR* gene had been replaced with the *kan*^{r} cassette (Δ*tolR::kan*^{r}) were selected on Luria-Bertani (LB) plates containing 25µg/mL kanamycin. Gene replacement was confirmed by PCR amplification of the genomic DNA of the mutant colonies using primers tolR7 and tolR8 (Table 3) annealing upstream and downstream of *tolR*.

Inactivation of *wbaP* gene with short PCR product

Inactivation of *wbaP* by PCR was done as described in(Datsenko & Wanner, 200 . The chloramphenicol-resistance gene (*cat*) was amplified by PCR from pKD3 using primers wbaP1 and wbaP2 (Table 3), which contained 45 bp extensions that were homologous to the regions adjacent to the *wbaP* gene in *S.* Typhimurium and *S.* Enteritidis. 1µg of the PCR product were then transformed into electrocompetent strains expressing the phage λ red recombinase encoded on pSIM18 and successful transformants where *wbaP* was replaced for *cat* (Δ*wbaP::cat*) selected on LB plates containing chloramphenicol. Gene deletion was confirmed by PCR screening of the genomic DNA of the mutant colonies using primers wbaP3 and wbaP4, which annealed upstream and downstream of *wbaP*.

Table 1. Bacterial strains used in this study.

Bacterial strain	Description	Source
S. Typhimurium strain LT2	Attenuated strain.	Novartis Master Culture Collection (McClelland et al., 2001)
S. Typhimurium strain SL1344	Virulent strain.	WTSI (unpublished; NCBI Ref Seq: NC_003197.1)
S. Typhimurium strain M525	Moderately virulent strain.	WTSI (Hormaeche, 1979)
S. Enteritidis PT4 strain P125109	Broad host range.	WTSI (Thomson et al., 2008)
E. coli MC4100 *katF13::Tn10*	Contains tetracycline resistance in Tn10.	WTSI (Broadbent, Davies, & van der Woude, 2010)
S. Typhimurium LT2Δ*tolR::kan*	GMMA overproduction	This study
S. Typhimurium SL1344Δ*tolR::kan*	GMMA overproduction	This study
S. Typhimurium SL1344Δ*wbaP::cat*	Lacks Oag expression	This study
S. Typhimurium SL1344Δ*tolR::kan*Δ*wbaP::cat*	GMMA overproduction; lacks Oag expression	This study
S. Typhimurium SL1344Δ*tolR::kan*Δ*wbaP::cat*Δ*msbB::tet*	GMMA overproduction; lacks Oag expression; penta-acylated lipid A	This study
S. Enteritidis P12109Δ*tolR::kan*	GMMA overproduction	This study
S. Enteritidis P125109Δ *wbaP::cat*	Lacks Oag expression	This study
S. Enteritidis P125109Δ*tolR::kan*Δ*wbaP::cat*	GMMA overproduction; lacks Oag expression	This study

Table 2. Plasmids and primers used in this study.

Plasmid	Antibiotic resistance	Source
pBluescript II KS (-)	Ampicillin	Qiagen
pUC4K	Kanamycin	(Taylor & Rose, 198
pKD3	Chloramphenicol	Datsenko and Wanner, 2000
pAJD434	Trimethoprim	(Maxson & Darwin, 200
pSim-18	Hygromycin	(Chan et al., 200

Primer name	Sequence
tolR1	CTAGTCTAGAGCCATCATTATCCAGCGAAC
tolR2	AGCTTGATATCGGCTTACCCCTTGTTGCTTTC
tolR3	AGCTTGATATCAGTCTGCGTCCCGTTGGCTT
tolR4	CCGGGTACCGTTGCGCCAGTTTTGCCGCT
tolR5	AGCTTGATATCAAAGCCACGTTGTGTCTCAAAATCTC
tolR6	AGCTTGATATCTGAGGTCTGCCTCGTGAAGAAG
tolR7	CGCCGCTGCGCGTGAGGCGG
tolR8	CCGCTTTCTTCTTCGCGTCC
wbaP1	CGCAGGCTAATTTATACAATTATTATTCAGTACTTCTCGGTAAGCGTGTAGGCTGGAGCTGCTTCG
wbaP2	CTTAATATGCCTATTTTATTTACATTATGCACGGTCAGAGGGTGACATATGAATATCCTCCTTAG
wbaP3	ATCTGTTGCTTGGACATTAC
wbaP4	GCGCGCTTCTGGTAATGCAC
msbB1	AGGTAGTACAGGGTTTGTCAGCATAAAGCCTCTCTTACGAGAGGCTTTATTTAAGACCCACTTTCACATT
msbB2	AGACGTCGCTACACTATTCACAATTCCTTTTCGCGTCAGCAGACCCTAAGCACTTGTCTCCTG
msbB3	GAACGCGCCGAAGCGGTATG
msbB4	TGTCGCGCGAGATGCTGGAT

Inactivation of *msbB* with short PCR product

The one-step inactivation of *msbB* was done as described in Datsenko and Wanner, 2000, but with slight modifications. A short DNA fragment containing a tetracycline resistance cassette (*tet*^{r}) was amplified by PCR from *E.* coli MC4100 *katF13::Tn10* (Broadbent *et al*., 2010) genomic DNA using primers msbB1 and msbB2 (Table 3), which contained 45 bp extensions that were homologous to the regions adjacent to the *msbB* gene in *S.* Typhimurium. 1µg of the PCR product was transformed into electrocompetent S. Typhimurium cells expressing the phage λ red recombinase encoded on pSim-18 (Chan et al., 2007) and successful recombination was checked by PCR on the transformant colonies grown on selective medium with LB supplemented with tetracycline.

Extraction and analysis of LPS content of *Salmonella* strains and mutants

1.1 x 10^9 cfu of *Salmonella* in mid exponential phase (A_{600} = 1) were washed and resuspended in PBS, incubated at 100ºC for 10 mins, and incubated in the presence of 250 µg of proteinase K (Promega) at 60ºC for 16h. Saturated phenol pH 8.0 was added as 1:1 (v/v), the mixture was incubated for 30 min at 70ºC and centrifuged at 10,000 x *g* for 1h. The top water phase was recovered and mixed with 100% ethanol 1:2 (v/v). LPS was precipitated for 1h at -80ºC and centrifuged at 12,000 x *g* for 30 min. The pellet was lyophilised with a speed-vac and resuspended in 500µL H_2O. 5µL of a 1:100 dilution in H_2O (v/v) in LDS loading buffer were loaded onto a 10% Bis-Tris polyacrylamide gel (Invitrogen) and gel was run at 25mA in MOPS buffer (Invitrogen). The gel was silver-stained with SilverQuest Silver Staining Kit (Invitrogen) according to the manufacturer's instructions.

TCA-precipitation of *Salmonella* culture supernatants

30 mL of culture supernatant from *S.* Typhimurium LT2Δ*tolR* grown in LB to A_{600} of 0.8 were TCA-precipitated in 1% TCA 0.04% DOC for 1 hour at 4ºC and centrifuged at 14,000 rpm for 10 mins, The remaining pellet was washed three times in 100% ethanol, lyophilised with a speed-vac, and finally resuspended in Tris-HCl before loading onto a 10% Bis-Tris polyacrylamide gel (Invitrogen) that was run at 25mA in MOPS buffer (Invitrogen) and stained with Coomassie R-250. Protein bands were identified as described in section 2.13.

Preparation of GMMA

GMMA overproducing-strains were grown in a liquid culture of LB, LB supplemented with 200uM bipyridyl (low iron conditions), or high yeast media (30g/L yeast extract (Difco), 5g/L KH_2PO_4, 20g/L K_2HPO_4, 2mL/L of 1M $MgSO_4$, 15g/L glycerol in a rotary shaker at 180 rpm to reach A_{600} = ~1.5 (LB) or A_{600} = ~7 (high yeast media). Bacteria were collected by centrifugation at 4,000 x g to isolate the culture supernatant. The supernatant was filtered through a bottle with a 0.22uM-pore size filter (Millipore, Bedford, MA) and concentrated using a stirred ultrafiltration cell with a 100 kDa membrane (Millipore). The concentrated filtrates were ultracentrifuged (Beckman-Coulter, Ti45 rotor) at 200,000 x g for 2 hours at 4ºC to generate a GMMA pellet, washed in PBS and ultracentrifuged for another 2 hours at 200,000 x g for 2 hours at 4ºC. The GMMA pellet was resuspended in ~1mL PBS.

GMMA visualisation by negative staining electron microscopy

GMMA were analysed by electron microscopy as described previously (Berlanda et al., 2008). Briefly, GMMA were fixed overnight in 2.5% glutaraldehyde in PBS and then washed and resuspended in the same buffer. A drop of the suspension was placed on Formyar/carbon-coated grids and GMMA were adsorbed for 5 minutes. The grids were washed with distilled H_2O, blotted with filter paper, and then treated with 2% uranyl acetate for 1 min. After air-dried, the GMMA were viewed with a Jeol JEM 1200 EXII electron microscope operating at 80 kV.

Quantification of GMMA proteins

Proteins were quantified using the Bio-Rad Protein Assay (Bio-Rad), based on the Bradford method, with slight modifications to the manufacturer's instructions. Briefly, GMMA were mixed 1:1 (v/v) with 6M guanidine hydrochloride, incubated at 100ºC for 10 min, mixed with Bradford reagent

at different dilutions and absorbance of the samples was measured at 595nm. The concentration of protein was determined against a BSA standard curve.

Mono-dimensional protein gel electrophoresis

10µg of GMMA were denatured for 10 minutes at 98ºC in LDS Sample buffer (Invitrogen) containing TCEP or β-mercaptoethanol. Samples were loaded onto Bis-Tris polyacrylamide gels (Invitrogen) and ran in MOPS buffer (Invitrogen). Gels were stained with Coomassie Blue R-250.

Two-dimensional protein gel electrophoresis

200 µg of GMMA proteins from S. Typhimurium SL1344Δ*tolR* were precipitated for 1 hour in 1% TCA 0.04% DOC, washed three times in 100% ethanol, and resuspended in reswelling buffer (7M urea, 2M thiourea, 2% CHAPS, 2% ASB-14, 0.1% DTT, 20mM Tris base, and bromophenol blue) for separation in the first dimension on non-linear pH 3-11 gradient IPG strip (GE Healthcare). Proteins were then separated in the second dimension on a 4-12% Bis-Tris gel (Invitrogen) in MOPS buffer (Invitrogen), and stained with Coomassie Blue-G250. Gel was destained in ethanol and acetic acid.

Protein isolation, digestion and mass spectrometry analysis by MALDI-TOF

Protein spots were excised from the gel using a glass Pasteur, bleached with 50mM ammonium bicarbonate and acetonitrile (1:1, v:v), washed in acetonitrile, and air dried until acetonitrile fully evapourated. The remaining pellets were digested with trypsin (Promega) in 5mM ammonium bicarbonate for 1 hour at 37ºC, before loading 0.6µL onto a matrix PAC target (Prespotted AnchorChip 96, set for proteomics, Bruker Daltonics). Each spot was washed with 1µL of washing solution (70% ethanol, 0.1% trifluoroacetic acid). Mass spectra were acquired on an Ultraflex MALDI-

TOF-TOF mass spectrometer (Bruker Daltonics) in reflectron, positive mode in the mass range of 900–3500 Da and MS spectra were analysed using flexAnalysis (version 2.4, Bruker Daltonics). flexAnalysis default parameters were used to automatically annotate monoisotopic peaks in each MS spectrum, followed by manual revision and annotation. Proteins were identified from the MS peak list using Mascot (Mascot server version 2.2.01, Matrix Science). Mascot was run on publicly available *Salmonella* sequences accessible from the NCBInr database (National Center for Biotechnology Information non-redundant). We used the following search parameters: fixed modifications, propionamide (Cys); variable modifications, oxidation (Met); cleavage by trypsin (cuts C-terminal side of Lys and Arg unless next residue is Pro); mass tolerance, 300 ppm; missed cleavage, 0; mass values, MH^+ monoisotopic. Known contaminant ions (trypsin, m/z = 842.509400, 1045.563700, 1165.585300, 1179.601000, 1300.530200, 1713.808400, 1716.851700, 1774.897500, 1993.976700, 2083.009600, 2211.104000, 2283.180200, and 2825.405600) were excluded.

Bioinformatic analysis of protein localisation

Prediction of localisation was carried out using the PSORTb v3.0 algorithm (Yu et al., 2010).

Mice, immunisations and infections

Female wild-type CD1 or C57BL/6 mice (6-8 week old) were obtained from Charles River Italy (CD1 mice) or Charles River UK (C57BL/6 mice). In the study with *S.* Typhimurium LT2 Δ*tolR* GMMA (high Fe), three groups of nine CD1 female mice each were immunised subcutaneously on days 0 and

14 with 100µL of 0.1µg, 1µg, or 10µg of GMMA. Sera were collected at day 0, 14, and 32. In the dose-ranging study with *S.* Typhimurium SL1344 Δ*tolR* GMMA (low Fe), five groups of five C57BL/6 mice each were immunised twice at three week intervals (day 0 and 21) with 1µg SL1344Δ*tolR*, 1µg SL1344Δ*tolR*Δ*wbaP*, or 0.1 µg, 1µg, or 10µg SL1344Δ*tolR*Δ*wbaP*Δ*msbB* GMMA. Sera were collected at day 0, 21, and 49. In the study to assess intranasal immunogenicity of *S.* Typhimurium GMMA (low Fe), three groups of two C57BL/6 mice each were immunised intranasally three times on days 0, 7 and 21 with 1µg or 5µg of *S.* Typhimurium SL1344 Δ*tolR* or 1µg of *S.* Typhimurium SL1344 Δ*tolR*Δ*wbaP*. Sera were collected on day 28. In the study to assess splenocyte population and cytokine responses, C57BL/6 mice were subcutaneously immunised twice with a one-week interval (days 0 and 7), and splenocytes were harvested one week after the last immunisation (day 14). In the immunisation and infection study, female C57BL/6 mice were immunised orally on day 0 with $1x10^9$ *S.* Typhimurium SL3261 or subcutaneously on days 0, 7 and 21 with GMMA from *S.* Typhimurium SL1344Δ*tolR*, *S.* Typhimurium SL1344 Δ*tolR*Δ*wbaP*, or *S.* Enteritidis P125109Δ*tolR*Δ*wbaP*. Five weeks after the last GMMA immunisation (day 63), mice were infected with 1 x 10^6 S. Typhimurium M525 for three days.

Bacterial enumeration in spleen and liver

Mice were sacrificed by cervical dislocation. Spleens and livers were aseptically removed and homogenised in a Colworth stomacher in 5mL PBS. Viable counts were performed by plating 50µl of 10^0, 10^{-1}, 10^{-2} and 10^{-3} dilutions of homogenate on LB agar.

Bacterial enumeration in blood

Mice were sacrificed by cervical dislocation. Blood was aseptically removed by cardiac puncture and placed in heparin-containing blood collection tubes. Viable counts were performed with 150µL of blood on LB pour plates.

Serum immunoglobulin (Ig) measurement by ELISA

Flat bottom or round bottom Maxisorp plates were coated overnight at 4ºC with *Salmonella* GMMA or *S.* Typhimurium LPS in 100µL of PBS, quickly inverted, and blocked with 200µL of PBS 0.05% Tween-20 (PBST) 3% milk for 1h at room temperature. Plates were washed three times with PBST and incubated with sera diluted in PBS for 2h at room temperature. Plates were further washed three times with PBST and incubated with alkaline phosphatase (AP)- or horse radish peroxidase (HRP)-linked anti-mouse Ig, anti-mouseIgG, anti-mouse IgG1, anti-mouse IgG2a or anti-mouse IgA (1:10,000) for 1 hour at room temperature. 4-nitrophenyl phosphate (pNPP) disodium hexahydrate substrate or o-phenylenediamine dihydrochloride (OPD) peroxidase substrate were added for 1h or 30 min respectively, and absorbance was measured (490nm - 405nm for pNPP disodium hexahydrate or 495nm for OPD-peroxidase). When endpoint Ig titres were calculated, a cutoff of two times the standard deviation of the negative control was used to determine the sera dilution just above the cutoff. Ig titres were established as the reciprocal of the dilution. Competitive ELISA were performed using a similar protocol, in the presence of increasing concentrations of *S.* Typhimurium LPS added contemporaneously with the sera.

Flow cytometry assay for *Salmonella*-specific IgG

1 x 10^7 *S.* Typhimurium or *S.* Enteritidis in 5µL of PBS were incubated for 20 min with 45µL of immune sera at different dilutions in PBS. *Salmonellae* were washed twice in PBS and incubated for 15 min with FITC- or APC-conjugated polyclonal rabbit anti-mouse IgG. *Salmonellae* were further washed in PBS and resuspended in PBS 1% formaldehyde. Sample data

were acquired on a FACSCanto II (BD Biosciences). *Salmonellae* were identified by their light scatter characteristics and 20,000 events were recorded for each sample.

Immunoblotting

Gels were transferred to nitrocellulose membranes using the iBlot dry transfer system (Invitrogen), blocked overnight in PBS 3% milk, and washed three times in PBST. The membranes were incubated with GMMA immune sera (1:1,000 in PBST 3% milk), washed three times in PBST, and incubated with HRP-linked anti-mouse IgG (1:10,000) in PBST. The membranes were further washed three times in PBST and detection was carried out using Amersham ECL Western Blotting Detection reagents (GE Healthcare) with photographic film (1D immunoblots) or electronically with Imagequant (GE Healthcare) (2D immunoblots). Different lenghts of exposure were attempted in both cases.

Isolation of splenocytes

For isolation of splenocytes, aseptically removed spleens were placed in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, penicillin and streptomycin, and macerated. Single cells were separated using a 100µM cell strainer (BD Biosciences) and washed once. ACK lysis buffer was added to thecell pellets for 10 min at room temperature to lyse the red blood cells. Cells were then washed twice in supplemented RPMI and finally resuspended in supplemented RPMI. Cells were counted with trypan blue (1:1, v/v) using a haemocytometer. The single cell suspensions were resuspended to a final concentration of 2 $x10^6$ cells per mL or 5 x 10^6 cells per mL in supplemented RPMI.

Staining of splenocytes for phenotyping

Single cell suspensions from the spleens of individual mice at 2 x10^6 cells/mL (see Section 2.22) were used to isolate 2 x 10^5 cells per sample for staining. Each sample was stained with mAb cocktail 1 or mAb cocktail 2 (see below). The mAb's used for flow cytometry were purchased from BD Biosciences unless stated otherwise. mAb cocktail 1 contained CD4 with PE conjugate, CD8 with PerCP Cy5.5 conjugate, CD3 with APC conjugate, NK1.1 with FITC conjugate; mAb cocktail 2 contained CD45R/B220 with PerCP conjugate, Gr-1 with PE conjugate, CD11b with Alexa Fluor 647 conjugate, and F480 with FITC conjugate. Post labeling, cells were washed twice with blocking buffer and finally resuspended in 0.2 mL 1% paraformaldehyde in PBS and stored at 4ºC overnight in the dark. Analysis is described in section 2.25.

