Mechanisms of antibody and complement-dependent immunity against nontyphoidal *Salmonella* in Africa

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Abstract

Nontyphoidal *Salmonella* (NTS) are a major cause of fatal bacteremia in Africa. Developing a vaccine requires an improved understanding of the relevant mechanisms of immunity. Antibodies are important for protection against NTS in both man and mouse, and a potentially important role for antibody in cell-free complement-mediated bactericidal activity against *Salmonella* in Africans has been reported. We demonstrated that although BALB/c, C3H and C57BL/6 mice produced antibody responses to immunisations with an African invasive *S*. Typhimurium isolate and laboratory strains of *S*. Typhimurium, their sera failed to effect antibody-dependent complement-mediated killing against the African *S*. Typhimurium isolate. Supplementation with human complement showed that the lack of serum-killing was due to the weaker complement activity of mouse serum. Therefore, the protective effect of antibodies could be underestimated by the mouse model.

To investigate the possibility that lack of serum-killing of NTS in the mouse influences bacterial spread from the blood, we created an *in vitro* model of *Salmonella* infection in human blood. Phagocytosis overlapped *Salmonella* survival in serum which allowed a proportion of *Salmonellae* to escape from complement-fixing bactericidal antibodies. However, remaining *Salmonellae* were deposited with membrane attack complex C5b-9 and killed. While *Salmonella* serum-susceptibility varied with growth conditions and bacterial growth phase, serum had at least a bacteriostatic effect. Antibody-dependent complement-mediated bactericidal activity could stop sustained and fatal bacteremia developing *in vivo*. Antibodies alone might halt fatal development of the salmonellosis in the absence of a strong cellular immune response. The data is consistent with clinical descriptions of IFNγ-deficient patients who suffer from serious extra-intestinal, yet non-fatal NTS disease.

Abstract

The description of inhibitory anti-O-antigen (OAg) antibodies in a subset of HIV-infected patients cast doubt on the suitability of OAg as a vaccine agent. We used a range of invasive African *Salmonella* isolates, laboratory *Salmonella* strains and *Salmonella* LPS mutants to adsorb human serum in order to investigate the targets of bactericidal complement-fixing antibodies. Adsorption with wild type *Salmonellae* produced an effective serovar-specific removal of both antibodies and bactericidal activity, without adversely affecting the activity of complement. In contrast, mutants that lacked OAg failed to remove *Salmonella* serovar-specific antibodies and killing against their parent strains. *S*. Typhimurium and *S*. Enteritidis OAg chimera strains, expressing either native or heterologous OAg (O:4 or O:9) demonstrated that OAg, and not serovar, was the principal determinant of the ability of *Salmonellae* to remove specific antibodies and serum-killing. These data indicate that anti-OAg antibodies are major effectors of complement-mediated killing of invasive African nontyphoidal *Salmonella* by normal human serum. This supports the development of an OAg based vaccine.

We used transmission electron microscopy to explore the mechanism of inhibition in some HIV-infected sera. Consistent with viable counts from serum bactericidal assays, HIV-infected inhibitory serum showed substantially less damaged and lysed cells than bactericidal serum. IgG, complement component C3 and C5b-9 were all observed on both the outer and inner membranes, as well as distal sites from the bacterial membrane of *Salmonellae* in both inhibitory and bactericidal sera. However, whereas damaged *Salmonellae* all had C3 and C5b-9 deposition on the OM, many healthy cells did not. Bactericidal serum deposited greater quantities of C3 and also C5b-9 on the *Salmonellae* than inhibitory serum. This suggests that the failure of inhibitory serum to kill *Salmonellae* is due to a reduced ability to deposit bactericidal complement.

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ABBREVIATIONS

AP	Alkaline phosphatase
BD	Becton Dickinson
BSA	Bovine serum albumin
EDTA	Ethylene-di-amine-tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Flow analysis cell sorter
FITC	Fluorescein isothiocyanate
GMFI	Geometric mean fluorescence intensity
HIV	Human immunodeficiency virus
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
LB	Luria Bertani (Lysogeny broth)
LPS	Lipopolysaccharide
NTS	Nontyphoidal *Salmonella*
Omp	Outer membrane protein
PBS	Phosphate buffered saline
SEn	*Salmonella enterica* serovar Enteritidis
STm	*Salmonella enterica* serovar Typhimurium
TNF-α	Tumour necrosis factor alpha
Th1	Helper T 1 cells
Th2	Helper T 2 cells
WT	Wild type

1. Introduction

1.1 Theme and overview

The main theme of this thesis is the potential role of bactericidal antibody and complement in immunity against nontyphoidal *Salmonellae* (NTS). Investigated within are: the kinetics of the antibody-dependent complement-mediated killing *in vitro*; influence of growth conditions of bacteria on their sensitivity to this killing; the relevance of this modality of immunity in mice models; the mechanism by which it achieves killing of NTS; and the antigen targets of such bactericidal antibodies.