Splenocyte flow cytometry analysis

To perform the flow cytometric analyses and measure relative fluorescence intensities, a FACSAria 11 cytometer (BD Biosciences) and BD Diva software (BD Biosciences) were used. For each sample 10,000-20,000 events were recorded. The percentage of cells labelled with each mAb was calculated in comparison with cells stained with similarly labeled isotype control antibody. Background staining was controlled by labelled isotype controls and fluorescence-minus-one (FMO). The results represent either the percentage or the total number of positively stained cells in the whole cell population exceeding the background staining signal.

Statistical Analysis of Data

Experimental results were plotted with Microsoft Excel or Prism4 software and analysed for statistical significance with Prism4 software (GraphPad Software Inc., San Diego, USA).

Ethics Statement

For the animal experiments carried out at the Wellcome Trust Sanger Institute (Cambridge, UK), all animal procedures were performed in accordance with the United Kingdom Home Office Inspectorate under the Animals (Scientific Procedures) Act 1986. Ethical approval for these procedures was granted by the Novartis Animal Ethical Review Committee. For the animal experiments carried out at Novartis Vaccines & Diagnostics (Siena, Italy), approval was granted by the Novartis Animal Ethical Review Committee.

Chapter 3

Generation of *Salmonella* GMMA

3.1 Introduction

Salmonella, like other Gram-negative bacteria, naturally release GMMA during growth (Yoon et al., 2011; Kitagawa et al., 2010). The deletion of one of the Tol-Pal proteins, a system involved in inner-outer membrane stability, has been shown to increase the release of GMMA in *E*. coli (Bernadac et al., 1998). We have chosen to delete one of the Tol-Pal proteins, *tolR*, to increase the release of GMMA in *Salmonella*.

These GMMA, however, contain high levels of O antigen (Oag), a very immunodominant component of *Salmonella* LPS that generates a serospecific response and potentially masks more conserved antigens on the GMMA surface (Nagy, Dobrindt, Hacker, & Emody, 2004; Nagy et al., 2008; Russo et al., 2007). For this reason, we decided to generate *Salmonella* lacking Oag by deleting the gene *wbaP* (Ilg et al., 2009; Wang, Liu, & Reeves, 1996), which encodes a glycosyl transferase involved in the first steps of O antigen biosynthesis in *Salmonella*. In this way, we are hoping to optimise the delivery of protein antigens by GMMA in a way that is not serovar-specific and enhance their immunogenicity to generate a broad-spectrum immune response.

A further consideration with regards to the potential use of GMMA as a vaccine in humans is the reactogenicity of *Salmonella* LPS. With the aim of

reducing this effect, we introduced a deletion in the gene *msbB* (Ki*m et al*., 2009; Le*e et al*., 2009), which encodes a lipid A biosynthesis acyltransferase that hexa-acylates lipid A and increases its reactogenicity. By leaving lipid A in its penta-acylated form, we are hoping to decrease the reactogenicity of *Salmonella* GMMA (Lee *et al.*, 2009).

To introduce the above deletions (*tolR*, *wbaP* and *msbB*) into *S.* Typhimurium and *S.* Enteritidis, several strategies are available. One of the most straightforward approaches used for site-specific gene deletion is based on the homologous recombination system for bacteriophage λ, λ-Red. This system allows for dsDNA fragments generated by PCR to be targeted to specific chromosomal locations, using regions of homology that flank the gene to be deleted. Successful recombination can be procured by introducing selectable markers, such as antibiotic-resistance genes, to replace the gene of interest (Karlinsey, 2007; Datsenko & Wanner, 2000). The DNA fragment is electroporated into *Salmonella* containing a plasmid, such as pAJD434 (Maxson & Darwin, 2004) or pSim-18 (Chan *et al.*, 2007), expressing the λ-Red recombination proteins (Gam, Bet, Exo) (Y*u et al*., 2000). Successful replacement of the gene (eg. *tolR*) with the antibiotic resistance cassette (eg. *kan*^{r}) can be selected by their antibiotic resistance (Fig. 1).

For the deletion of *tolR*, we used PCR to generate a DNA fragment with long regions (500 bp) of homology to the upstream and downstream regions of *tolR* with restriction sites at the 5' and 3' end that were ligated to a kanamycin-resistance cassette using pBluescript18 as a vector. The genes *wbaP* and *msbB* were deleted with a similar approach, but using a single PCR step to generate a DNA fragment containing an antibiotic-resistance cassette with short homology regions (50 bp) to the upstream and

downstream regions of the target genes (Datsenko & Wanner, 2000). We used different antibiotic-resistance markers for the different gene deletions we were trying to achieve. For *tolR* we used a kanamycin-resistance cassette (kan^r) amplified from pUC4K (Taylor & Rose, 1988); for *wbaP* we amplified a chloramphenicol-resistance cassete (*cam*^{r}) from pKD3 (Datsenko & Wanner, 2000); and for *msbB* we used a tetracyline-resistance cassette (*tetRA*)-containing strain, *E.* coli MC4100 *katF13::Tn10* (Broadbent *et al*., 2010). These procedures are described in more detail in Chapter 2, Materials and Methods.

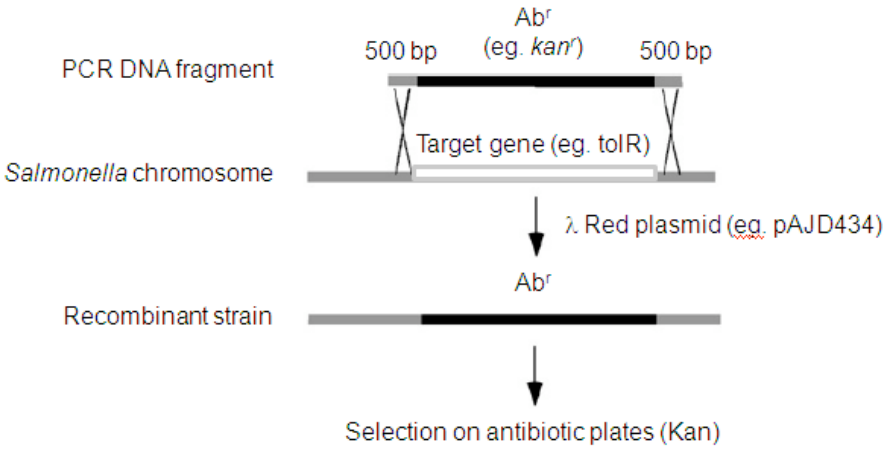

Figure 1. General strategy for gene deletion in *S. enterica* using λ Red homologous recombination system. The antibiotic-resistance cassette (Ab^r) is generated by PCR and is flanked by 50 bp of homology to the flaking regions of the gene to be deleted. λ Red recombination proteins are expressed from a λ Red plasmid, such as pAJD434 or pSim-18, and enable the recombination of Abr into the chromosome to replace the target gene. Recombinants are selected on antibiotic plates. Adapted from (Karlinsey, 2007).

The generation of a potentially broad-spectrum immune response by GMMA will rely on the presence of antigens that are conserved across serovars. One such example is a subset of *Salmonella* outer membrane proteins, the iron-regulated outer membrane proteins (IROMPs), which are essential for sequestering iron from the environment to enable growth and survival (Payne, 1988). IROMPs have previously shown protection against *S.*

Typhimurium and *S.* Typhi infection in murine models (Sood, Rishi, Dhawan, Sharma, & Ganguly, 2005). The expression of IROMPs is induced by iron limitation *in vivo*, but these conditions can be recreated *in vitro* with the use of an iron-chelating reagent, such as bipyridyl (Rabsch et al., 2003; Hantke, Nicholson, Rabsch, & Winkelmann, 2003). Using this strategy, we hypothesised that the expression of IROMPs would be increased on *Salmonella* GMMA with the aim of increasing the ability of GMMA to protect against heterologous infection.

3.2 Generation of *Salmonella* Δ*tolR* mutants

The entire *tolR* coding sequence was deleted in *S.* Typhimurium LT2 and replaced with a kanamycin-resistance cassette (*kan*^{r}) using phage λ red recombination (described in section 3.1) to generate *S.* Typhimurium LT2 Δ*tolR::kan*^{r} (LT2Δ*tolR*). The deletion of *tolR* was checked by PCR amplification of genomic DNA from the mutant colonies using primers annealing upstream and downstream of the regionstargeted for homologous recombination. The PCR products obtained were ~1,600 bp for LT2 wild type and ~2,400 for LT2 Δ*tolR::kan*^{r} mutants, corresponding to the 720 bp difference between *tolR* (480 bp) and *kan*^{r} (1,209 bp) (Fig. 2).

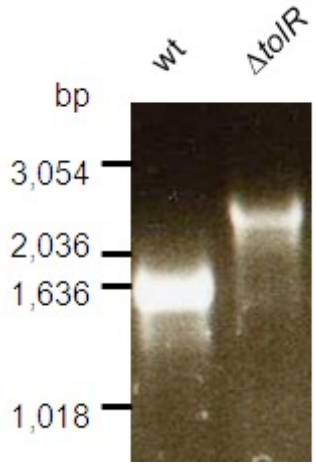

Figure 2. ***tolR* deletion in *S.* Typhimurium was confirmed by PCR.** A PCR reaction with primers annealing upstream and downstream of *tolR* was carried out with LT2 wild type and LT2 Δ*tolR::kan*^{r} genomic DNA and generated 1,600 bp-long and a 2,400 bp-long products, respectively, confirming the substitution of *tolR* (480 bp) with *kan*^{r} (1,209bp) in the mutant.

tolR deletion mutants of *S.* Typhimurium SL1344 and *S.* Enteritidis P125109 were also generated by replacing *tolR* with *kan*^{R} using the same long DNA fragment containing 500 bp homologous regions, as the *tolR* coding sequence and flanking regions are homologous across *Salmonella enterica* species. To determine whether the Δ*tolR* mutants released increased amounts of GMMA relative to wild type strains, these strains were grown in LB to early log phase (0.5 OD_{600}) and the protein content of the culture supernatants was TCA-precipitated and analysed by SDS-PAGE (Fig. 3*A* and *B*). A large number of protein bands were visible in the supernatant of LT2Δ*tolR* and P125109Δ*tolR*, with the most intense bands found in the 37kDa region. Only a small number of bands were present in the supernatant of the wild type strain, a likely result of proteins secreted during growth, including naturally released GMMA, and those originating from cell lysis. Mass spectrometry analysis confirmed that some of the most intense bands in the LT2Δ*tolR* supernatant corresponded to some of *S.* Typhimurium outer membrane porins, such as Outer Membrane Protein A or C (Fig. 3*A*). To

verify that the increased protein was likely due to the release of GMMA, we purified the GMMA from the supernatant of SL1344Δ*tolR* by ultracentrifugation. We obtained a translucent pellet, which was resuspended in PBS and analysed by transmission electron microscopy, revealing homogeneous spherical particles about 40 nm in size (Fig. 3*C*).

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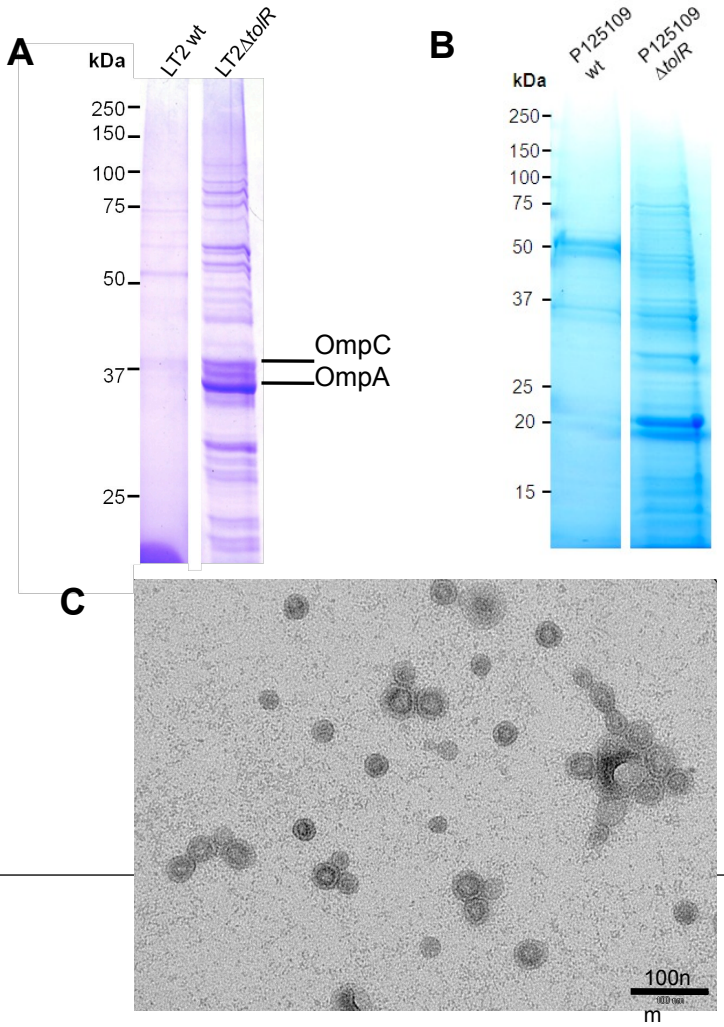


Figure 3. *S.* Typhimurium and *S.* Enteritidis *tolR* deletion mutants that have increased GMMA production. Culture supernatants of (*A*) wild type (LT2 wt) and *tolR* mutant (LT2Δ*tolR*) of *S.* Typhimurium LT2 or (*B*) wild type (P125109 wt) and *tolR* mutant (P125109Δ*tolR*) of *S.* Enteritidis P125109 were precipitated with TCA and 10 μg of protein were separated on a 12% SDS-PAGE and stained with Coomassie Blue. Some of the main bands were identified as outer membrane proteins A (OmpA) and C (OmpC) by mass spectrometry. (*C*) LT2Δ*tolR* GMMA were harvested from the culture supernatants by ultracentrifugation, fixed overnight in 2.5% glutaraldehyde in PBS, prepared for negative staining, and viewed by transmission electron microscopy. Bar scale is 100nm.

3.3 Deletion of O antigen in *Salmonella* overproducing-mutants

With the aim of abrogating the expression of the Oag in *Salmonella* GMMA, we deleted the gene *wbaP* (Ilg et al., 2009; Wang et al., 1996) in *S.* Typhimurium SL1344 and *S.* Enteritidis P125109 Δ*tolR* mutants. Strains expressing phage λ red recombinase were transformed with a PCR product (described in section 3.1) to generate *Salmonella* Δ*tolR::kan*^{r}Δ*wbaP::cat*^{r} double mutants (SL1344Δ*tolR*Δ*wbaP* and P125109Δ*tolR*Δ*wbaP*). Successful recombination and substitution of *wbaP* for *cat*^{r} was checked by PCR amplification using genomic DNA of transformant colonies (Fig. 4*A*). This showed a shorter PCR product in the mutant strain relative to wild type (~1,400 bp and 1,800bp, respectively).

The absence of Oag expression in Δ*wbaP* mutants was confirmed by silver-stained SDS-PAGE gels of LPS extracted from wild type and *wbaP* mutants (Fig. 4*B*). Sensitivity assays with phage P22, which is Oag-specific, were

also carried out on *S.* Typhimurium strains and showed that *S.* Typhimurium Δ*tolR*Δ*wbaP* was resistant to P22 infection as expected, confirming the absence of Oag (not shown). We also purified GMMA from *S.* Typhimurium Δ*tolR*Δ*wbaP* (Fig. 4*C*) and *S.* Enteritidis Δ*tolR*Δ*wbaP* (results not shown) and analysed their Oag content by western blotting with anti-O:4,5 and anti-O:9 antibody, respectively, to show that they did not contain any detectable Oag, compared to GMMA from single Δ*tolR* mutants.

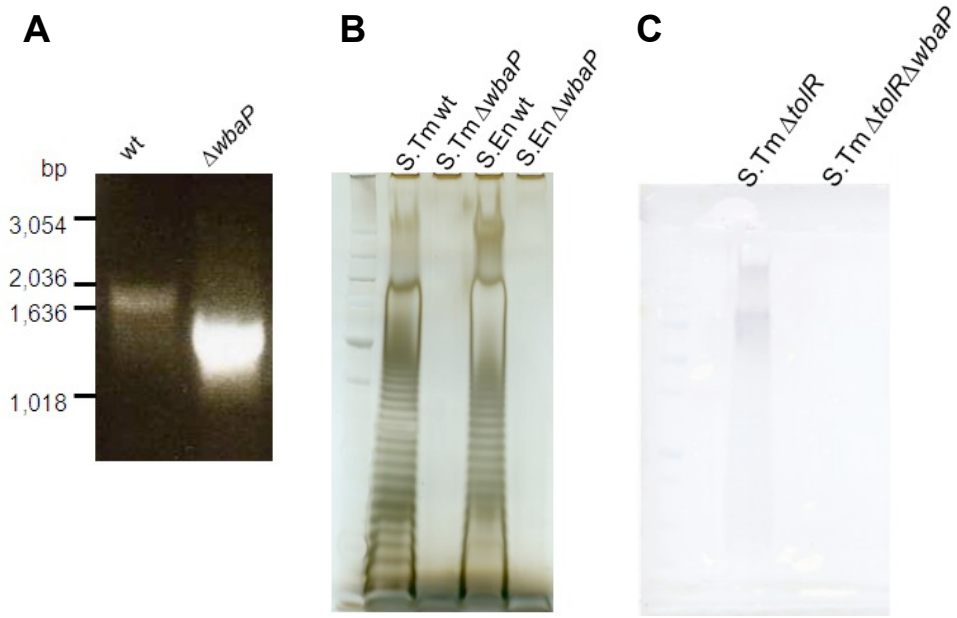


Figure 4. Deletion of *wbaP* generates *Salmonella* mutants lacking O antigen. (*A*) PCR amplification with primers annealing upstream and downstream of deleted region generated PCR products with 1,800bp for wt and ~1400bp for Δ*wbaP* (*B*) LPS was extracted from wild type *S.* Typhimurium SL1344 (*S.*Tm wt), *S.* Typhimurium SL1344Δ*wbaP* (*S.*TmΔ*wbaP*),

wild type *S.* Enteritidis P125109 (*S.*En wt), and *S.* Enteritidis P125109Δ*wbaP* (*S.*EnΔ*wbaP*), separated by 10% SDS-PAGE and silver-stained, confirming the lack of O antigen expression in Δ*wbaP* strains. (*C*) GMMA from S. Typhimurium SL1344Δ*tolR* and S. Typhimurium SL1344Δ*tolR*Δ*wbaP* were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane for probing with anti-O:4,5, confirming the absence of Oag in *S.* Typhimurium SL1344Δ*tolR*Δ*wbaP* GMMA (S.TmΔ*tolR*Δ*wbaP*).

3.4 Generation of *Salmonella* Δ*msbB* mutants

With the aim of reducing LPS reactogenicity, we deleted the *msbB* gene (Kim et al., 2009; Lee et al., 2009) in *S.* Typhimurium using a PCR product with 50bp homology to the *msbB* flanking regions that contained a tetracycline resistant (Tc^r) element, *tetRA*, and used it to replace *msbB* for *tetRA* (Datsenko & Wanner, 2000; Karlinsey, 2007) as described in section 3.1. The generation of a triple mutant strain *S.* Typhimurium SL1344 Δ*tolR::kan*^{r}Δ*wbaP::cat*^{r}Δ*msbB::tetRA* (SL1344Δ*tolR*Δ*wbaP*Δ*msbB*) was confirmed by PCR analysis. Transformant colonies generated products that were ~3,500 bp long, corresponding to *tetRA* (1,910 bp) and *msbB* flanking regions (1,575 bp total upstream and downstream), compared to wild type DNA products that were ~2,500 bp long, corresponding to *msbB* (972 bp) and *msbB* flanking regions (1,575 bp) (Fig. 5).

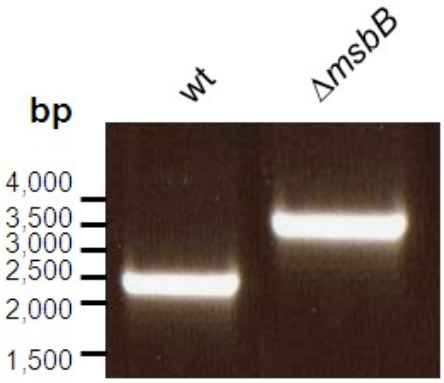


Figure 5. Construction of a *msbB* deletion mutant of *S.* Typhimurium SL1344 Δ*tolR* and Δ*tolR*Δ*wbaP*. Deletion of *msbB* and substitution with *tetRA* in *S.* Typhimurium was confirmed by PCR using primers annealing upstream and downstream of *msbB* region, which showed a ~2,500 bp-long product for wild type (wt) and a ~3,500 bp-long product for *msbB::tetRA*.

3.5 Generation of GMMA under low Fe^{2+} conditions

We isolated GMMA originating from the different mutants of S. Typhimurium (Δ*tolR*, Δ*tolR*Δ*wbaP*, Δ*tolR*Δ*wbaP*Δ*msbB*) and compared their protein content by SDS-PAGE (Fig. 6, *left panel*). The presence of Oag in Δ*tolR* GMMA seemed to interfere with protein separation, as the protein bands showed reduced mobility compared to the other two mutants and individual bands were not distinct in the 37-75 kDa region. In GMMA from Δ*tolR*Δ*wbaP*Δ*msbB*, we observed a reduction in the intensity of one of the outer membrane porins (around 37kDa), likely outer membrane protein A

(OmpA), indicating that the introduction of cumulative mutations gave rise to minor changes in the content of the GMMA.

In order to increase the expression of IROMPs (Rabsch et al., 2003; Hantke et al., 2003), which are highly conserved across *Salmonella enterica* serovars, we cultured the *S.* Typhimurium GMMA-overproducing mutants under low Fe^{2+} conditions (LB in the presence of 200 μM bipyridyl) and analysed the purified GMMA by SDS-PAGE (Fig. 6, *right panel*). As expected, a number of bands were upregulated in the 75kDa region (indicated by arrows), corresponding to the expected size of the *Salmonella* IROMPs Cir (74kDa), IronN (78kDa) and FepA (83kDa).

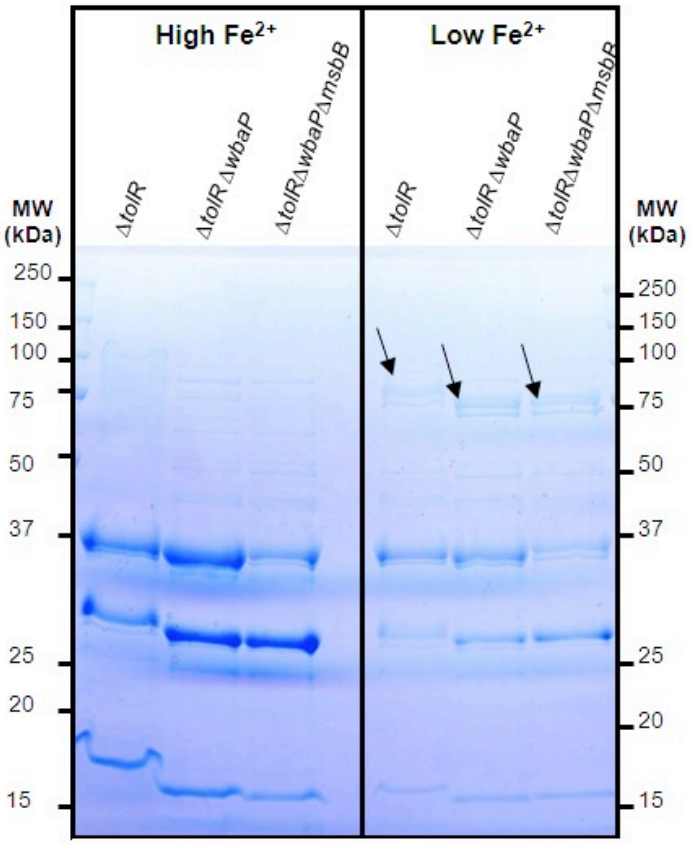


Figure 6. Generation of GMMA under low Fe^{2+} conditions upregulates the expression of proteins in the 75kDa region. 10µg of GMMA protein from different *S.* Typhimurium SL1344 mutants grown under high Fe^{2+} and low Fe^{2+} conditions were separated on a 12% SDS-PAGE. Up-regulated proteins under low Fe^{2+} are indicated by arrows.

3.6 Discussion

We have successfully generated GMMA from two different serovars of NTS, *S.* Typhimurium and *S.* Enteritidis, and optimised them for protein-antigen delivery by deleting the Oag. Although *Salmonella* GMMA have previously been studied and their possible use as vaccines suggested (Alaniz et al., 2007), this is the first time that Oag is deleted with the aim of overcoming a possible serospecific immune response. Furthermore, and as a consideration for their possible use in humans, we have created an *msbB* mutant that generates GMMA with penta-acylated LPS, which has been reported to be less toxic than the wild-type hexa-acylated form. With the aim of increasing the possible generation of broad-spectrum antibodies against GMMA proteins, we have successfully upregulated the expression of the IROMPs. This subset of proteins has previously shown to generate a protective response against *Salmonella* infection and we hypothesise that their overexpression in the GMMA will improve the protective capacity of GMMA.

The characterisation of the GMMA we have generated and described in this Chapter is described in the following chapters of this Ph.D. thesis.

Chapter 4

Proteomic analysis of *Salmonella* GMMA

4.1 Introduction

With the aim of characterising the protein content of *Salmonella* GMMA, we carried out a proteomic analysis of *S.* Typhimurium SL1344 Δ*tolR* GMMA using two-dimensional gel electrophoresis (2D-GE). 2D-GE provides a powerful tool in proteomic analysis (Gorg, Weiss, & Dunn, 2004) and is one of the most common approaches in the analysis of GMMA protein contents used to date (Ferrari et al., 2006; Berlanda et al., 2008). This technique enables the separation of complex protein mixtures according to pI, molecular weight, solubility, and relative abundance (Gorg et al., 2004). Proteins are initially separated in the first dimension by isoelectric focusing, and then in the second-dimension with SDS-PAGE. One of the main advantages of 2D-GE is that a map of intact proteins is obtained, and this map can be used to compare protein expression levels under different conditions.

We hypothesise that the proteomic analysis of *S.* Typhimurium GMMA will reveal a large number of outer membrane and periplasmic proteins. Cytoplasmic and inner membrane proteins, on the other hand, should be absent or present in very small amounts. This would be in agreement with the release of GMMA from the outer membrane of bacteria.

4.2 53 GMMA proteins were identified by two-dimensional gel electrophoresis

To characterise the content of S. Typhimurium SL1344 Δ*tolR* GMMA, we separated 10µg or 200µg of GMMA proteins by one- and two-dimensional gel electrophoresis, respectively, to obtain a comprehensive map of the GMMA proteome (Fig. 1*A* and *B*). In the one-dimensional gel, we observed two strong bands, one in the 37kDa region and another in the 30kDa region, likely corresponding to the most abundant protein on the *Salmonella* outer membrane, OmpA, and its degradation products. We also hypothesised that OmpA was the most intense protein spot in the two-dimensional (2D) gel.

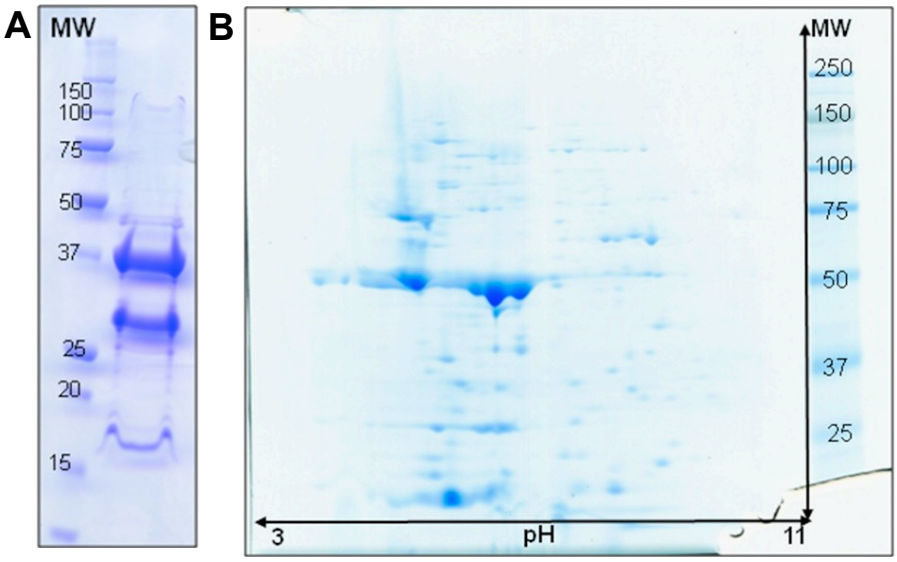


Figure 1. *S.* Typhimurium SL1344 Δ*tolR* GMMA were separated by one- and two-dimensional gel electrophoresis. (*A*) 10µg of GMMA protein were separated in a 10% polyacrylamide gel and stained with Coomassie Blue. (*B*) 200 µg of GMMA proteins were focused on a non-linear pH 3-10 gradient and separated on a 10% polyacrylamide gel. Gel was stained with Coomassie Blue. Spots were picked and identified by peptide mass fingerprinting and MALDI-TOF mass spectrometry.

In order to obtain the identity of each protein on the 2D-gel, we excised spots visible after Coomassie staining and digested them with trypsin. Tryptic peptides were analysed by MALDI-TOF MS. Protein identification was carried out using the publicly available *Salmonella* sequences from NCBI. If more than one protein was identified, the one with the match to *S.* Typhimurium LT2 was picked, as *S.* Typhimurium SL1344 was not available on the database at the time. If a match was not found in LT2 but in a different *Salmonella* strain, that strain was picked instead. We analysed a total of 119 spots and we were able to identify 79 of those, which corresponded to 53 unique proteins. The identity of these proteins can be found in Appendix 1 of this thesis.

Although we were able to find many matches to *S.* Typhimurium genomes, some spectra could only be matched to *S.* Typhi genomes. Moreover, 40 of the picked spots remains unidentified either due to lack of peptide mass fingerprint match or lack of a good quality spectrum.

4.3 Predicting the subcellular localisation of GMMA proteins

We had initially hypothesised that *Salmonella* GMMA contained a large number of outer membrane and periplasmic proteins due to the fact they are shed from the outer membrane. To confirm this, we carried out a prediction of the subcellular localisation for each of the 53 proteins identified using PSORTb (Yu et al., 2010) (Fig. 2).

The analysis showed a majority of outer membrane and periplasmic proteins (53%), accounting for 34% and 19% of the total proteins by identity, respectively. We also identified some predicted extracellular proteins (4%),

cytoplasmic proteins (25%), and some with unknown localisation (17%). Although we identified more cytoplasmic proteins than initially anticipated for a GMMA sample, we expect that these proteins are present in small amounts and are a result of cell lysis and not of direct association with the GMMA. These GMMA from *S.* Typhimurium SL1344 had been generated in a rich yeast media and harvested in stationary phase, thus increasing the likelihood of cell lysis and cytoplasmic contamination.

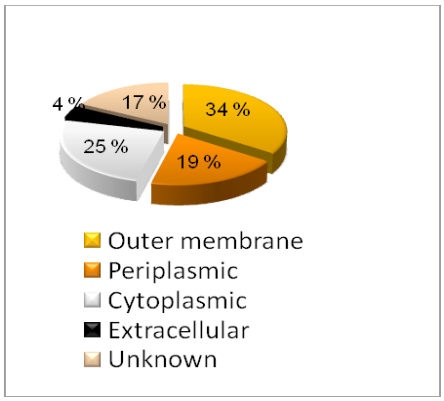


Figure 2. S. Typhimurium Δ*tolR* GMMA contain a large number of outer membrane and periplasmic proteins. *S.* Typhimurium Δ*tolR* GMMA proteins were identified by mass spectrometry and their subcellular localisation was predicted using PSORTb (Yu et al., 2010).

4.4 Discussion

The presence of a large number of outer membrane and periplasmic proteins confirms that *Salmonella* GMMA are formed by a shedding process of the outer membrane. Whereas OMP are imbedded in the membrane, periplasmic proteins are likely found in the centre of the GMMA particle. This is in agreement with a previous report with *E. coli* Δ*tolR* mutants (Berland*a et al*., 2008), which also showed that GMMA generated by these mutants had a high content of periplasmic proteins and OMP.

Contrary to what was initially expected, we identified a small number of cytoplasmic proteins during our analysis of the GMMA proteome. As explained, these are likely a result of cell lysis during the culture of S. Typhimurium SL1344 Δ*tolR*. In subsequent proteomic studies (not described in this thesis), we have harvested the GMMA at a lower OD in an attempt to avoid cytoplasmic contamination.

Proteomic analyses of GMMA also provide a good strategy for identifying potential protein vaccine candidates (Ferrari et al., 2006; Berlanda et al., 2008). The outer membrane proteins that compose GMMA play a crucial role in the interaction between the bacteria and the environment. Due to their location, they are readily exposed to the immune system and pose a target for antibodies. By characterising the protein content of *Salmonella* GMMA, we are also potentially identifying candidates for vaccine antigens.

Chapter 5

Immunological characterisation of *Salmonella* GMMA

5.1 Introduction

Effective protective immunity against *Salmonella* infection requires both cell-mediated and humoral immunity. Although antibody alone or T cells alone can confer low levels of protection, a combination of cell-mediated and humoral immunity is required for high levels of resistance against *Salmonella* (Mastroeni, 2006). *Salmonella* is an intracellular organism and cellular responses play a role during this phase, in particular through CD4+ T cells and IFN-γ production. However, *Salmonellae* is transiently present in the extracellular compartment while spreading to new foci of infection (Sheppard et al., 2003). Moreover, *Salmonella* can also cause bacteraemia in both mice and humans (Collins, 1969; Tacket et al., 1992) which, as described in Chapter 1, is a common feature of NTS disease in Sub-Saharan Africa. During these extracellular phases of *Salmonella* infection, antibodies play a crucial role at neutralising the bacteria. In humans, this is known to happen either through cell-dependent opsnophagocytic mechanisms or cell-independent complement-mediated killing (MacLennan et al., 2008; Gondwe et al., 2010).

In this chapter we describe the humoral and cellular immune response elicited by the *Salmonella* GMMA. We start by looking at the ability of different GMMA to elicit antibodies by different immunisation routes – subcutaneous and intranasal – and characterise that Ab response. We use the sera generated by *S.* Typhimurium GMMA immunisation to assess the

reactivity of the antibodies on live *Salmonella*, where we evaluate both homologous (*S.* Typhimurium) and heterologous (*S.* Enteritidis) reactivity. Cross-reactivity of *S.* Typhimurium antibodies with *S.* Enteritidis is particularly interesting, as it reveals the ability of GMMA proteins to elicit potentially broad-spectrum antibodies. We also make an attempt at identifying the GMMA proteins eliciting antibody. Moreover, we examine the cell populations in the spleen following GMMA immunisation in order to determine whether GMMA can activate a cell-mediated response.

5.2 *S.* Typhimurium GMMA elicit a strong antibody response

In order to assess the ability of *Salmonella* GMMA to generate an antibody response, a dose ranging study in mice was performed with *S.* Typhimurium LT2 GMMA with Oag (LT2Δ*tolR*). Groups of mice were immunised subcutaneously on days 0 and 14 with 0.1µg, 1µg or 10 µg of GMMA and GMMA-specific serum IgG on day 0, 14, and 32 was measured by ELISA (Fig. 1). IgG levels were low following the first immunisation (day 14) in all three groups, but increased greatly after booster immunisation (day 32). The increase in the IgG level response at day 32 was dose-dependent. Immunization with 0.1µg of GMMA resulted in significantly lower GMMA-specific IgG levels than immunization with 1µg. However, there was no significant difference between mice immunised with the 1µg and the 10µg dose. These data show that *S.* Typhimurium GMMA with Oag are highly immunogenic, and that a 1µg is sufficient to generate a strong IgG response that is not further increased by higher doses. Instead, two of the mice immunised with10µg of GMMA died following the second immunisation,

indicating that at this dose *S.* Typhimirium GMMA with Oag are too reactogenic.

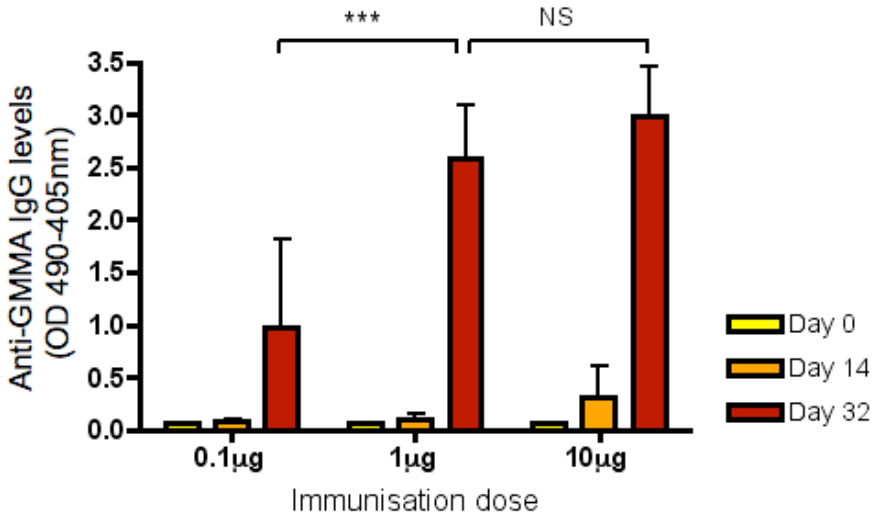


Figure 3. *S.* Typhimurium LT2 Δ*tolR* GMMA generated high levels of GMMA-specific antibody. Mice were immunised with 0.1µg, 1µg or 10 µg of LT2Δ*tolR* GMMA on days 0 and 14 and GMMA-specific IgG levels were measured for individual mice on day 0, 14 and 32 by ELISA using GMMA with Oag (LT2Δ*tolR*) as coating. Data are presented as means with standard deviation. Statistical comparisons of groups highlighted by the bars were performed using Student's t-test. ***, $p<0.001$, NS, not significant.