This chapter will discuss *Salmonella,* the diseases it causes and their epidemiology, its pathogenesis and the immune responses involved in control and protection from infection. In addition, the current situation regarding vaccines and potential future vaccines for *Salmonellae* is addressed. Finally, the specific aims and hypotheses of this study are detailed.

1.2 *Salmonella*

Salmonella is a genus of rod shaped, Gram-negative, non-spore forming and predominately pathogenic bacteria of the Enterobacteriaceae family. *Salmonellae* are approximately 0.5 to 1.5 μm wide and 1 to 5 μm in length and are facultative intracellular organisms (Fields et al., 1986) that are mostly motile, due to flagella which project in all directions. *Salmonellae* are chemoorganotrophs, obtaining energy from oxidation and reduction reactions using organic sources, and are facultative anaerobes.

1.2.1 Taxonomy

The nomenclature is complicated and has developed greatly over the last 40 years. Currently, the Kauffman-White scheme is employed and official listings are maintained

by the World Health Organisation (WHO) (Grimont, 2007). There are two recognised species: *S. enterica* and *S. bongori,* which are thought to have diverged from a common ancestor with *Escherichia coli* around 100 million years ago (Doolittle et al., 1996). *S. bongori* is predominantly associated with cold-blooded animals, it shares a basic set of ancestral *Salmonella* virulence genes with *S. enterica* but lacks many genes important for infection in humans. However, infection in man is possible and has been recorded (Nastasi et al., 1999). In evolutionary terms, *S. bongori* likely lies between *E.coli* and *S. enterica* (Fookes et al., 2011). *S. enterica* has six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *hotenae* (IV), and *indica* (VI). Within these six subspecies are over 50 serogroup classifications defined by the carbohydrate structures found in lipopolysaccharide (LPS), and over 2,500 serovars distinguished by flagella antigens (Popoff et al., 2004, Grimont, 2007). Serovar designation is commonly shortened in the literature (and herein). For example, the full designation of *Salmonella enterica* subspecies *enterica* serovar Typhimurium commonly becomes simply '*S.* Typhimurium'. *S. enterica* subspecies *enterica* contains the vast majority of medically important pathogens as well as those that affect farming industry, serovars such as *S.* Enteritidis, *S.* Paratyphi, *S.* Typhi and *S.* Typhimurium (Su and Chiu, 2007, Jones et al., 2008).

1.2.2 *Salmonellae* as pathogens

S. enterica infections are generally acquired through the faecal-oral route and most commonly take on the form of one of three diseases: enteric fever, bacteraemia or enterocolitis. Chronic asymptomatic carriage is also possible (Coburn et al., 2007). The form of disease is dependent on the serovar, the host species and the host's immune state. *Salmonellae* display a varying degree of host restriction at the serovar level. Some serovars, such as *S.* Typhi (humans) and *S.* Gallinarum (chickens) are exclusively

confined to one host species, whereas other serovars are 'promiscuous' and can infect a range of species. These generalist serovars, such as *S*. Typhimurium, cause a greatly differing 'typical' disease dependent on host. In genetically susceptible *SLC11A1*^{s} (previously NRAMP-1) mice, *S*. Typhimurium infection causes a fatal enteric fever with symptoms similar to those observed after infection of humans with *S*. Typhi (Eisenstein, 1999). Whereas in man it mostly results in a self-limiting diarrhoeal disease (Jones et al., 2008, Zhang et al., 2003), typically an enterocolitis, with a lack of gastritis (Boyd, 1985). NTS are one of the most common causes of death from diarrhoeal diseases associated with microorgansims in the US (Olsen et al., 2000, Lynch et al., 2006). More rarely in man the infection can become systemic.

Invasive NTS disease was first recognised in American children in the 1950s (Saphra and Winter, 1957). The problem became apparent in adults, with the onset of opportunistic NTS infections in AIDS patients in Africa during the 1980s (Feasey et al., 2010, Clumeck et al., 1983). Since then, culture-based surveillance for invasive bacterial infections in sub-Saharan Africa identified that NTS infections rivalled *Haemophilus influenzae* type b (which is now increasingly controlled by widening implementation of an effective vaccine (Gessner, 2009)) and *Streptococcus pneumonia* infections in frequency and severity amongst children (Graham et al., 2000b, Morpeth et al., 2009). A recent meta-analysis of the whole of Africa found that over 15% of all child bloodstream infections were due to NTS (Reddy et al., 2010). Despite this, invasive NTS (particularly among the HIV-infected), is currently an under-recognised emerging infectious disease problem in Sub-Saharan Africa (Gordon, 2008).

1.3 Epidemiology and outline of nontyphoidal *Salmonella* infections

1.3.1 NTS in the developed world

NTS are the leading cause of foodborne disease outbreaks in the USA (Mead et al., 1999, Olsen et al., 2000). They cause an estimated 1.4 million illnesses in the United States each year, resulting in an estimated 15,000 hospitalisations and 400 deaths (Voetsch et al., 2004). This translates to an economical cost of $0.5–2.3 billion annually in medical care, lost productivity and mortality (Frenzen et al., 1999). Worldwide, the annual global burden of NTS diarrhoeal disease is estimated to be around 94 million cases with over 150,000 deaths (Majowicz et al., 2010).

Salmonella infection of humans in industrialised countries is generally a foodborne zoonosis (Espie et al., 2005, Varma et al., 2005). The symptoms of NTS enterocolitis normally appear 12 to 72 hours after ingestion, and include fever, diarrhoea, abdominal cramps, nausea, and occasionally vomiting, though asymptomatic infections can occur. The illness lasts from between 4 and 7 days and the vast majority of cases typically resolve without treatment (Gill and Hamer, 2001). Systemic infections, such as bacteraemia, are infrequent (~5%) and generally confined to immunodeficient patients (Hohmann, 2001, Jones et al., 2008). Infection in children is commonly more severe and carries an increased risk of invasive complications, though bacteraemia in young children typically has an excellent outcome (Zaidi et al., 1999). Antibiotic treatment is required in neonates as a prophylaxis to prevent invasive disease (Coburn et al., 2007).

The prevalence of serovars varies dependent on location and some are even limited to certain regions of the world (Galanis et al., 2006). Worldwide, *S*. Enteritidis and *S.* Typhimurium cause the majority of human clinical cases, although globally the overall proportion for both serovars has decreased over time. Currently infections with *S*. Enteritidis represent 41.5% and with *S*. Typhimurium 15.0% of total world cases

(Hendriksen et al., 2011). The proportion of infections with serovars *S*. Choleraesuis and *S*. Dublin that result in invasive disease is around ten times higher than the average for NTS infections (Jones et al., 2008). However, because of the small number of total cases, these results might simply be due to particularly virulent strains rather than demonstrating serovar as a risk factor for invasive disease. In addition, in terms of overall cases S. Typhimurium and S. Enteritidis are also the most common causes of invasive disease in the US (Jones et al., 2008).

1.3.2 NTS in Africa

The World Health Organization (WHO) estimates that diarrheal diseases result in an estimated 2–3 million annual deaths among children in developing countries. However, in contrast with the spectrum of NTS disease in developed countries, NTS are also a leading cause of severe and invasive disease amongst children and immunocompromised individuals in sub-Saharan Africa (Reddy et al., 2010). They are estimated to be one of the most common causes of bacteraemia in Africa with case rates in excess of 500 000 per year (Morpeth et al., 2009). Presentation of NTS bacteraemia most commonly occurs in children below 2 years old (Brent et al., 2006, Graham et al., 2000b) and HIV-infected adults (Gordon, 2008).

Serovars *S*. Typhimurium and *S*. Enteritidis are the most frequent cause of bacteraemia in Malawi and much of tropical Africa (Brent et al., 2006, Graham et al., 2000b). Figure 1.2 shows countries in which NTS has been reported as a major cause of childhood bacteraemia. It should be noted that the appropriate studies have not been carried out in many African countries and so occurrence is likely to be even more widespread. Clinical NTS isolates from African cases of salmonellosis are different to those found in animal contacts, suggesting human-to-human spread of infection (Kariuki et al., 2002, Kariuki et al., 2006b).