Given the high levels of IgG antibody generated upon Δ*tolR* GMMA immunisation, we hypothesised whether the majority of this response had been elicited against the Oag portion of LPS. We carried out a competitive ELISA (Fig. 2*A*) in the presence of exogenous *S.* Typhimurium LPS and found that, by increasing its concentration, there was a decrease in absorbance from 3.2 to 1.7 OD (10µg group) and from 2.8 to 1 OD (1µg group). This indicated that a large proportion of the IgG antibody was directed at the LPS portion of the GMMA. In order to investigate what other antigens might be eliciting an IgG response, we carried out a two-dimensional immunoblot with GMMA proteins and probed it with sera from

the same immunogenicity study (Fig. 2*B*). The immunoblot revealed two strong bands, likely corresponding to OmpA - the most immunodominant protein in the *Salmonella* outer membrane – and its degradation products. We could also observe the presence of an Oag ladder on the left of the immunoblot that possibly precipitated with the proteins.

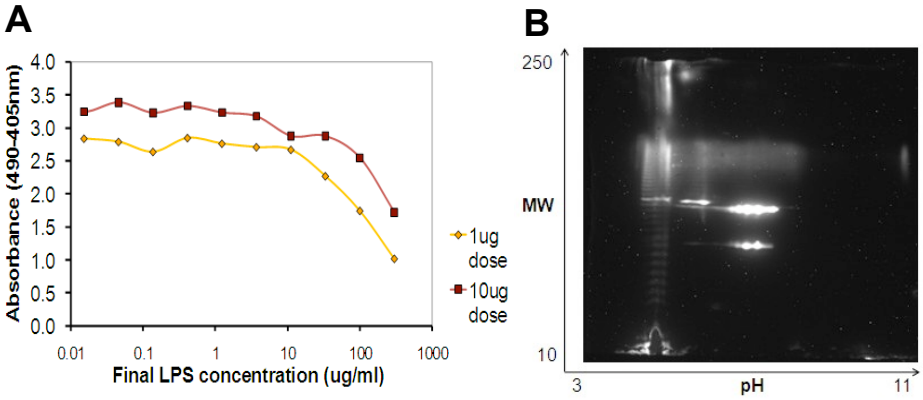


Figure 2. S. Typhimurium LT2 Δ*tolR* GMMA generated high levels of LPS-specific antibody. (*A*) Mice were immunised with 1µg or 10µg of LT2Δ*tolR* GMMA on days 0 and 14 and GMMA-specific IgG levels were measured for individual mice on day 32 by ELISA in the presence of increasing concentrations of *S.* Typhimurium LPS, using GMMA with Oag (LT2Δ*tolR*) as coating. (B) *S.* Typhimurium SL1344 Δ*tolR* GMMA were separated two-dimensionally, transferred to a nitrocellulose membrane, and probed with sera from mice immunised with 10µg. Membranes were incubated with anti-mouse IgG and developed by chemiluminescence.

In order to determine whether *Salmonella* GMMA lacking Oag (Δ*tolR*Δ*wbaP* and Δ*tolR*Δ*wbaP*Δ*msbB*) could elicit an antibody response as high as Δ*tolR* GMMA with OAg and abrogate the presence of LPS-specific antibodies, we carried out a dose-ranging immunogenicity study with the fully optimised (no Oag and detoxified LPS) *S.* Typhimurium SL1344 Δ*tolR*Δ*wbaP*Δ*msbB* GMMA (Δ*msbB* GMMA). Again we used a 0.1µg, 1µg and 10µg doses, as we expected these GMMA to be less reactogenic than the ones used in the previous study. In control groups, mice were immunised

with the intermediate dose of 1µg of *S.* Typhimirium SL1344 Δ*tolR* or S. Typhimirium SL1344 Δ*tolR*Δ*wbaP* GMMA. All GMMA used in this study were generated under iron-limiting conditions. Immunisations occurred on day 0 and 21.

In the group immunised with the highest dose of Δ*msbB* GMMA, we observed adverse effects (ruffled fur and inflammation at site of immunisation) following the first immunisation. Thus, the dose was decreased to 1µg in the second immunisation. GMMA-specific serum IgG levels on day 20 and 45 were determined by ELISA using either GMMA without Oag (SL1344Δ*tolR*Δ*wbaP*) (Fig. 3*A*) or GMMA with Oag (SL1344Δ*tolR*) (Fig. 3*B*). We were interested in seeing whether there would be a difference in these two coatings, as by using GMMA without Oag we would likely be measuring anti-protein and anti-LPS core antibodies only, whereas by using GMMA with Oag, we also expected to detect anti-Oag antibodies.

Using GMMA without Oag as coating, we observed high protein IgG levels in all groups following two immunisations. There were three low responders in the Δ*tolR* group, two in the 0.1µg Δ*msbB* group, and one in the1µg Δ*msbB* group. As observed previously, the booster immunisation was essential to generate a stronger immune response, leading to differences in IgG levels between day 20 and day 45. There were no significant differences in IgG levels in mice immunised with 0.1 or 1µg of GMMA and 1 or 10µg of Δ*msbB* GMMA. However, a significant difference was observed between the groups immunised with 0.1 and 10 µg. At the 1.0µg dose, the Δ*wbaP* GMMA gave rise to significantly higher protein-specific IgG levels than the Δ*tolR* GMMA ($p<0.01$), indicating that in the absence of the Oag layer GMMA proteins are more immunogenic.

When we measured the IgG response to GMMA with Oag, we observed high IgG levels in the Δ*tolR* group but not the other immunisation groups. We did observe an exception in one outlier mouse in the 1µg Δ*msbB* group. Antibodies elicited by GMMA lacking Oag were not able to bind their cognate antigens on GMMA with OAg, indicating that the presence of Oag in the GMMA used for the ELISA coating created a shielding effect, or steric hindrance, that prevented the binding of antibodies closer to the GMMA surface. This effect had been previously observed with *E. coli* porin monoclonal antibodies in a cytofluorimetry assay (Bentley & Klebba, 1988), where an intact Oag blocked adsorption of porin antibodies, but as the LPS structure become shorter the porin surface epitopes were increasingly recognised. With regards to the use of GMMA without Oag or GMMA with Oag as ELISA coating antigens, we can conclude that whereas in the first case protein and LPS core epitopes will be detected, that will not occur in the second case, as only Oag antibodies will be detected,.

We observed an inverse correlation between anti-Oag antibodies and anti-protein antibodies. The two mice in the Δ*tolR* group that were identified as high responders on GMMA without Oag coating (Fig. 2*A*) showed the lowest anti-Oag IgG levels (Fig. 2*B*). Similarly, the mouse with the lowest anti-protein IgG levels is the high responder in the 1µg Δ*msbB* group in Fig. 2*A* corresponds. This was surprising, as an Oag response was not expected in this group and none of the other mice immunised with the Δ*msbB* GMMA developed this response.

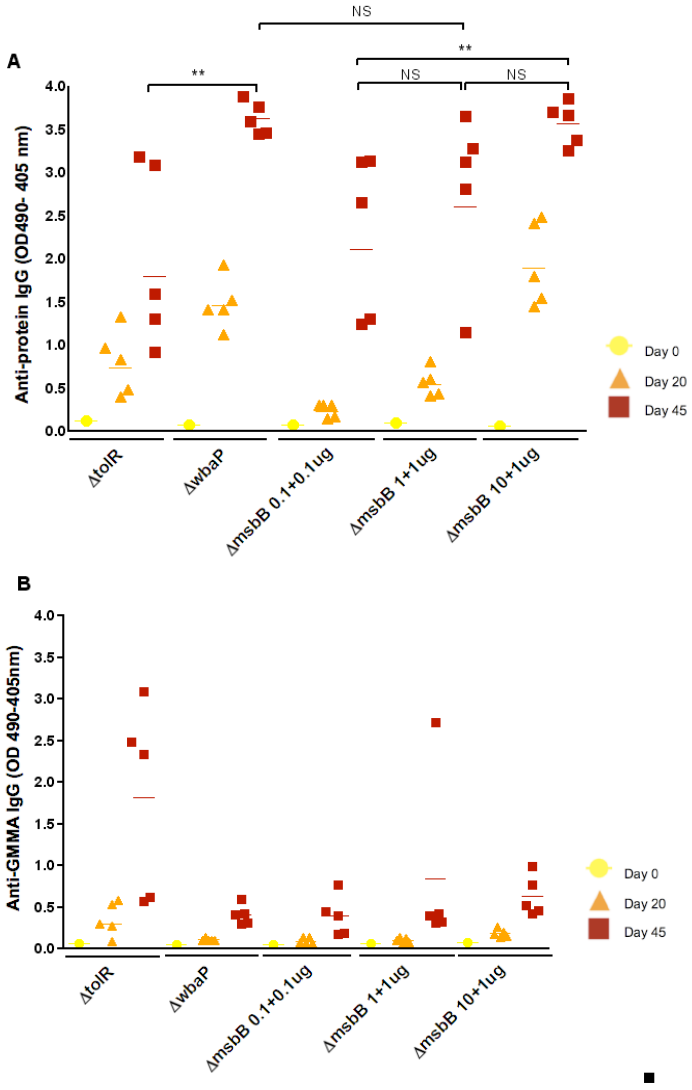


Figure 3. *Salmonella* GMMA elicit high anti-protein and anti-Oag IgG levels following subcutaneous immunisation. Mice were immunised with of SL1344 Δ*tolR* (1ug & 1ug on the first and second immunisation, respectively), Δ*tolR*Δ*wbaP* (1ug + 1ug), and different dosages of Δ*tolR*Δ*wbaP*Δ*msbB* GMMA (0.1ug + 0.1ug, 1ug + 1ug, or 10ug + 1ug) on days 0 and 21. Anti-protein IgG response on day 0, 20, and 45 measured by ELISA using (*A*) GMMA without O antigen (SL1344Δ*tolR*Δ*wbaP*) as coating and (*B*) using GMMA with O antigen (SL1344Δ*tolR*) as coating. Geometric mean is shown as a bar. Groups highlighted by bars were compared using using the Mann-Whitney test. **, $p<0.01$, NS, not significant.

Despite the high immunogenicity of Δ*msbB* GMMA and ability to generate protein antibodies, we used GMMA from Δ*tolR*Δ*wbaP* mutants in the remainder of this project. *S.* Typhimurium Δ*tolR*Δ*wbaP* mutants produced higher amounts of GMMA per litre of culture than Δ*tolR*Δ*wbaP*Δ*msbB*, and allowed us to get a higher yield of GMMA for subsequent studies. Furthermore, the difference between the fully toxic hexa-acylated lipid A and the penta-acylated lipid A generated by the *msbB* deletion is not detected by mice.

5.3 Characterisation of the antibody subclasses activated by *S.* Typhimurium GMMA

To better understand the type of antibody response elicited by *Salmonella* GMMA immunisation, we assessed LPS-specific and protein-specific serum Ig, IgG1, IgG2a and IgA titres by ELISA following three immunisations with GMMA from *S.* Typhimurium SL1344 with OAg (STm Oag+ GMMA) or without OAg (STm Oag- GMMA) (Fig. 4).

STm Oag- GMMA elicited higher protein-specific total Ig levels than STm Oag+ GMMA (Fig. 4*A*), likely resulting from the unmasking of protein antigens on the GMMA surface as described above. As expected, LPS-specific Ig was higher in the STm Oag+ group (Fig. 4*B*). However, we also detected some anti-LPS Ig in the STm Oag- group, which might have resulted from anti-LPS core antibodies, contamination of LPS by protein, or unspecific binding in the ELISA. Immunisation with either type of GMMA elicited protein-specific IgG1 at similar levels (Fig. 4*C*), indicating that STm Oag- GMMA can elicit IgG1 in the absence of Oag. High titres of LPS-specific IgG1 were detected following Oag+ but not Oag- GMMA immunisation, indicating a role for Oag in IgG1 production (Fig. 4*D*). STm

Oag+ elicited higher anti-protein IgG2a titres than STm Oag+ (Fig. 3*E*). However, LPS IgG2a titres were also detected after Oag- GMMA immunisation. STm Oag+ GMMA gave rise to an IgA response directed at both proteins (Fig. 3*G*) and LPS (Fig. 3*H*), expected due to the presence of LPS in the GMMA. We were not able to detect any IgA in STm Oag- GMMA-immunised mice sera at the lowest dilution used (1:100).

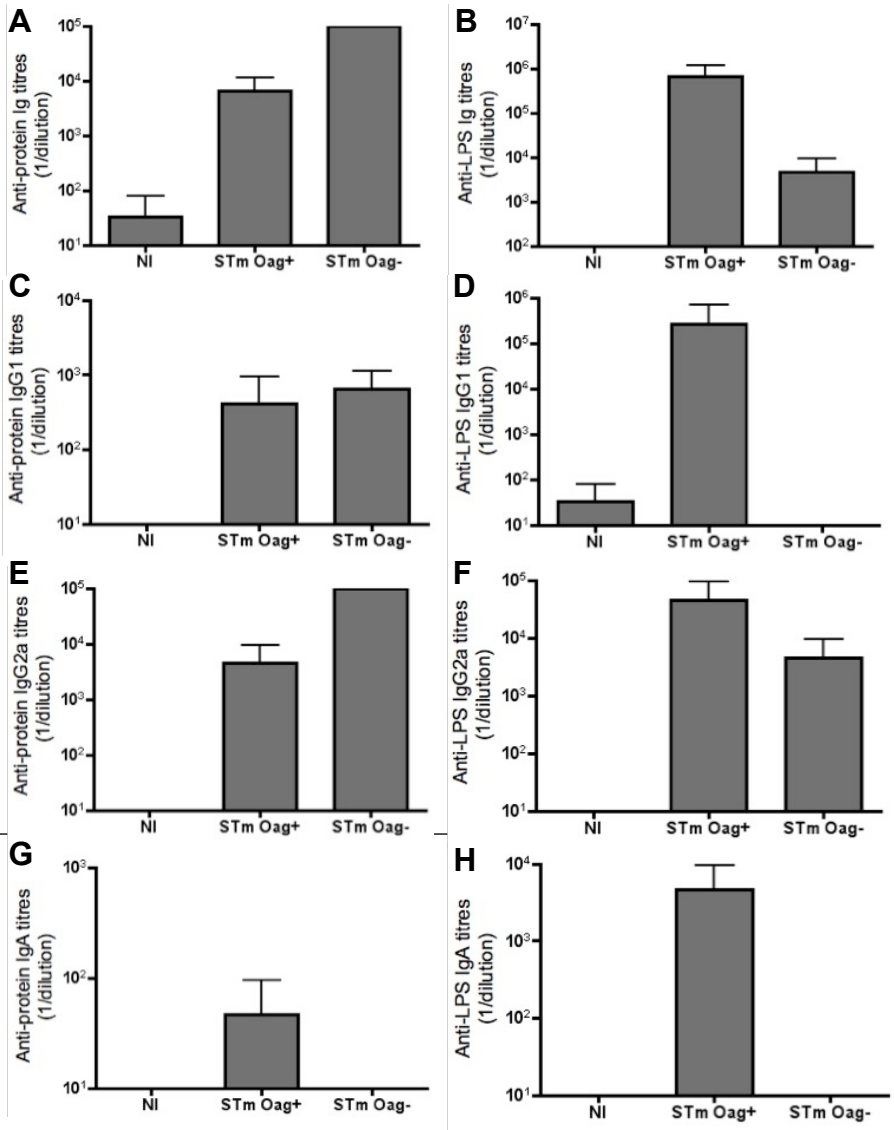


Figure 4. *S.* Typhimurium GMMA elicit different Ig subclasses following subcutaneous immunisation. Five mice per group were immunised three times on days 0, 7 and 21 with *S.* Typhimurium SL1344 Δ*tolR* GMMA (STm Oag+) or S. Typhimurium SL1344 Δ*tolR*Δ*wbaP* GMMA (STm Oag-) and protein-specific and LPS-specific Ig (*A*, *B*), IgG1 (*C*, *D*), IgG2a (*E*, *F*) and IgA (*G*, *H*) titres were measured by ELISA one week after the last immunisation by calculating using the mean of the negative control plus 2 standard deviations as the cut-off for absorbance. Data as presented as arithmetic means with standard deviation.

4.4 GMMA are immunogenic by intranasal route

As *Salmonella* GMMA with and without Oag generated under low iron conditions were highly immunogenic by subcutaneous route, we were interested in determining whether administration of GMMA through a mucosal route would be safe and would also elicit high serum antibody titres in mice, as a possible alternative to subcutaneous immunisation. *Salmonella* is a mucosal pathogen, and by stimulating mucosal immunity, we were hoping to activate protective immune responses suited to this entry route. Two mice per group were immunized intranasally with 1µg or 5µg of *S.*

Typhimurium SL1344 GMMA from Δ*tolR* mutants (STm Oag+) or Δ*tolR*Δ*wbaP* (STm Oag-) on days 0, 7 and 21. GMMA with Oag were well tolerated at both GMMA doses, whereas GMMA without Oag were only tolerated well at the lower dose. Serum IgG titres were determined by ELISA one week after the last immunisation (Figure 4).

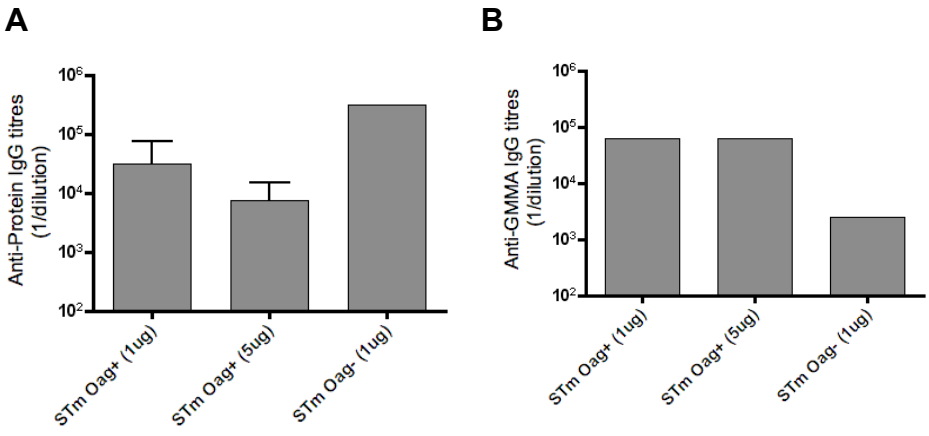


Figure 4. *S.* Typhimurium GMMA elicit high serum IgG titres by intranasal immunisation. Four groups of two mice each were immunised with 1µg or 5µg of SL1344Δ*tolR* (STm Oag+) or SL1344Δ*tolR*Δ*wbaP* GMMA (STm Oag-) on days 0, 7 and 21. Serum IgG levels were measured on day 28 by ELISA using (*A*) GMMA without Oag or (*B*) GMMA with Oag as coating.

Immunisation with either STm Oag+ GMMA or STm Oag- GMMA elicited the production of protein-specific (Fig. 4*A*) and Oag-specific (Fig. 4*B*) IgG. It should be noted that group numbers were very small (two mice per group) and more mice would be required to get a more accurate measurement of IgG titres. Nonetheless, we can infer from these results that *S.* Typhimurium GMMA are immunogenic by intranasal route and that at a 1µg dose they are safe for use in mice.