Genotype of *Salmonella* is not normally considered a risk factor. However, in Africa, multilocus sequence analysis of *S*. Typhimurium causing invasive infections in Malawi and Kenya has identified an overwhelming dominant type, termed ST313, which is rarely reported outside of Africa. Whole genome analysis of one of the most common African *S.* Typhimurium isolates, D23580, revealed a distinct prophage repertoire and a composite genetic element encoding multi drug resistance genes. In addition, there was genome degradation involving some genes previously implicated in *S*. Typhimurium virulence. A number of these chromosomal deletions and pseudo-gene formations involved genes which are either non-functional or absent in the systemic invasive pathogen, *S.* Typhi (Kingsley et al., 2009). These factors could point towards a more invasive genotype of S. Typhimurium as a risk factor for invasive disease. But, it may simply be a case that this genotype is the most common in the area and so therefore causes the greatest amount of invasive disease. Epidemiological studies of diarrhoeal *Salmonella* disease in Africa would help clarify the situation, though comprehensive data is not yet available.

As well as bacteraemia, invasive NTS disease can present as meningitis, pneumonia or septic arthritis (Graham, 2002). Indeed, NTS are the second most common cause of neonatal meningitis in Malawi (Molyneux et al., 2000). Symptoms of NTS bacteraemia may include fever, vomiting, cough and diarrhoea, but there is frequently an absence of gastrointestinal symptoms (Graham et al., 2000b). The lack of specific clinical presentation of NTS bacteraemia makes diagnosis difficult (Graham et al., 2000b). Often only a fever is present and so NTS bacteraemia is commonly misdiagnosed as malaria. Almost half of all NTS disease cases seen fulfil the WHO criteria for pneumonia diagnosis, a diagnosis for which antibiotics ineffective against many strains of antibiotic-resistant NTS, such as penicillin, may be administered (Brent et al., 2006). Resistance to

ampicillin, chloramphenicol, streptomycin, and sulphonamides has been reported in NTS across the world. It developed countries where there is greater use of antibiotics, resistance is very common and widespread (Vandenberg et al., 2010, Morpeth et al., 2009). In addition, development of resistance to quinolones and third-generation cephalosporins has been identified in numerous locations (Chimalizeni et al., 2010).

Case fatality rates from NTS bacteraemia in Africa are as high as 24% in children and around 50% in HIV infected adults, even when culture facilities and appropriate antibiotics are available (Brent et al., 2006, Graham et al., 2000b). The majority of those who die do so during their acute hospital admission. These time-restraints mean the current 'gold standard', diagnosis of blood culture is far from ideal because it is a relatively slow process. There is no available vaccine against NTS for use in humans. Increasing development of multidrug resistance to NTS (Graham, 2002), a lack of new targets for drug development (Becker et al., 2006) and current treatment difficulties highlight the pressing need for a vaccine. For this an understanding of protective immune mechanisms against NTS bacteraemia is needed.

1.4 Risk factors of invasive disease

The incidence of invasive NTS disease in Africa is much higher than that in Europe or North America. The strongest data currently available suggest the primary reason is due to host factors. It is probable that pathogen and environmental aspects also contribute.

A number of clinical associations have been reported, including: HIV (Gordon, 2008, Gordon et al., 2002), malaria (Graham et al., 2000a), severe anaemia (Brent et al., 2006), malnutrition (Graham et al., 2000c), and sickle cell anaemia (Williams et al., 2009). Young age is also a significant risk factor (Graham et al., 2000c). All of the medical conditions above are more common in Africa than the developed world and so

they may help to explain the geographical disparity seen in systemic disease. Whether these clinical risk factors are co-existent or causative is not always known.

1.4.1 Young age as a risk factor

Children and infants <2 years old are particularly at risk for invasive NTS disease (Fig 1.1) (Walsh et al., 2000, Gordon et al., 2008, Berkley et al., 2005, Sigauque et al., 2009, Brent et al., 2006). Young age is also associated with an increased risk of death (Graham et al., 2000b). A study of around 350 cases of NTS bacteraemia between 2003 and 2004 in Malawi showed NTS was commonest among young children under 24 months (MacLennan et al., 2008). These data also showed a relative sparing of infants less than 4 months old. This sparing is notable as maternal IgG aids immunity during this period. Sera from children under 16 months old lacked *Salmonella*-specific antibody and failed to kill NTS, whereas sera from older children both contained this antibody and killed NTS. These results for age-incidence and serum-killing of NTS suggest that antibody is important in immunity against NTS. The absence of *Salmonella*-specific antibodies in young children could be a key factor in the prevalence of invasive NTS disease in this age group. However, because invasive salmonellosis is much less common in developed countries, other risk factors are likely, for example increased exposure due to environmental factors.

1.4.2 Malaria and Severe anaemia as a risk factors

A link between invasive *Salmonella* infections and malaria was originally reported in the late 1920s in Africa (Giglioli, 1929). Since then further studies have strengthened the association of NTS bacteraemia and malarial parasitaemia (Mabey et al., 1987) (Graham et al., 2000). These concurrent infections with malaria and NTS lead to increased morbidity for children under the age of 5 years.

Severe anaemia, in which plasma haemoglobin levels are lower that 5g dl^{-1}, is strongly linked with NTS in African children (Brent et al., 2006, Bronzan et al., 2007) (Mabey et al., 1987, Calis et al., 2008). Malaria is a common cause of severe anaemia in tropical African countries (Biemba et al., 2000). Severe anaemia that can develop due to malaria infection may be a key reason for the association between malaria and NTS (Bronzan et al., 2007, Graham et al., 2000). Malaria infection results in haemolysis, which prompts host cell production of heme oxygenase-1 (HO-1). HO-1 production could be important for protection against malaria (Seixas et al., 2009). However, HO-1 has recently been reported to increase susceptibility to NTS bacteraemia, probably through suppression of oxidative burst in granulocytes (Cunnington et al., 2012).

As well as the effect of anaemia, Roux and colleagues recently reported that malaria parasite-specific factors contributed prominently to the increased systemic dissemination and/or replication of *S*. Typhimurium in mice with malaria (Roux et al., 2010). Malaria infection leads to an inhibition of Fc receptor-mediated phagocytosis, a system that is potentially important in immunity against NTS (Graham et al., 2000a). Moreover, patients with malaria are known to have lower levels of complement with lessened haemolytic activity (Nyakoe et al., 2009). These factors could increase the likelihood of invasive bacterial infection (Morgan and Walport, 1991). Studies in the mouse have shown that antibody-induced haemolysis prior to infection with *S*. Typhimurium results in a more rapid death of the mice (Kaye and Hook, 1964, Kaye et al., 1967). This is probably due to enhanced erythrophagocytosis of parasitised and non-parasitised red cells interfering with anti-microbial macrophage functions (Schwarzer et al., 1992, Gill et al., 1966, Kaye et al., 1967).