5.5 S. *Typhimurium* GMMA antibodies are cross-reactive in S. *Enteritidis*

5.5.1 Assessing antibody binding to live *Salmonella in vitro*

Having confirmed the ability of *S.* Typhimurium GMMA proteins to elicit a strong antibody response in mice, we wanted to assess the ability of these antibodies to bind live *S.* Typhimurium and to investigate the cross-reactivity of these antibodies with other NTS serovars. Using a flow cytometry-based assay, we stained *S.* Typhimurium SL1344 or *S.* Enteritidis P125109 with immune sera raised against STm Oag+ and STm Oag- GMMA to measure the binding of the antibodies to the live bacteria. We used anti-mouse IgG labelled with APC or FITC for fluorescence detection. Different dilutions were tested in order to assess the binding of the antibodies. Pre-immune sera were used to define the base level (Fig. 5).

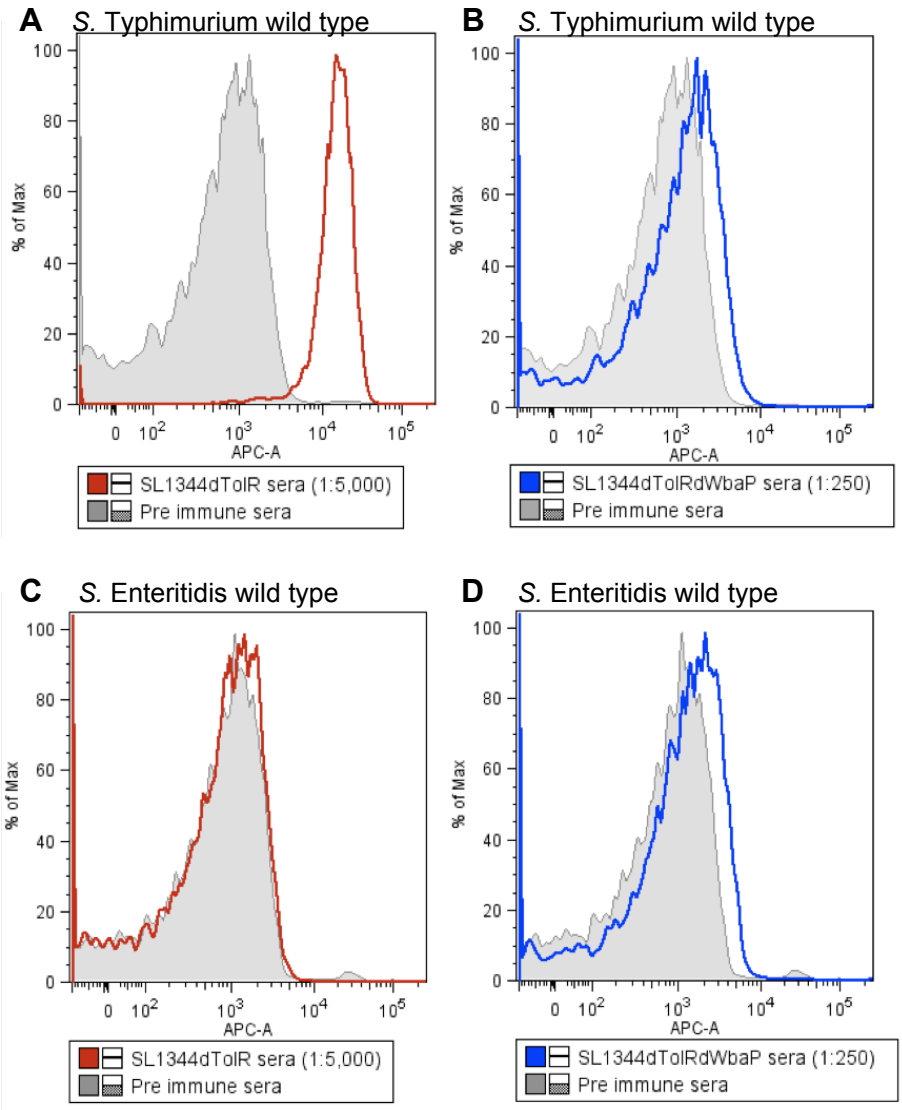

A
S. Typhimurium wild type
% of Max
APC-A
SL1344dTolR sera (1:5,000)
Pre immune sera
B
S. Typhimurium wild type
% of Max
APC-A
SL1344dTolRdWbaP sera (1:250)
Pre immune sera
C
S. Enteritidis wild type
% of Max
APC-A
SL1344dTolR sera (1:5,000)
Pre immune sera
D
S. Enteritidis wild type
% of Max
APC-A
SL1344dTolRdWbaP sera (1:250)
Pre immune sera

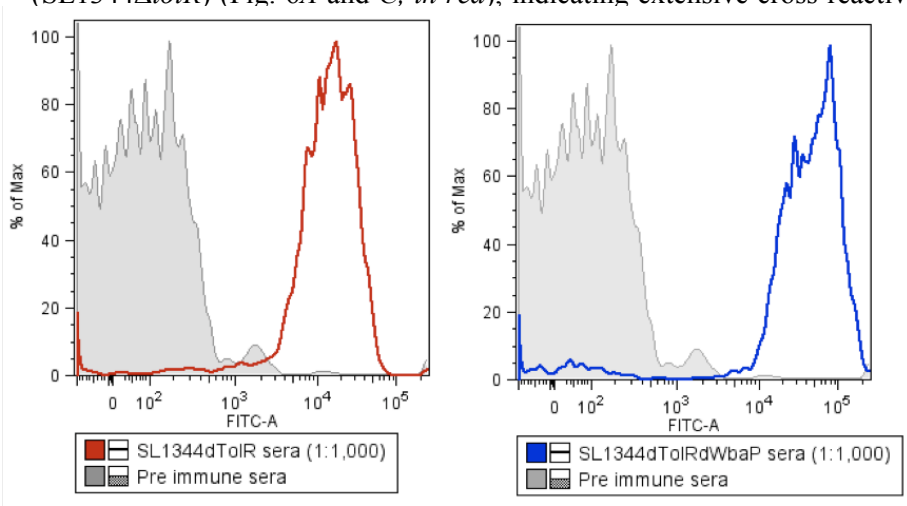
Figure 5. O antigen LPS layer hinders the binding of anti-GMMA protein antibodies to the surface of bacteria. *S.* Typhimurium SL1344 (*A* and *B*) and *S.* Enteritidis P125109 (*C* and *D*) wild type strains were incubated with pooled sera from mice immunised with STm Oag+ (SL1344d*tolR, A* and *C*, in red) or STm Oag- (SL1344d*tolR*d*wbaP*, *B* and *D*, in blue) GMMA, and then incubated with APC-labeled-anti-IgG secondary antibody. APC intensity in each sample was analysed by flow cytometry.

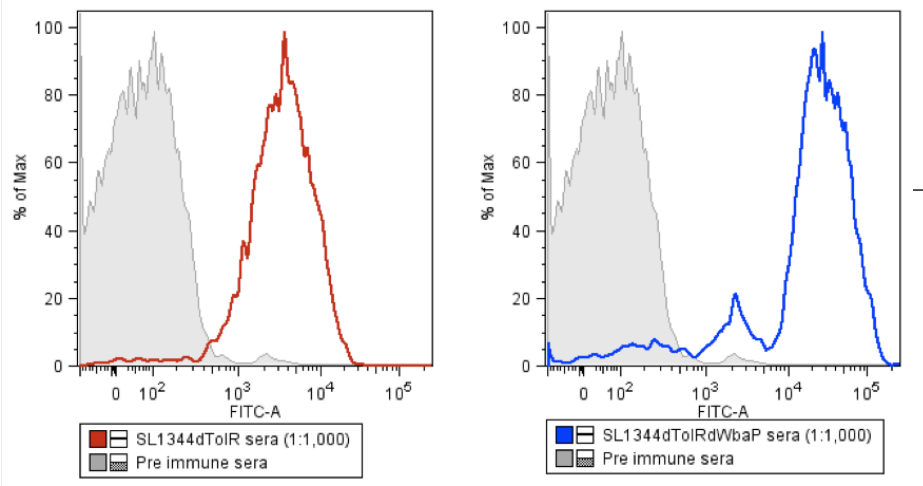
We observed strong binding of STm Oag+ GMMA serum IgG to *S.* Typhimurium at a 1:5,000 dilution compared to the pre-immune sera (Fig. 5*A*, *in red*). When we used sera from Oag- GMMA, a signal was only detected at a high sera concentration (1:250) (Fig. 5*B, in red*), indicating that the presence of full length LPS did not allow for the binding of the protein antibodies to their antigens on the bacterial surface. Not surprisingly, we did not observe a fluorochrome signal when we assessed the binding of Oag+ GMMA sera to *S.* Enteritidis (Fig. 4*C* and *D*). Whereas the O:4-specific antibodies in Oag+ sera were unable to bind *S.* Enteritidis O:9 (Fig. 4*C*), the protein antibodies in Oag+ and Oag- sera could not access their cognate antigens due to steric hindrance of the Oag layer. We did observe slight binding of Oag- GMMA sera at high sera concentrations (1:250), indicating

that some protein antibodies were able to bind at these concentrations. This effect has been observed previously in our ELISA (see Fig. 2*B*) and also in studies with *E. coli* porin antibodies in a flow cytometry assay (Bentley & Klebba, 1988).

Although we observed that protein antibodies were not able to bind live *Salmonella in vitro* due to steric hindrance by full-length LPS, this effect might not occur *in vivo*. We tested the ability of Oag- GMMA to protect against homologous and heterologous infection at a later stage. Please refer to Chapter 6 of this thesis.

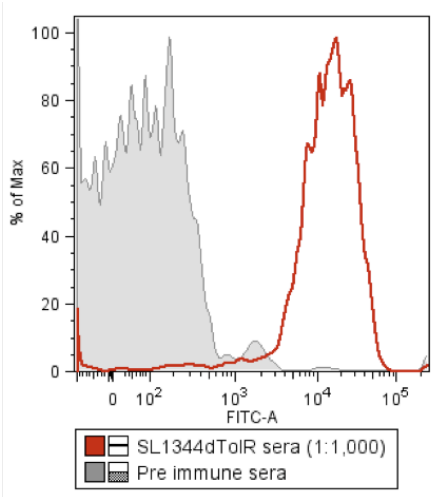
To analyse the binding of protein sera to different serovars, strains lacking Oag were used. We analysed *S.* Typhimurium SL1344 and *S.* Enteritidis P125109 mutants lacking Oag (Δ*wbaP*) using same flow cytometry assay. A strong signal was obtained when *S.* Typhimurium Δ*wbaP* and *S.* Enteritidis Δ*wbaP* were stained with sera raised against STm Oag+ GMMA (SL1344Δ*tolR*) (Fig. 6*A* and *C, in red*), indicating extensive cross-reactivity



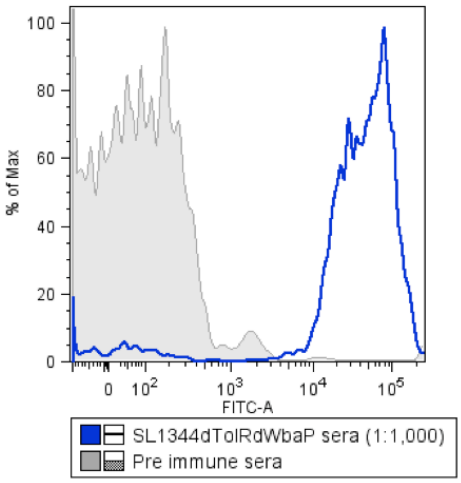


Proteomic characterisation and immunoprecipitation assays to determine the

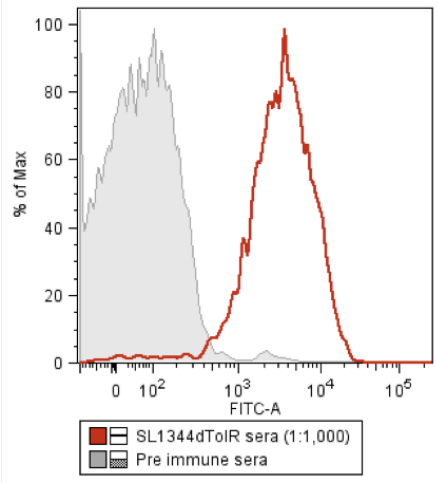
A *S.* Typhimurium rough (Oag-)



B *S.* Typhimurium rough (Oag-)



C *S.* Enteritidis rough (Oag-)



D *S.* Enteritidis rough (Oag-)

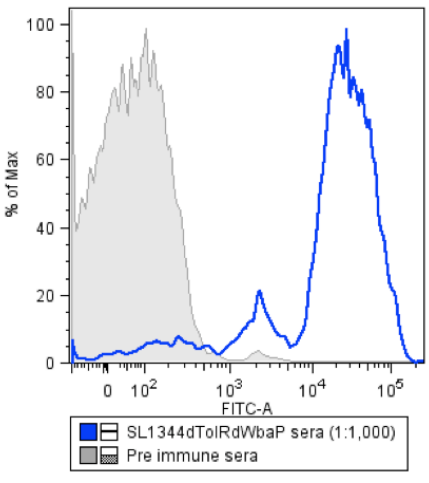


Figure 6. Antibodies raised against *Salmonella* GMMA show cross-reactivity in NTS. *S.* Typhimurium (*A* and *B*) and *S.* Enteritidis (*C* and *D*) rough mutants were stained with pooled sera from mice immunised with STm Oag+ (SL1344d*tolR, A* and *C*, in red) or STm Oag- (SL1344d*tolR*d*wbaP*, *B* and *D*, in blue) GMMA, and then incubated with FITC-labeled anti-IgG secondary antibody. FITC intensity of each sample was assessed by flow cytometry.

5.5.2 Characterisation of antibody targets

Given the high protein-specific serum antibody levels seen in the ELISA and the extensive cross-reactivity observed by flow cytometry, we investigated which GMMA proteins elicited antibody responses. Immunoblots were performed using sera from subcutaneous and intranasal immunisations with *S.* Typhimurium Oag- GMMA (low iron) to probe *S.* Typhimurium SL1344 or *S.* Enteritidis P125109 GMMA proteins separated by gel electrophoresis (Figure 7).

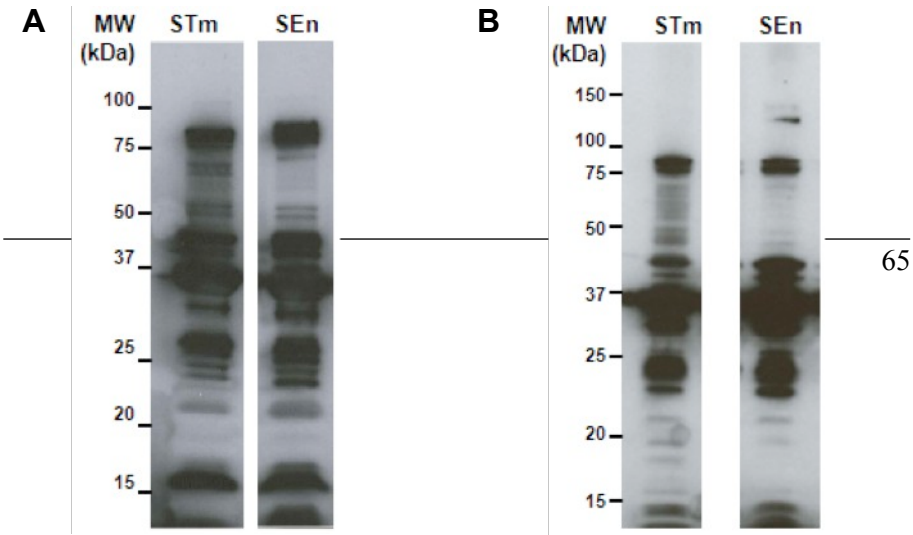


Figure 7. *S.* Typhimurium Δ*tolR*Δ*wbaP* GMMA raise antibodies against a large number of proteins and these are cross-reactive in *S.* Enteritidis. (*A*) 2.5μg or (*B*) 1μg of STm OAg- GMMA (*left lane*) or SEn Oag- GMMA (*right lane*) proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with pooled sera from mice immunised subcutaneously (A) or intranasally (B) three times with STm Oag- GMMA (Δ*tolR*Δ*wbaP*).

The immunoblots showed that sera from subcutaneous and intranasal STm Oag- GMMA immunisation react with a large number of proteins in *S.* Typhimurium (Fig. 7*A*, *left lane*) and *S.* Enteritidis (Fig. 7*A*, *right lane)*. (Fig. 7*A*, *right lane*). The patterns of reactive proteins in *S.* Typhimurium and *S.* Enteritidis showed great similarity, indicating that protein contents of the two GMMA are similar. The most predominant bands, in all cases, are situated in the 75kDa, 45kDa, 37kDa and 25kDa region. We did not identify these proteins by mass spectrometry. The band visible at 37kDa corresponds in size to the *Salmonella* porins OmpA, OmpC, and OmpD. OmpA is the most immunodominant protein target on the Salmonella surface, partly due to its abundance, and for this reason we would expect it to be highly immunogenic in a GMMA context. The 75kDa band might correspond to the

outer membrane iron-regulated proteins, as the other main band in that region corresponding to YaeT is found at 89kDa.

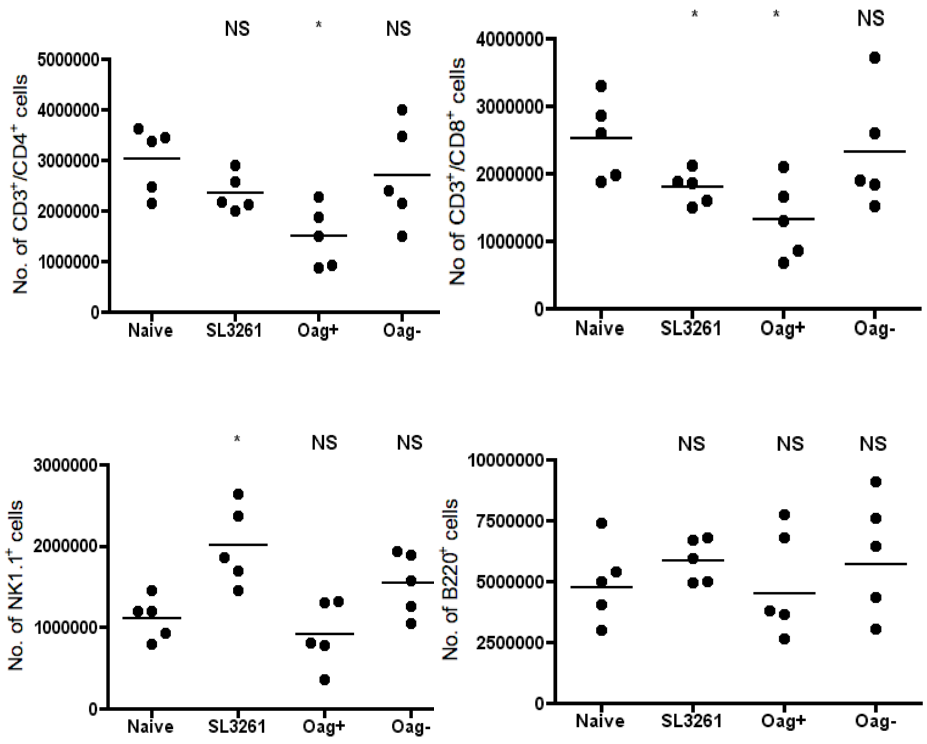
Despite the cross-reactivity of *S.* Typhimurium sera with *S.* Enteritidis proteins, there are nonetheless some regions in the SEn lane with less reactivity than in the STm lane. For example, in the region between 50 and 75kDa, whereas there are four clearly defined bands in STm, only three of these are present in SEn. In the intranasal blot, we also observed a reactive protein in *S.* Enteritidis at around 120 kDa that was not visible in the *S.* Typhimurium lane. Although we can assume that this protein was present in *S.* Typhimurium GMMA because the antibodies were generated, it is possible that it is present in larger amounts in *S.* Enteritidis.