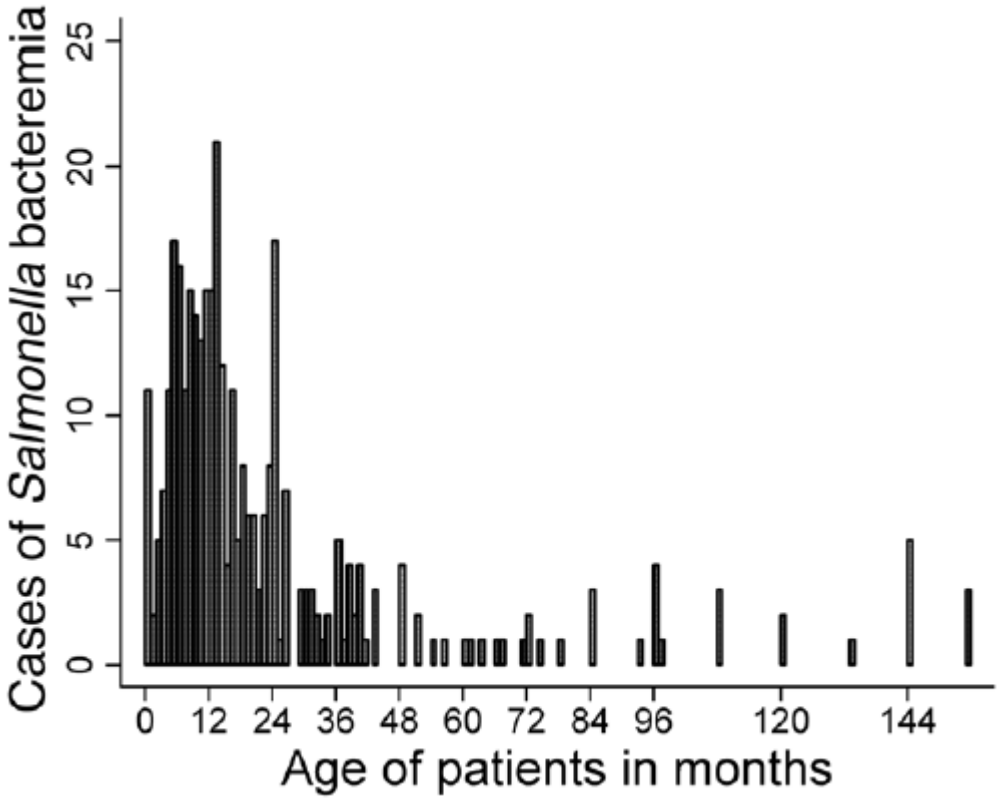


Figure 1.1: Cases of NTS bacteraemia in Malawian children 352 cases of blood culture-confirmed NTS bacteraemia by age among children reported at QECH, Blantyre, Malawi between 2003 and 2004. (Figure taken from MacLennan et al., J Clin Inv. 2008)

1.4.3 HIV-infection as a risk factor

As stated in section 1.2.2, the link between *Salmonella* infections and HIV-infection and AIDS has been known since the early 1980s (Clumeck et al., 1983). Since then numerous studies have strengthened this association (Gilks et al., 1990, Gilks, 1998, Gordon et al., 2001, Kiratisin, 2008). The association is so strong that recurrent invasive NTS infection is a WHO determinant of HIV/AIDS stage IV and requires commencement of anti-retroviral therapy (Hung et al., 2007). A HIV-infected adult with a CD4 count of less than 200/µl is around 50 times more likely to have invasive NTS disease than an uninfected adult (Ikumapayi et al., 2007, Gordon et al., 2008). In the US, the risk of

invasive NTS in HIV-infected patients has been reported to be up to 100 times higher than in the general population (Gruenewald et al., 1994).

HIV-infection causes the death of CD4+ T cells and macrophages which results in severe suppression of cell-mediated immunity. It is thought that this immunosuppression leads to increased susceptibility to NTS infections in HIV-infected patients (Celum et al., 1987). Studies using rhesus macaques reported that T helper type 17 (T_H17) responses were prominent in response to NTS infection. Prior infection of monkeys with simian immunodeficiency virus was detrimental to the T_H17 response and resulted in an increased systemic dissemination of *S*. Typhimurium from the gut (Raffatellu et al., 2008).

In addition to suppression of cell-mediated immunity, dysregulation of humoral immunity may contribute to the susceptibility of HIV-infected patients to invasive NTS. High titres of antibodies that inhibit serum-killing of NTS *in vitro* have been identified in a proportion of HIV-infected patients and could play a role in increased NTS incidence among HIV-infected patients (Maclennan, 2010). Interestingly, the co-incidence of invasive NTS disease and HIV infection in children is of a much lower proportion than in adults (Graham et al., 2000c), though comprehensive data is not yet published on this matter.

1.4.4 Environmental factors

Perhaps the most important environmental factor that may contribute to invasive NTS disease is water sanitation. The source and transmission of NTS in Africa is unknown. Though, as no animal food sources have been identified (Kariuki et al., 2006a, Kariuki et al., 2006b) contaminated water and human to human spread are likely to be important. Overcrowding contributes to hygiene problems and probably aids transmission,

particularly during wet seasons. The observation that NTS infection rises during the rainy season is consistent with this. Though, the incidence of diseases that are associated risks for NTS disease, such as malaria and malnutrition also peak in this period (Gordon, 2008, Brent et al., 2006, MacLennan et al., 2008).

1.4.5 Pathogen factors

As discussed in sections 1.3.1 and 1.3.2, *Salmonella* genotype is not typically considered a risk factor in invasiveness of NTS, but there is limited data to suggest it may play a role. Some researchers believe the presence of *spv* genes are a possible contributor to the ability of *Salmonella* to cause invasive disease (Guiney and Fierer, 2011, Fierer, 2001, Fierer et al., 1992). All isolates of serovars *S*. Dublin and *S*. Choleraesuis have the highly conserved *spv* operon that is encoded on virulence plasmids that are otherwise unique to each serovar (Gulig et al., 1993). The most important virulence gene in the operon is *spvB*, an ADP-ribosylating toxin that targets G-actin and prevents polymerisation to F actin in macrophages, thereby disrupting the actin cytoskeleton (Lesnick et al., 2001). It has previously been reported that clinical isolates of *S*. Typhimurium from blood cultures are twice as likely to carry *spv* genes as are isolates from stool (Fierer et al., 1992), although other groups have not found this (Chiu et al., 2000a). In experimental mice, mutation of *spvB* decreases the LD50 of isogenic strains as much as 1000-fold (Gulig and Curtiss, 1987). Serovars *S*. Abortusovis and *S*. Gallinarum also carry the virulence plasmid yet are not associated with invasive infection in humans (Rychlik et al., 2006) and it is likely that chromosomal genes are more important in determining both serovar specificity and type of infection (Chiu et al., 2000b).

1.5 Pathogenesis of infection

1.5.1 Systemic infection

In vivo studies of *S*. Typhimurium infection in genetically susceptible *SLC11A1*^{s} (previously NRAMP-1) mice have given insights into the likely pathogenesis of *Salmonella*. Following ingestion, *Salmonellae* that survive the low pH of the stomach reach the small intestine. Here they adhere to and colonise the epithelium before crossing the intestinal barrier. There is controversy over the exact route *S*. Typhimurium takes after ingestion, but the ingested *Salmonellae* seem to either invade epithelial or M cells or alternatively are engulfed by resident macrophages in the submucosa (Clark et al., 1996, Jepson and Clark, 2001). The coordinated activity of *Salmonella* pathogenicity island 1 (SPI-1) type III secretion system (T3SS) (TTSS-1) effector proteins induces bacterial uptake by activating intracellular signalling cascades and cytoskeletal machinery (Coburn et al., 2005).

Salmonellae moving into gut-associated lymphoid tissue spread into the lymph via the circulatory system which leads to a transient bacteraemia. This primary bacteraemia is rapidly cleared by CD18+ phagocytes to reach an intracellular location in the bone-marrow, spleen or liver (Biozzi et al., 1960, Dunlap et al., 1991, Worley et al., 2006). These phagocytes are able to kill the *Salmonella* and there is an initial net reduction in *Salmonellae* number. However, *Salmonella*e are capable of intracellular survival within phagocytes and, by preventing normal formation of the phagosome, survive and replicate in the protected environment of the *Salmonella*-containing vacuole (Salcedo et al., 2001). The *Salmonellae* then instigate phagocyte death (Hueffer and Galan, 2004), which allows escape and infection of a new phagocyte. SPI-2 is crucial for systemic infection in the mouse (Hensel et al., 1995) because it directs the evasion of the anti-microbial phagocyte oxidase response (Vazquez-Torres et al., 2000).