These results indicate that the antibodies generated by *S.* Typhimurium GMMA immunisation cover a wide range of *S.* Typhimurium and *S.* Enteritidis proteins.

5.7 Cell-mediated response to GMMA immunisation

Given the important role played by cellular immunity during the intracellular stages of *Salmonella* infection, we investigated the cell-mediated response one week after GMMA immunisation. Mice were immunised on days 0 and 7 with *S.* Typhimurium GMMA with or without Oag, with *S.* Typhimurium SL3261 (SL3261) on day 0 or not immunised. SL3261 is an established live attenuated vaccine against *S.* Typhimurium infection in mice and the correlates of protection induced by this strain have been described (Harrison, Villarreal-Ramos, Mastroeni, Demarco de, & Hormaeche, 1997). As a result, we used it as the positive control in these experiments. However, we cannot discard the possibility that GMMA are associated with different correlates of protection, given that they are a subunit and not a live vaccine like SL3261.

We analysed the composition of the splenocyte population by looking at T cells, NK cells, B cells, neutrophils and macrophages in individual mice by staining of spleen cells and analysis by flow cytometry (Fig. 8). In the SL3261, we observed elevated levels of NK1.1+ cells (NK and NKT cells), Ly6G+ (neutrophils) and F480+ (macrophages), and reduced levels of CD3+/CD8+ (CD8+ T cells) cells when compared to splenocytes of naive mice. Surprisingly, we found no significant differences in CD3+/CD4+ (CD4+ T cells) levels between SL3261 and naive mice. STm Oag+ GMMA, on the other hand, led to reduced levels of CD4+ and CD8+ T cells, and similarly to SL3261, increased neutrophil levels. Immunisation with STm Oag- GMMA elicited a cellular response that differed from Oag+ GMMA, leading to an increase in neutrophils and macrophages only.



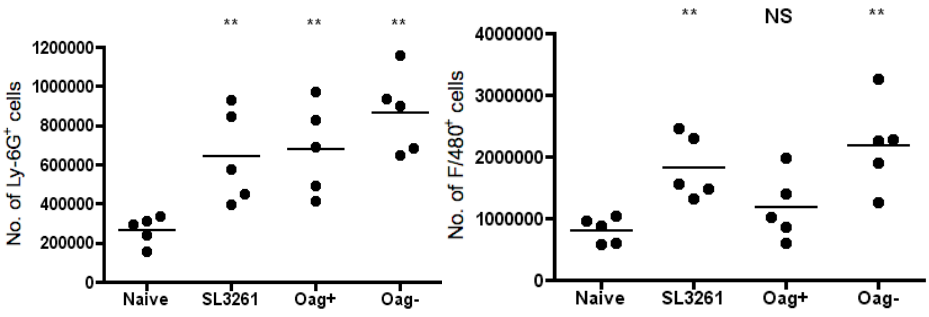


Figure 8. Composition of the splenocyte populations following GMMA immunisation. Mice were immunised with *S.* Typhimurium GMMA with Oag (Oag+) or without Oag (Oag-) on days 0 and 7, with *S.* Typhimurium SL3261 (SL3261) on day 0, or not immunised (naive). Spleens were harvested and splenocytes were stained for (*A*) CD4+ T cells, (*B*) CD8+ T cells, (*C*) NK and NKT cells, (*D*) B cells, (*E*) neutrophils, and (*F*) macrophages.

5.9 Discussion

The immune response elicited by *S.* Typhimurium GMMA with Oag has been previously characterised and determined to stimulate antigen-presenting cells, to have strong pro-inflammatory properties and to activate a B and a T cell response (Alaniz et al., 2007). In this chapter we have further characterised the immunogenicity of *Salmonella* GMMA. In our analyses we have included both GMMA with and without Oag and we have assessed both antibody and cell-mediated responses elicited upon immunisation. GMMA with Oag and without Oag were very immunogenic, generating high Ab titres both subcutaneously and intranasally. The impaired ability of protein-based antibodies to bind live *Salmonella* in vitro was surprising, but

we cannot exclude that this is simply an *in vitro* effect. The immunoblots used for characterisation of the antibody response against *S.* Typhimurium GMMA showed that there were an extensive number of reactive proteins in both *S.* Typhimurium and *S.* Enteritidis. Although some attempts were made at identifying these bands based on molecular weight, further analyses are required. A suitable approach to use for the identification of reactive proteins would be immunoprecipitation of *Salmonella* proteins using GMMA sera. Moreover, we carried out an analysis of the splenocyte populations following GMMA immunisation. We found that GMMA with and without Oag elicit distinct groups of immune cells between them, both they both lead to an increase in the neutrophil population. The cellular response for the GMMA was also different from the live attenuated strain SL3261, but this is not surprising as we were comparing a subunit vaccine with a live strain.

Chapter 6

Protection studies with *Salmonella* GMMA

6.1 Introduction

Previous studies with *Salmonella* GMMA showed these were protective against homologous infection in a murine model of acute *Salmonella* infection (Alaniz et al., 2007). However, the study failed to take into account the ability of GMMA to generate a broad-spectrum response against other serovars if they are to be used as an anti-*Salmonella* vaccine. Similarly, recent studies with subunit vaccines against *Salmonella* (Gil-Cruz *et al*., 2009) only assessed the ability to protect against homologous infection.

In our study, the expression of Oag in *Salmonella* GMMA was abrogated, which unmasks conserved protein antigens and avoids an anti-Oag serospecific response, in order to test the ability of GMMA to generate an immune response that is protective against homologous and heterologous serovars.

The study by *Alaniz et al.* with *Salmonella* GMMA used i.p. immunisation with i.p. infection in *Salmonella*-susceptible and *Salmonella*–resistant mice (C3H/HeJ and C3H/HeN respectively). We used a similar model of acute *Salmonella* infection in *Salmonella*-susceptible mice (C57BL/6), but GMMA were administered subcutaneously. Mice were immunised with GMMA from either one of two serovars, *S.* Typhimurium or *S.* Enteritidis. By immunising with GMMA from different NTS serovars we were able to

test their ability to generate a protective and/or a cross-protective immune response. As positive controls we used an established live vaccine against S. Typhimurium infection in mice, *S.* Typhimurium SL3261. *S.* Typhimurium M525, a strain of intermediate virulence, was used as a challenge strain. This strain is routinely administered intravenously at 1 x 10^3 c.f.u. per dose.

It is established that protectively immunised mice inhibit bacterial replication during live *Salmonella* challenge (Mastroeni, Villarreal-Ramos, & Hormaeche, 1992), so we used organ colonisation as a measure of protection against infection. Whereas analysis of bacterial numbers in the spleen and liver is the gold standard in *Salmonella* vaccine studies for assessment of vaccine effectiveness, blood is not commonly used as a measure of protection. However, given that porin-immunised mice showed abrogation of bacteraemia compared to non-immunised mice (*Gil-Cruz et al.*, 2009), we hypothesised whether the same effect would be observed in GMMA-immunised mice.

The role of the humoral and the cellular response following GMMA immunisation and *S.* Typhimurium infection was also investigated.

6.2 Assessing the ability of *Salmonella* GMMA immunisation to impair *S.* Typhimurium infection

Previous studies with *S.* Typhimurium GMMA showed that subcutaneous immunisation with GMMA could impair S. Typhimurium intraperitoneal infection in the spleen, liver, and mesentheric lymph nodes of mice (Alaniz et al., 2007). We used a similar model in mice susceptible to *Salmonella* infection (Nramp1-negative) and combined subcutaneous GMMA immunisation with intraperitoneal challenge in order to determine whether

protein GMMA (i.e. those lacking Oag) from *S.* Typhimurium and *S.* Enteritidis could protect against *S.* Typhimurium intraperitoneal infection. In this way we could assess the ability of GMMA to elicit a homologous and/or heterologous protection.

Five groups of five mice were immunised subcutaneously with *S.* Typhimurium GMMA with (STm Oag+) or without Oag (STm Oag-), *S.* Enteritidis GMMA without Oag (SEn Oag-), or orally with *S.* Typhimurium SL3261 (SL3261) and infected with 1 x 10^4 *S.* Typhimurium M525 for 3 days. We examined the bacterial burden in the spleen, liver and blood. We expected to see a reduction in colonisation of spleen and liver of GMMA-immunised mice to indicate protection. However, organ colonisation was very low in all groups (results not shown) and it was not possible to distinguish between protectively-immunised mice and those not protectively immunised. We concluded this was due to too low an infection dose of *S.* Typhimurium M525 when administered by intraperitoneal route, so we carried out dose titration studies on age-matched non-immunised mice to determine the optimal infection dose of M525 for subsequent studies. We concluded that 1 x 10^6 c.f.u. M525, a two log increase relative to before, led to non-lethal but sufficient organ colonisation by day 3 of infection (results not shown).

In a subsequent study, five groups of ten mice were immunised subcutaneously with STm Oag+, STm Oag-, SEn Oag-, or orally with SL3261 and infected with 1.1 x 10^6 *S.* Typhimurium M525 for 3 days. As previously, we assessed the bacterial burden in the spleen (Figure 1*A*) and liver (Figure 1*B*) in individual mice as a measure of protective immunity.

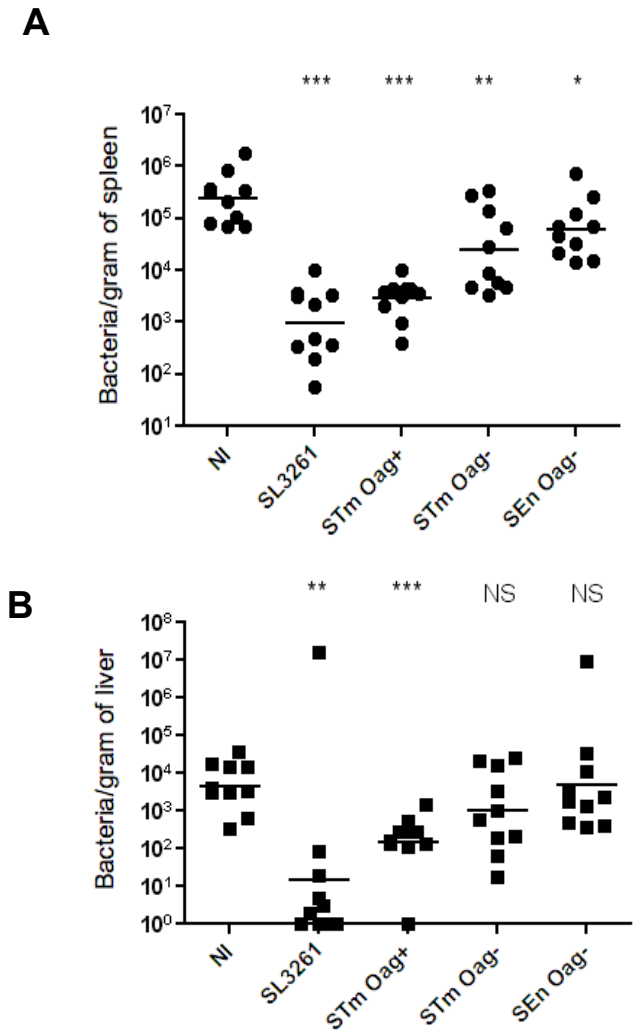


Figure 9. *Salmonella* GMMA impair *S.* Typhimurium infection in mice. Mice were immunised with *S.* Typhimurium SL1344 Δ*tolR* GMMA (STm Oag+), *S.* Typhimurium SL1344 Δ*tolR*Δ*wbaP* GMMA (STm Oag-), or *S.* Enteritidis P125109Δ*tolR*Δ*wbaP* GMMA (SEn Oag-) on days 0, 7 and 21, or with 1.1 x 106 *S.* Typhimurium SL3261 (SL3261), or not immunised (NI) and infected intraperitoneally with 1.1 x 10^6 *S.* Typhimurium M525 for 3 days. Bacterial numbers were measured in the (*A*) spleen and (*B*) liver by direct culturing. Data are represented as individual mice with the geometric mean as a bar. The Mann–Whitney test was applied to compare the statistical significance between the immunised

groups and non-immunised mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$; NS, not significant.

Mice immunised with STm Oag- had significantly less bacteria in the spleen than non-immunised mice ($p<0.01$), with a one log reduction on average. Although this reduction was less pronounced than for STm Oag+ or the live attenuated control SL3261, it showed that GMMA lacking Oag can impair *S.* Typhimurium infection. Furthermore, immunisation with SEn Oag- GMMA also led to a reduction of M525 colonisation in the spleen ($p<0.05$), showing these GMMA were able to impair the infection of a heterologous *Salmonella* serovar.

In the liver we observed similar colonisation patterns to those of the spleen. SL3261-immunised mice showed the biggest reduction in colonisation relative to non-immunised mice ($p<0.01$), with the exception of a heavily colonised mouse. Immunisation with STm Oag+ GMMA was also very effective at reducing bacterial burden in the liver ($p<0.0001$), as this group showed a 1.5 log10 decrease relative to non-immunised mice. Half of the mice immunised with STm Oag- GMMA also showed lower bacterial numbers than non-immunised mice, but given the other half was more heavily colonised, there was no significant difference between these two groups. There were no differences in the levels of colonisation of mice immunised with SEn Oag- GMMA and non-immunised mice, indicating that albeit the impairment of colonisation of a heterologous strain in the spleen, this might not have been a widespread effect.

We also collected blood from individual mice in order to assess bacteraemia and to better understand the role of an anti-GMMA response in hindering circulation of *Salmonella* in the blood. The number of S. Typhimurium in the blood was variable between and within immunisation groups (Figure 2). We did not detect any bacteria in the blood of mice immunised with SL3261 or STm Oag+ GMMA. In mice immunised with STm Oag- GMMA bacterial

numbers ranged from 5 x 10^2 to undetected, with no significant differences relative to non-immunised mice. A similar colonisation pattern was seen in the blood of mice immunised with SEn Oag- GMMA, indicating that anti-protein antibodies were less efficacious at controlling bacterial spread in the blood. This indicates a possible role for an anti-Oag response in hindering bacteraemia.

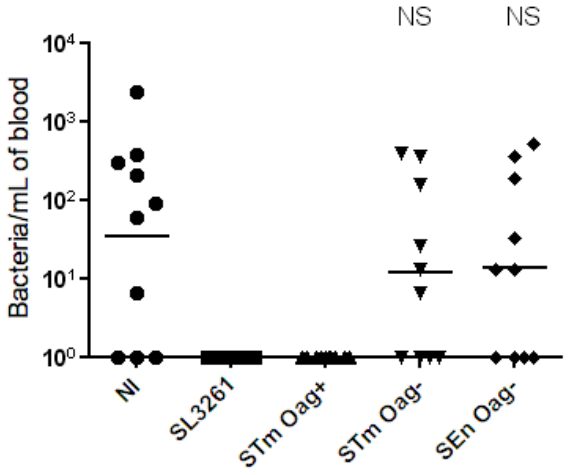


Figure 2. *Salmonella* GMMA lacking Oag did not impair *S.* Typhimurium M525 blood bacteraemia. Mice were immunised with *S.* Typhimurium SL1344 Δ*tolR* GMMA (STm Oag+), *S.* Typhimurium SL1344 Δ*tolR*Δ*wbaP* GMMA (STm Oag-), or *S.* Enteritidis P125109Δ*tolR*Δ*wbaP* GMMA (SEn Oag-) on days 0, 7 and 21, or with 1.1 x 10^6 *S.* Typhimurium SL3261 (SL3261), or non-immunised (NI) and infected intraperitoneally with 1.1 x 10^6 *S.* Typhimurium M525 for 3 days. Bacterial numbers were measured in the blood by direct plating on ampicilin-containing plates three days after blood was harvested. Detection limit was 6 c.f.u. per mL of blood. Data are represented as individual mice with the geometric mean as a bar. The Mann–Whitney test was applied to compare the statistical significance between the STm Oag+ and STm Oag- groups and non-immunised mice. NS, not significant.

6.3 Evaluating the role of antibody in *S.* Typhimurium organ colonisation

We wanted to investigate the role played by antibody immunity in the colonisation results described in section 5.2. With this in mind, we measured individual protein- and LPS-specific serum IgG antibody titres by ELISA one week prior to *S.* Typhimurium infection. Anti-protein IgG (Figure 3*A*) was high across all immunisation groups and in all cases significantly different from the non-immunised mice ($p<0.0001$). Less protein-specific IgG were detected in the SEn Oag- group, likely reflecting the differences in proteomic content and reactivity between *S.* Enteritidis GMMA and the *S.* Typhimurium GMMA used as a coating antigen. Anti-LPS titres (Figure 3*B*) were highest in the SL3261- and the STm Oag+-immunised groups as expected ($p<0.0001$), given that both these immunising agents contain high levels of STm LPS and immunogenic Oag. Some anti-LPS antibodies were also detected in the STm Oag- group and to a lesser extent in the SEn Oag- group, probably corresponding to antibodies directed at the core portions of the LPS also present in Oag- GMMA, but these were not significantly different from non-immunised mice titres.

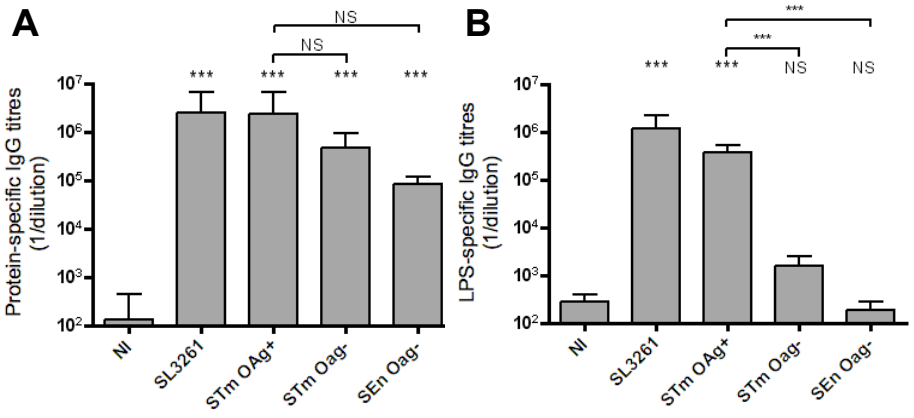


Figure 3. GMMA-specific serum IgG levels are still elevated one week prior to *S.* Typhimurium M5252 infection. Mice were immunised with *S.* Typhimurium SL1344 Δ*tolR*

GMMA (STm Oag+), *S.* Typhimurium SL1344 Δ*tolR*Δ*wbaP* GMMA (STm Oag-), or *S.* Enteritidis P125109Δ*tolR*Δ*wbaP* GMMA (SEn Oag-) on days 0, 7 and 21, or with 1.1 x 10^6 *S.* Typhimurium SL3261 (SL3261), or non-immunised (NI). Serum IgG titres for individual mice were measured one week prior to infection with *S.* Typhimurium M525 using ELISA. Plates were coated with (*A*) STm Oag- GMMA or with (*B*) *S.* Typhimurium LPS in order to determine STm LPS-specific and protein-specific titres, respectively. Data are represented as means + SEM. The Mann–Whitney test was applied to compare the statistical significance between the immunised groups and non-immunised group (lower representation) or groups connected by lines. ***, $P < 0.0001$; NS, not significant.

IgG levels seemed to reflect the colonisation patterns of the spleen and the liver, with the SL3261- and the STm Oag+ GMMA-immunised groups exhibiting the highest protein- and LPS-specific titres and the lowest c.f.u. counts. The STm Oag- GMMA- or the SEn Oag- GMMA-immunised groups had lower LPS- and protein-specific IgG (albeit not significantly different in the latter case) and higher organ colonisation.

However, when we plotted protein and LPS IgG titres for individual mice against the corresponding colonisation numbers in the spleen, liver and blood and calculated the correlation coefficient for each set of data, we did not find any strong correlations between the two in any of the cases (Figure 4). All of the six correlation coefficients were lower than 0.2, indicating a weak correlation between IgG titres and colonisation of the different organs.

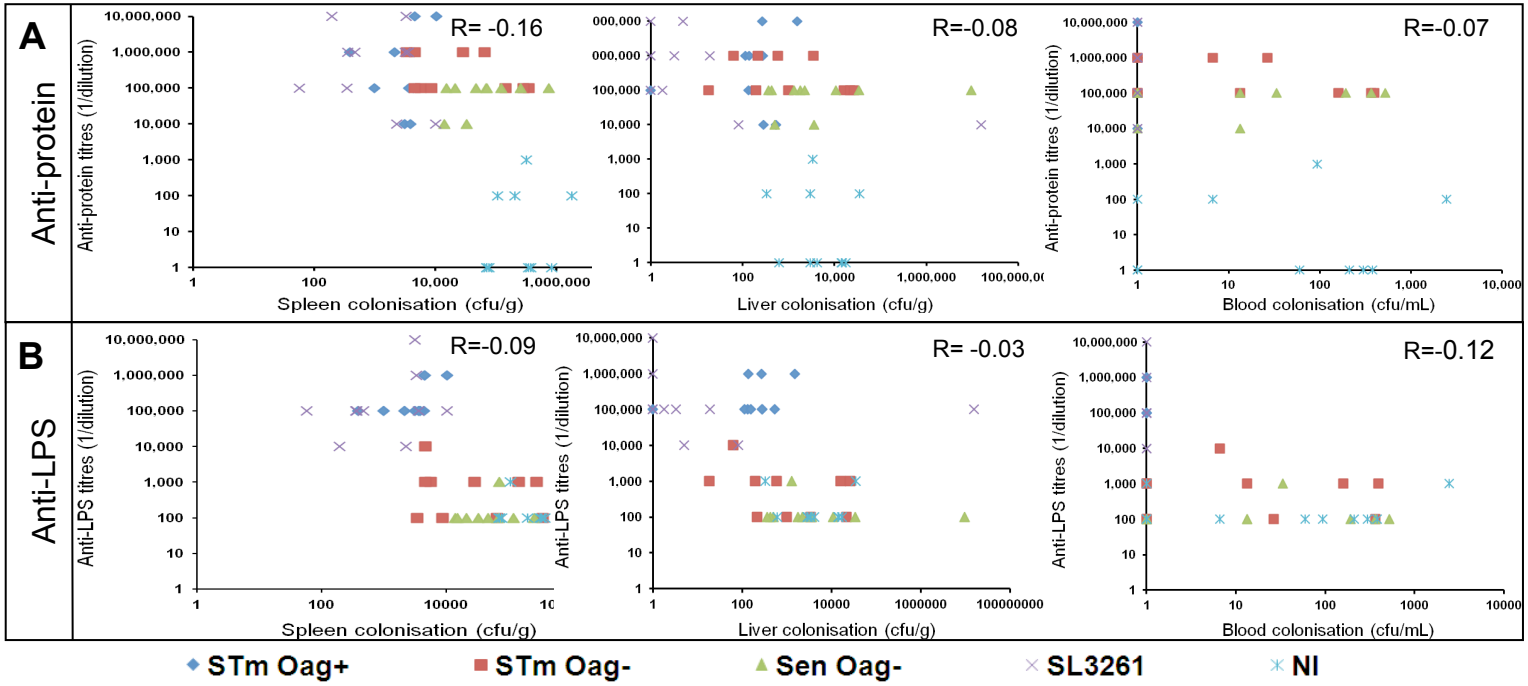


Figure 10. There is no strong correlation between individual serum IgG titres and bacterial colonisation of the spleen, liver, and blood. Mice were immunised with *S.* Typhimurium SL1344Δ*tolR* GMMA (blue), *S.* Typhimurium SL1344Δ*tolR*Δ*wbaP* GMMA (red), or *S.* Enteritidis P125109Δ*tolR*Δ*wbaP* GMMA (green) on days 0, 7 and 21, or with 1.1 x 10^6 *S.* Typhimurium SL3261 (purple) on day 0, or were not immunised (light blue). Serum IgG titres for individual mice were measured one week prior to infection with *S.* Typhimurium M525 using ELISA. Plates were coated with (*A*) STm Oag- GMMA or with (*B*) *S.* Typhimurium LPS in order to determine *S.* Typhimurium protein-specific and LPS-specific, respectively. These titres plotted with bacterial numbers in the spleen (*left*), liver (*middle*) and blood (*right*). The correlation coefficient (R) was calculated for each set of data.

6.4 Cell-mediated immunity following GMMA immunisation and challenge

Effective immunity against *Salmonella* infection in mice requires a combination of humoral and cell-mediated immunity. Whereas antibody plays an important role in neutralising *Salmonella* in extracellular spaces, cell-mediated immunity is crucial to clear *Salmonella* during the intracellular phases of pathogenesis. To determine the impact of GMMA immunisation on cell-mediated immune responses to *S.* Typhimurium infection, spleens from non-immunised mice or from mice immunised with *S.* Typhimurium GMMA, *S.* Enteritidis GMMA, or *S.* Typhimurium SL3261 were examined by flow cytometry three days after infection with *S.* Typhimurium M525. Examination of the spleen populations of lymphocytes, CD3+ (T cells), CD3+/NK1.1+ cells (natural killer T cells), B220+ (B cells), NK1.1+ (natural killer and natural killer T cells), CD11b+ (phagocytic cells), Gr-1 (neutrophils), F4/80+ (macrophages) revealed differences in immune cell populations between immunised and the non-immunised-mice controls (Figure 5).

Immunisation with STm Oag+ GMMA led to the most significant changes in splenocyte populations. Compared to non-immunised mice, the overall leukocyte population was significantly lower ($p<0.001$). In particular, we observed significant lower NK and NKT cells ($p<0.01$), B cells ($p<0.01$) and CD11b+ cells ($p<0.05$), including neutrophils ($p<0.001$) and macrophages ($p<0.001$). In the STm Oag- GMMA group we observed similar changes in the spleen populations, but with less prominent reductions. In contrast, splenocytes from the SEn Oag- group did not exhibit any significant changes

in any of the specific cell populations relative to non-immunised mice. Despite similar colonisation results in the spleen to the STm Oag+ GMMA group, we observed a unique cell population pattern in SL3261-immunised mice. There were significant lower CD11b+ cells and neutrophils, but no significant changes in the T cell, B cell, NK cell or macrophage populations compared to non-immunised mice.

The patterns observed in the STm Oag+ GMMA, STm Oag- GMMA and SL3261 appear to indicate that impairment of *S.* Typhimurium M525 infection in mice was associated with the migration of certain cell populations away from the spleen in protected mice and/or an influx of cells into the spleen in non-immunised mice. Given that we did not assess the dynamics of cell-mediated immunity by measuring cellular responses in other organs (eg. mesenteric lymph nodes), we cannot say which of these two possibilities, or if both, apply.

Interestingly, although SL3261 and STm Oag+ GMMA immunisation gave the highest levels of protection against *S.* Typhimurium infection, they did so by triggering different cellular responses. Differences in immunisation route and/or vaccine type (live vs. killed) are likely to explain these differences.

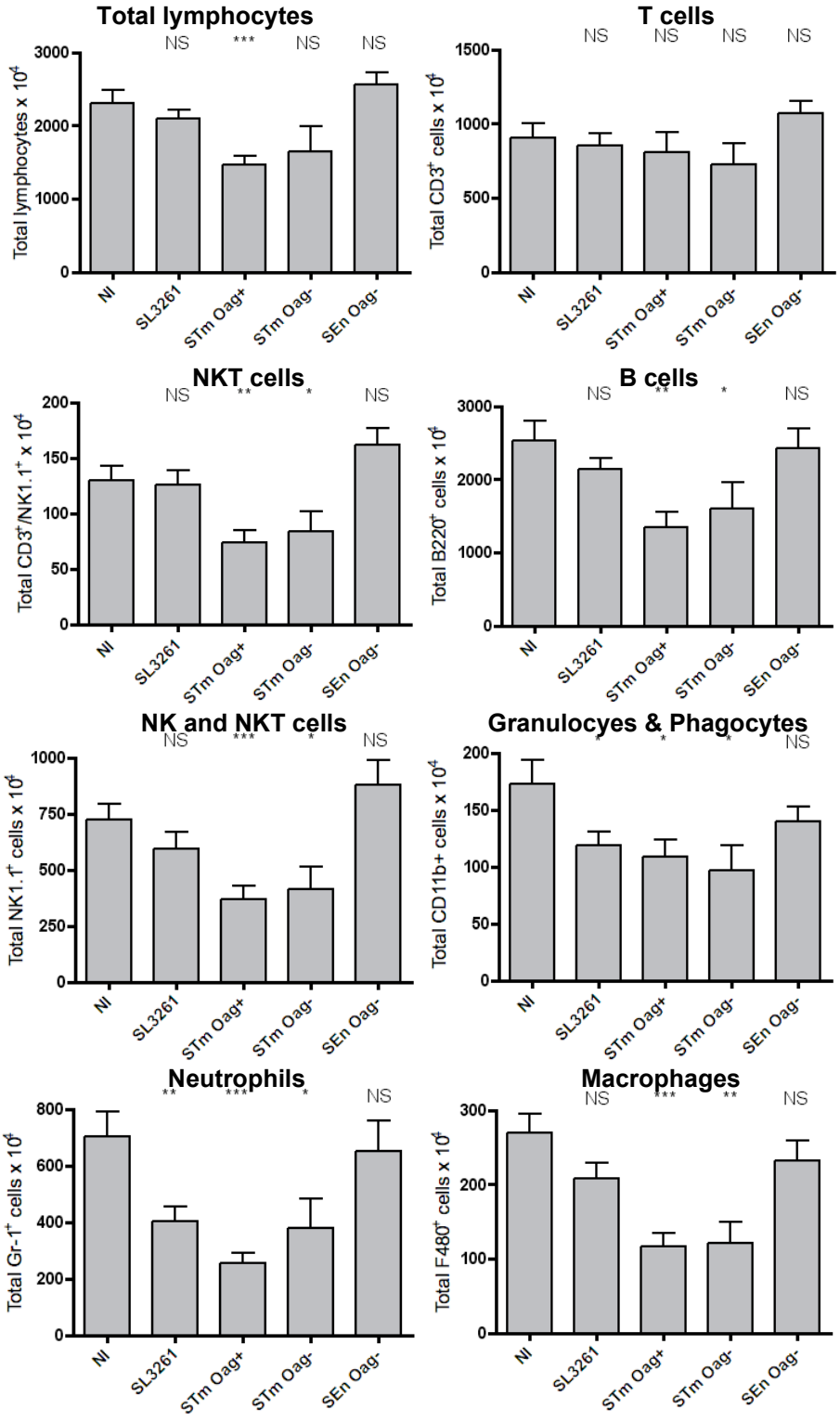

Total lymphocytes
Total lymphocytes x 10⁴
NS
***
NS
NS
T cells
Total CD3⁺ cells x 10⁴
NS
NS
NS
NS
NKT cells
Total CD3⁺/NK1.1⁺ x 10⁴
NS
**
*
NS
B cells
Total B220⁺ cells x 10⁴
NS
**
*
NS
NK and NKT cells
Total NK1.1⁺ cells x 10⁴
NS
***
*
NS
Granulocyes & Phagocytes
Total CD11b+ cells x 10⁴
*
*
*
NS
Neutrophils
Total Gr-1⁺ cells x 10⁴
**
***
*
NS
Macrophages
Total F480⁺ cells x 10⁴
NS
***
**
NS
NI
SL3261
STm Oag+
STm Oag-
SEn Oag-

Figure 11. *Salmonella* GMMA induce differential cell-mediated responses in the spleen following infection with *S.* Typhimurium. Cells were isolated from spleens after immunisation with *S.* Typhimurium SL3261 (SL3261), *S.* Typhimurium GMMA with Oag (STm Oag+), *S.* Typhimurium GMMA without Oag (STm Oag-), *S.* Enteritidis GMMA without Oag (SEn Oag-), or not immunised, and infected intraperitoneally with *S.* Typhimurium M525 for three days. Cells were stained with flurochrome-labeled mAb to the specific surface markers and analysed by flow cytometry in which 10,000 events were recorded. Columns represent the total number + SEM. Significance differences between each immunised group and non-immunised mice were determined by Mann-Whitney test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; NS, not significant.

Not surprisingly, both types of *S.* Typhimurium GMMA – with and without Oag – triggered similar responses in the spleen. Although the presence of Oag in STm Oag+ GMMA might have stimulated different cell groups, this was not in the case. Instead, we observed changes in the same cell types, with the presence of Oag in STm Oag+ GMMA leading to more marked reductions in all cases. This similarity in cell-mediated immune responses like arises from the fact they are both non-living vaccines from the same *Salmonella* serovar, and thus of similar composition.

Similarly, the differences observed between STm Oag- and SEn Oag- GMMA likely arose from differences in protein composition, as both of these GMMA lacked Oag. The lack of significant differences in splenocyte populations between the SEn Oag- group and non-immunised mice is likely related to the colonisation of spleen and liver results described previously. Previous exposure to *S.* Enteritidis proteins appears not to have triggered a strong cell-mediated response following exposure to *S.* Typhimurium infection, which resulted in an impaired immune response.

6.5 Discussion

Overall these results corroborate our hypothesis and indicate that GMMA lacking Oag were able to elicit an immune response that can hinder *S.* Typhimurium infection in the mouse and lead to impaired colonisation of the spleen. Although only in the spleen, through SEn Oag- GMMA immunisation we obtained evidence that Oag- GMMA can also impair the infection of a heterologous *Salmonella* serovar and indicating that GMMA have the potential to generate a cross-protective response. Differences in colonisation dynamics between spleen and liver might explain why the results were only significant in the spleen. Colonisation levels were however not as low as those of Oag-containing GMMA or the live vaccine SL3261, indicating an important role for Oag in generating a protective immune response, as reported previously in studies with *Salmonella* rough mutant vaccines. It was nonetheless interesting that STm Oag+ GMMA, a non-living vaccine, was able to reach protection levels very similar to those of SL3261.

Even though high levels of *S.* Typhimurium specific IgG (anti-LPS and anti-protein) were present in all groups one week prior to S. Typhimurium infection, there was no correlation between antibody levels and organ colonisation. It is known that antibodies play an important role at the site of infection – the peritoneum, in this case – right before *Salmonella* uptake by macrophages and neutrophils. However, this uptake happens quickly and *Salmonellae* enter an intracellular phase while cells migrate to the mesenteric lymph nodes, spleen and liver. At this point, cellular responses, rather than antibody, become crucial to inhibit intracellular infection and replication of *Salmonella*.

In fact, the analysis of cell-mediated responses in the spleen three days after infection with *S.* Typhimurium provided some insight into the correlates of protection. In mice immunised with SL3261 and STm Oag+, where the lowest spleenic and liver bacterial burden was observed, there were significantly less total CD11b+ cells, which includes granulocytes and macrophages. In the STm Oag+ and STm Oag- GMMA groups, impairment of infection appeared to also be correlated with reduced B, NK, and NKT total cell numbers per spleen.

Most importantly, it should be noted that this study represents a snapshot of *S.* Typhimurium infection dynamics following different immunisation regimes. Day three post-infection was chosen for the analysis as, based on previous infection studies with *S.* Typhimurium M525 by the same group, sufficient organ colonisation by S. Typhimurium should be observed. No other time points were analysed due to time- and size of study-restrictions, but would ideally have been carried out in order to obtain the full picture of colonisation of the spleen, liver, and possibly other organs over time, as well as the concomitant changes in cellular responses.

Chapter 7

General discussion

7.1 Summary of results

With the aim of developing a broad-spectrum protein-based vaccine against non-typhoidal *Salmonella* (NTS), we used an outer-membrane particle based approach. These outer membrane particles are naturally released by *Salmonella* and other Gram-negative bacteria during growth. Although they are usually known in the literature as outer membrane vesicles, we named them GMMA, or Generalised Modules for Membrane Antigens, so as to distinguish them from detergent-extracted OMV, which differ in composition and immunogenicity (van de Waterbeemd et al., 2010).

GMMA are produced as a result of membrane budding and represent the protein composition of the bacterial outer membrane and periplasmic components (Bernadac et al., 1998; Yem & Wu, 1978). Therefore, they have the potential to be developed as a protein-based vaccine against NTS due to their high protein content. Outer membrane proteins (OMP), in particular, are considered good vaccine antigens due to their surface exposure and immediate accessibility to antibodies during *Salmonella* infection.

We selected strains from the two most prevalent NTS serovars in an African setting, *S.* Typhimurium and *S.* Enteritidis, and investigated their ability to produce GMMA. The deletion of one of the Tol-Pal proteins, TolR, led to increased production of GMMA in a manner similar to *E. coli* (Bernadac et

al., 1998). Although we are not able to discard the possibility that the *tolR* deletion changes the architecture of naturally-released vesicles, GMMA from S. Typhimurium Δ*tolR* were analysed by transmission electron microscopy and found to be highly homogenous in size and structure, with a clearly defined outer membrane surrounding periplasmic space. Furthermore, it has been shown that, in GMMA from *E. coli* Tol-Pal mutants, OMP are exposed on the outer surface in their natural conformation (Bernadac et al., 1998).

Because GMMA are representative of the outer membrane components of the *Salmonella* bacterial surface, they also contain high levels of lipopolysaccharide (LPS), which includes O-antigen (Oag) polysaccharide in its composition. Oag is a highly immunodominant feature of the *Salmonella* surface which, due to immune pressure, varies between *Salmonella* serovars (for example, O:4,5 is found in *S.* Typhimurium and O:9 in *S.* Enteritidis). Therefore, infection with one serovar of *Salmonella* will only generate protection against a homologous, but not a heterologous, serovar (Kenny & Herzberg, 1968). Furthermore, it has been shown that the presence of full length LPS hinders the access of antibodies to the bacterial cell surface (Bentley & Klebba, 1988). Therefore, we successfully abrogated the expression of Oag in the GMMA, by deleting the *wbaP* gene (Ilg et al., 2009), in order to generate a non serovar-specific immune response upon immunisation and increase the accessibility to the protein antigens on the GMMA surface.

Furthermore, we addressed the issue of LPS reactogenicity by introducing a third gene deletion, in *msbB*, in *S.* Typhimurium. This deletion is reported to reduce the adverse effects of LPS by generating penta-acylated lipid A rather

than the more toxic hexa-acylated form (Lee et al., 2009). The triple *S.* Typhimurium SL1344 Δ*tolR*Δ*wbaP*Δ*msbB* mutant was still viable and released GMMA with similar protein composition to those from the single Δ*tolR* or the double Δ*tolR*Δ*wbaP* mutant. We did observe a reduction in the intensity of the outer membrane protein A (OmpA) in the Δ*msbB* compared to other GMMA, indicating that this protein might be less abundant in GMMA released by the triple mutant.

During *Salmonella* infection *in vivo*, iron availability is low and bacteria overexpress a specific subset of iron-regulated outer membrane proteins (IROMPs) on their surface with the aim of scavenging iron from the environment (Faraldo-Gomez & Sansom, 2003). The sequence of these IROMPs is conserved across *S. enterica*, making this group of proteins an attractive vaccine target. In fact, *Salmonella* IROMPs they have previously been shown to protect against *Salmonella* infection in animal models (Sood et al., 2005) and some of these are currently used as veterinary vaccines. Further optimisation of the protein content of the GMMA was therefore achieved by growing the GMMA-overproducing strains in culture media with limited iron availability. When the proteome of these GMMA was analysed, we observed an increase in the intensity of the protein bands in the 75kDa range, likely corresponding to these proteins.

Characterisation of the full proteomic content of *Salmonella* GMMA by mass spectrometry using two-dimensional gel electrophoresis and MALDI-TOF MS allowed for the identification of a large number of proteins and confirmed presence of a large number of outer membrane and periplasmic proteins. This was in agreement with previous reports (Berlanda et al., 2008) and further confirmed that GMMA are shed from the outer membrane of *Salmonella* and are not a result of cell lysis.

Upon subcutaneous or intranasal immunisation of mice, *S.* Typhimurium GMMA with and GMMA without Oag elicited high serum antibody titres. These antibodies were raised against a large number of *S.* Typhimurium proteins and reacted extensively with *S.* Enteritidis proteins. Sera from GMMA without Oag were reactive against both live *S.* Typhimurium and *S.* Enteritidis, but only in strains lacking Oag due to steric hindrance caused by full length LPS (Bentley & Klebba, 1988), an effect which we also came across during the ELISA. Whether this happens *in vivo* or is an *in vitro* effect remains to be investigated.

With the aim of evaluating whether a protein-based GMMA vaccine would be able to protect against homologous and heterologous *Salmonella* infection challenge, we used an established murine model of acute *Salmonella* infection in *Salmonella*-susceptible mice (strain C57BL/6). Upon infection with *S.* Typhimurium, mice that were immunised with *S.* Typhimurium GMMA with Oag showed similar protection levels to *S.* Typhimurium SL3261-immunised mice in the spleen and liver, relative to non-immunised mice. Immunisation with either *S.* Typhimurium or *S.* Enteritidis GMMA lacking Oag (i.e. protein-GMMA) led to impaired *S.* Typhimurium infection in the spleen but not in the liver. These differences between the organs might result from different colonisation of the different organs by *Salmonella* or by this particular strain of *S.* Typhimurium. Although there results are indicative that protein-GMMA immunisation can yield some protection against homologous and heterologous *Salmonella* infection, protection levels are not as high as those yielded by GMMA containing Oag. This is not surprising, in the context of previous studies with heat-killed smooth strains and rough mutants (with and without Oag, respectively), which showed that the presence of Oag was crucial for the development of a protective response.

We found no strong correlation between IgG antibody titres and colonisation of the spleen or the liver. We would expect antibody to play an important role in protection against *Salmonella* infection by intraperitoneal route, whereby antibody would opsonise the bacteria immediately upon entry and before uptake by macrophages (Mastroeni, P., personal communisation). Even though this might have been the case, these differences were not reflected in bacterial viability at the organ level.

Analysis of cell-mediated responses in the spleen after immunisation and challenge showed, as expected, that higher levels of protection in the Oag+ GMMA group were associated with an overall reduction in overall splenocyte number in comparison to non-immunised mice. This reduction was also seen in mice immunised with Oag- GMMA, where a significant degree of protection had been observed. To an extent, this is in agreement with the fact that, in unprotected mice, *Salmonella* will colonise the spleen to an extent that will lead to the influx of most immune cell populations. In protected mice, however, *Salmonella* burden in the spleen will be lower or inexistent, leading to a decreased influx of cells into this organ. Surprisingly, this was not the case with the SL3261 group which, for most cell types, showed similar levels to non-immunised mice, the exception being granulocytes and phagocytes.

Taken together, these results show that the *Salmonella* GMMA we have generated and optimised for protein antigen delivery have the potential to be developed as a broad-spectrum protein-based vaccine against NTS. They elicited a strong antibody response and activated some of the subpopulations of cell-mediated immunity. The ability of *S.* Typhimurium and *S.* Enteritidis GMMA to impair *S.* Typhimurium infection in the spleen of mice further exemplified their potential as vaccines. Interestingly, *S.* Typhimurium GMMA provided a higher degree of protection than *S.* Enteritidis GMMA despite extensive overlap in their protein content. A more detailed analysis

of the proteomes might provide some insight as to what proteins might be leading to this differential immune response. Nonetheless, and despite these positive results, further optimisation of these GMMA is required to obtain levels of protection similar to those elicited by Oag-containing vaccines.

7.2 Implications for vaccine development

GMMA have shown great potential for development as vaccines against *Salmonella*, Shigella, *B. pertussis*, *V. cholerae* and other organisms based on results in animal models (Alaniz et al., 2007; Camacho et al., 2011; Roberts et al., 2008; Schild, Nelson, & Camilli, 2008). Moreover, detergent-extracted OMV from *N. meningitidis* serogroup B have been used in humans in Cuba, New Zealand and Norway and shown high levels of efficacy. However, the immune responses elicited by GMMA or detergent-extracted OMV tend to be serotype-specific, due to the presence of a polysaccharide component (Oag or capsule) that varies between species' serotypes.

We had hypothesised that by removing the serotype-specific component from GMMA, the Oag, we would be able to elicit a protective and a cross-protective immune response. In fact, immunisation with *S.* Typhimurium GMMA impaired homologous *S.* Typhimurium infection in the spleen and to some extent in the liver, and *S.* Enteritidis GMMA impaired heterologous *S.* Typhimurium infection in the spleen. Even though the protection levels were not as high as those of GMMA with Oag or the live attenuated strain, it indicates that the subset of proteins that is found in protein-GMMA is able to elicit antibodies that hinder *Salmonella* infection.

It is true that previous studies with killed rough mutants had shown that indeed these are less effective at protecting than killed smooth mutants. However, the treatment to which these bacteria (either heat or chemical

stress) were subjected might have interfered with protein structure and destroyed important epitopes. GMMA have the advantage that proteins are expressed in their natural conformation (Bernadac et al., 1998). Furthermore, protein-based vaccines have been previously shown to induce protection against *Salmonella*. In particular, a recent study with *Salmonella* outer membrane porins in a murine model (Gil-Cruz et al., 2009) showed that immunisation with these proteins induced protection levels similar to heat-killed bacteria and indicated a crucial role for OmpD in protection.

Interestingly, GMMA were shown to be rich in outer membrane porins, including those previously shown to be protective. However, the OMP in the above study were used at a 20µg dose, which is 20 times higher than the 1µg of protein used in our immunisations. It is thus possible that higher doses of GMMA might be even more effective against infection by generating a stronger antibody and cell-mediated response.

It has been recently highlighted that *Salmonella*-resistant mice are more suited to the assessment of efficacy of non-living vaccines, as is the case of GMMA (Simon, Tennant, Galen, & Levine, 2011). Instead, we used a mouse strain that is susceptible to *Salmonella* infection, C57BL/6 (Nramp -/-) and thus more suited to the testing of mucosally administered live attenuated strains (Simon et al., 2011).

Moreover, it is also important to keep in mind that we were using a mouse model to study a vaccine to be used for a human disease. Despite the widespread use of murine model to study *Salmonella* vaccines due to the overlap between *S.* Typhimurium infection in mice and invasive NTS in humans, including the fact they are both systemic, there are differences that might affect vaccine testing (Siggins et al., 2011). In particular, mouse serum is not able to effect cell-free complement-dependent killing of *Salmonella* due to the reduced ability of the complement to kill bacteria (Siggins et al.,

2011). Therefore, even though GMMA might have elicited antibody suitable for complement-fixing, the killing would have been impaired by this deficiency in the murine immune response.

In fact, it has been shown that antibody-mediated immunity, through cell-free complement-dependent bactericidal activity or cell-dependent opsonophagocytosis, is important for protection against *Salmonella* infection in Africans (Gondwe et al., 2010; MacLennan et al., 2008). In particular, it has been shown that *Salmonella* serum killing is induced by antibodies against outer membrane proteins (MacLennan et al., 2010). GMMA elicited a strong protein-specific antibody response against an extensive range of proteins. Therefore, despite not achieving as the same levels of protection as Oag-containing GMMA, protein GMMA might be worth pursuing as a protein-based vaccine nonetheless.

7.3 Future perspectives

It is likely that invasive non-typhoidal *Salmonellae* (iNTS) will continue to be one of the main causes of bacteraemia in children and immunocompromised adults in Africa. However, with increased surveillance and improved diagnostic tools, along with the introduction of vaccines for the other two main causes of bacteraemia - *Haemophilus influenzae* (von Gottberg et al., 2012; Gessner, 2009) and pneumococcal 7-, 10- and 13-valent vaccines (Roca et al., 2011) – it is probable that iNTS will gain even greater public health visibility, which will in turn highlight the need for an effective and affordable vaccine against the disease.

The work described in this Ph.D. thesis has shown that *Salmonella* GMMA have the potential to be developed as a protein-based vaccine against NTS in the future if they are further optimised.

A key aspect that was not assessed but is of crucial importance is the assessment of endotoxicity in GMMA generated from Δ*msbB* mutants. Even though the *msbB* deletion has been reported to reduce LPS reactogenicity in *Salmonella* and *E. coli* GMMA (Lee et al., 2009; Kim et al., 2009), GMMA generated as part of this study should be tested for their endotoxicity in an *in vitro* cytokine-release assay (Stoddard, Pinto, Keiser, & Zollinger, 2010). If they are not shown to be sufficiently detoxified, additional deletion can be introduced, such as *htrB* (Sunshine et al., 1997). The deletion of this gene generates a mutant that is, in theory, less toxic than the wild type or the *msbB* deletion mutants.

The functional characterisation of the antibodies generated against GMMA would also be of added interest. We would be particularly interested in the cell-free complement-mediated bactericidal activity of these antibodies, as well as in the cell-mediated opsonophagocytic activity. We carried out some assessments of the bactericidal activity of GMMA sera, but because they were only preliminary results, we have not included them in this thesis.

Exploring alternative immunisation and/or challenge routes might have yielded more insight into the immune response and potential protection mechanisms elicited by GMMA. Despite exhibiting an invasive phenotype in some cases (iNTS and *S.* Typhi), *Salmonella* is a mucosal pathogen and mucosal routes of immunisation might be more suitable. Although we assessed the immunogenicity and safety of intranasal GMMA immunisation, we did not test the ability to protect against oral or parenteral challenge.

Another issue to be addressed in the future is the extension of our findings to iNTS disease in Sub-Saharan Africa. Throughout this study we used diarrhoeagenic strains of NTS (*S.* Typhimurium SL1344, LT2 or M525 and *S.* Enteritidis P125109) as the source of GMMA and as the strains for *in vitro* and *in vivo* studies. However, as recently reported (Kingsley et al.,

2009), strains causing iNTS disease in Africa are genetically distinct. Although we did not observe any differences in the protein pattern of *S.* Typhimurium SL1344 and *S.* Typhimurium D23580 GMMA, it is possible that a closer analysis would unveil differences at the proteomic level.

Moreover, and as mentioned previously, further optimisation of the GMMA is required to enable their use as a protein-based vaccine against NTS. There are several possibilities, namely with the use of a higher dose or the use of adjuvants to stimulate a stronger cell-mediated response. Another alternative, which has been used extensively with *N. meningitidis* GMMA, is the overexpression of conserved antigens on the GMMA surface, either by placing them on a plasmid or integrated into the chromosome. This has resulted in improved immune responses to *N.* meningitidis GMMA (Koeberling, Welsch, & Granoff, 2007; Koeberling, Seubert, & Granoff, 2008) and it is likely that upon identification of suitable antigens for overexpression, this approach would likely work for *Salmonella* GMMA.

Appendix 1

	Protein ID	Accession number	Ref. strain	Predicted Location
1	outer membrane protein	16763614	*Salmonella* typhimurium LT2	Outer membrane
2	pyruvate dehydrogenase subunit E1	16763542	*Salmonella* typhimurium LT2	Cytoplasmic
3	outer membrane receptor	16763962	*Salmonella* typhimurium LT2	Outer membrane
4	Carboxy-terminal protease	16765186	*Salmonella* typhimurium LT2 str. 14028S	Cytoplasmic
5	ferrichrome outer membrane transporter	16763581	*Salmonella* typhimurium LT2	Outer membrane
6	putative transglycosylase	16766562	*Salmonella* typhimurium LT2	Cytoplasmic membrane
7	outer membrane protein W	16764479	*Salmonella* typhimurium LT2	Outer membrane
8	peptidoglycan-associated outer membrane lipoprotein	213582865	*Salmonella* enterica subsp. enterica serovar Typhi str. E98-8	Cytoplasmic membrane
9	Chain A, Feedback Inhibition Of Fully Unadenylylated Glutamine Synthetase From *Escherichia coli* By Glycine, Alanine, And Serine	1649034	*Salmonella* enterica subsp. enterica serovar Typhimurium	Cytoplasmic
10	putative lipoprotein	16764905	*Salmonella* typhimurium LT2	Unknown
11	protein phosphorylase	2168160	*Salmonella* typhimurium	Cytoplasmic
12	outer membrane transporter	16764815	*Salmonella* typhimurium LT2	Outer membrane
13	Chain A, Flagellin middle domain variant C150	116489824	*Salmonella* enterica subsp. enterica serovar Typhimurium	Unknown
14	putative outer membrane protein precursor	16764916	*Salmonella* typhimurium LT2	Outer membrane
15	outer membrane lipoprotein LolB	16763489	*Salmonella* typhimurium LT2	Unknown
16	DNA-binding protein Dps	213649900	*Salmonella* enterica subsp. enterica serovar Typhi str. J185	Cytoplasmic
17	galactosyltransferase protein G	16764005	*Salmonella* typhimurium LT2	Periplasmic
18	gmhC precursor	16764966	*Salmonella* typhimurium LT2	Periplasmic
19	hypothetical protein	16764728	*Salmonella* typhimurium LT2	Cytoplasmic
20	serine protease	16766643	*Salmonella* typhimurium LT2	Periplasmic
21	putative peptidase protein	213644853	*Salmonella* enterica subsp. enterica serovar Weltevreden	Outer membrane
22	pyruvate dehydrogenase E1 component	16764719	*Salmonella* typhimurium LT2	Cytoplasmic
23	survival protein SurA subunit precursor	261245325	*Salmonella enterica* subsp. *enterica* serovar Paratyphi A str. ATCC 9150	Periplasmic
24	bifunctional acetaldehyde-CoA/alcohol dehydrogenase	16765093	*Salmonella* typhimurium LT2 str. D23580	Cytoplasmic
25	putative hydrolase	16764885	*Salmonella* typhimurium LT2	Extracellular
26	phage head-like protein	16765924	*Salmonella* typhimurium LT2	Unknown
27	fructose-bisphosphate aldolase	16761083	*Salmonella* enterica subsp. enterica serovar Typhi str. CT18	Unknown
28	lpfB	829372	*Salmonella* enterica subsp. enterica serovar Typhimurium	Periplasmic
29	hypothetical protein Sb36	94317625	*Salmonella* enterica subsp. enterica serovar Typhimurium	Unknown
30	hypothetical protein Salmoneentericaenterica_237	213663870	*Salmonella* enterica subsp. enterica serovar Typhi str. E00-7866	Cytoplasmic
31	assembly factor YaeT	16762046	*Salmonella* typhimurium LT2	Outer membrane
32	subunit E1	213857854	*Salmonella* enterica subsp. enterica serovar Typhi str. M223	Outer membrane
33	hypothetical protein	267994497	*Salmonella* enterica subsp. enterica serovar Typhimurium str. 14028S	Unknown (multiple locations)
34	transporter	16765012	*Salmonella* typhimurium LT2	Outer membrane
35	protease	16764479	*Salmonella* typhimurium LT2	Outer membrane
36	peptidoglycan-associated outer membrane lipoprotein	213582865	*Salmonella* enterica subsp. enterica serovar Typhi str. E98-8	Cytoplasmic membrane
37	outer membrane protein X	16759751	*Salmonella* typhimurium LT2	Outer membrane
38	Chain A, Feedback Inhibition Of Fully Unadenylylated Glutamine Synthetase From *Escherichia coli* By Glycine, Alanine, And Serine	157884007	*Salmonella* enterica subsp. enterica serovar Typhimurium	Cytoplasmic
39	*Escherichia coli* peptidyl-prolyl cis-trans isomerase	16766742	*Salmonella* typhimurium LT2	Periplasmic
40	putative lipoprotein	16764905	*Salmonella* typhimurium LT2	Unknown
41	protein phosphorylase	2168160	*Salmonella* typhimurium	Cytoplasmic
42	outer membrane transporter	16764815	*Salmonella* typhimurium LT2	Outer membrane
43	flagellin	16763689	*Salmonella* typhimurium LT2	Extracellular
44	outer membrane protein	16765979	*Salmonella* typhimurium LT2	Outer membrane
45	outer membrane lipoprotein LolB	16763489	*Salmonella* typhimurium LT2	Unknown
46	DNA-binding protein from stationary phase	16764257	*Salmonella* typhimurium LT2 SL1344	Cytoplasmic
47	galactosyltransferase protein G	213649005	*Salmonella* enterica subsp. enterica serovar Choleraesuis str. SC-B67	Periplasmic
48	virulence membrane protein	16764600	*Salmonella* typhimurium LT2	Outer membrane
49	hypothetical protein	16764728	*Salmonella* typhimurium LT2	Cytoplasmic
50	putative peptidase protein	213644853	*Salmonella* enterica subsp. enterica serovar Weltevreden	Outer membrane
51	pyruvate dehydrogenase E1 component	16764719	*Salmonella* typhimurium LT2	Cytoplasmic
52	bifunctional acetaldehyde-CoA/alcohol dehydrogenase	16765093	*Salmonella* typhimurium LT2 str. D23580	Cytoplasmic
53	L-lactate dehydrogenase	213649540	*Salmonella* enterica subsp. enterica serovar Typhi str. E01-6750	Cytoplasmic

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