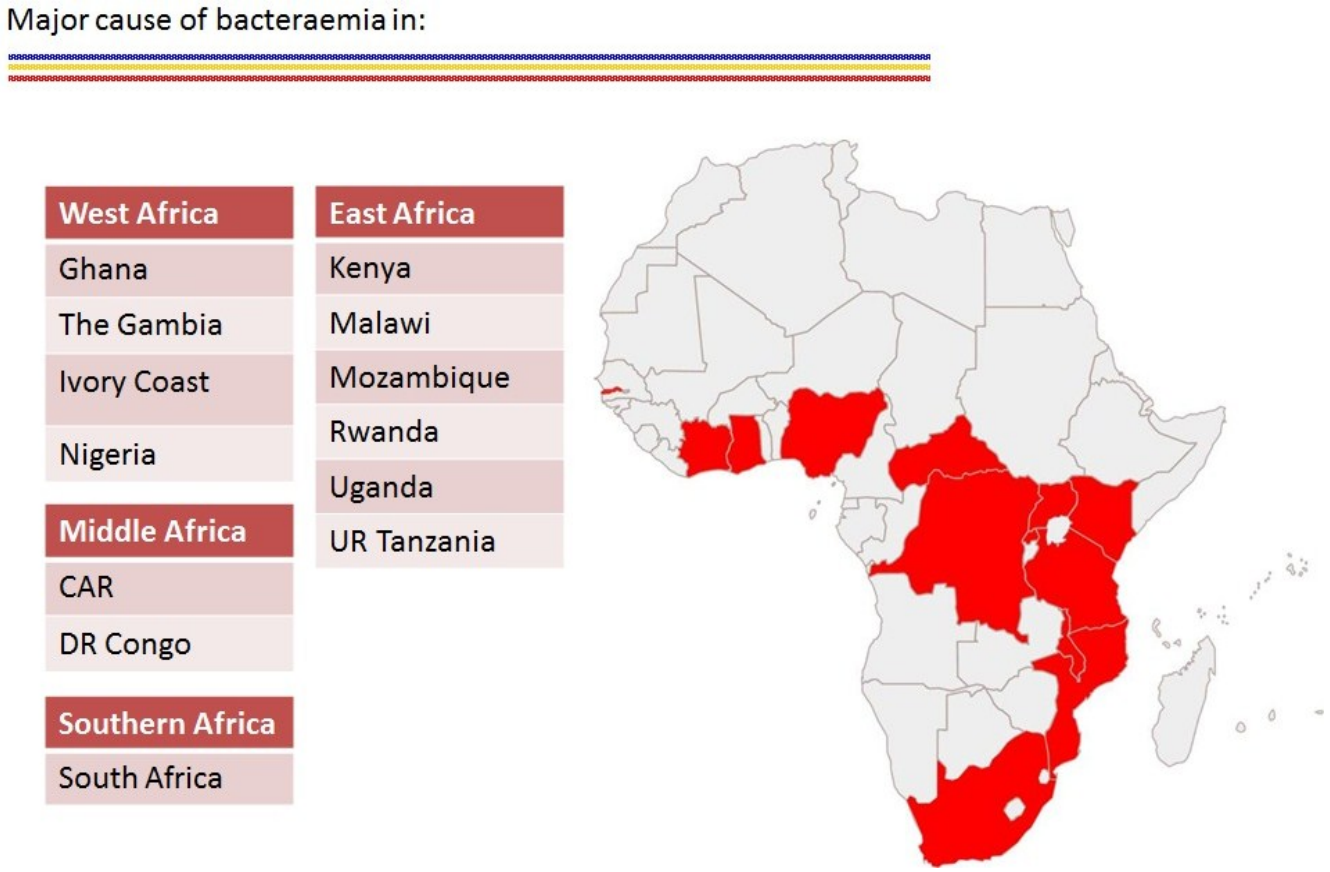


Figure 1.2: African countries in which NTS has been reported as a major cause of paediatric bacteraemia. Occurrence is likely to be more widespread than currently reported. Adapted from (Reddy et al., 2010)

Within 24 hours there is development of the infection to a second phase, in which *Salmonellae* numbers in the aforementioned organs increase. *Salmonella* levels in individual infected phagocytes remain low as the infection progresses; instead the increase translates to an increase in the number of infected foci (Mastroeni et al., 2009, Mastroeni and Sheppard, 2004, Sheppard et al., 2003). Eventually, the growth leads to onset of a third infection phase, occurring within 72 hours and signalled by the development of a secondary bacteraemia and endotoxic shock which kills the mouse (Mittrucker and Kaufmann, 2000).

Whether systemic *S*. Typhimurium infection in Africans follows the same progression as in mice is unknown. However, the fact that children with NTS bacteraemia typically have detectable anti-*Salmonella* IgG and IgM by the time of admission to hospital, in contrast to healthy children of the same age who tend to lack such antibody (MacLennan et al., 2008) suggests that the infection has developed over a number of days, as opposed to a rapid onset of bacteraemia.

1.5.2 Enterocolitis infection pathogenesis

As NTS does not naturally cause enterocolitis in mice, most work on *Salmonella* intestinal disease has been performed on bovine ileal loop inoculations and cultured intestinal epithelial cells (Coburn et al., 2005). Recently, a mouse enterocolitis model that uses pre-treatment of mice with streptomycin has been developed and may allow a furthering of understanding of the disease in humans (Barthel et al., 2003).

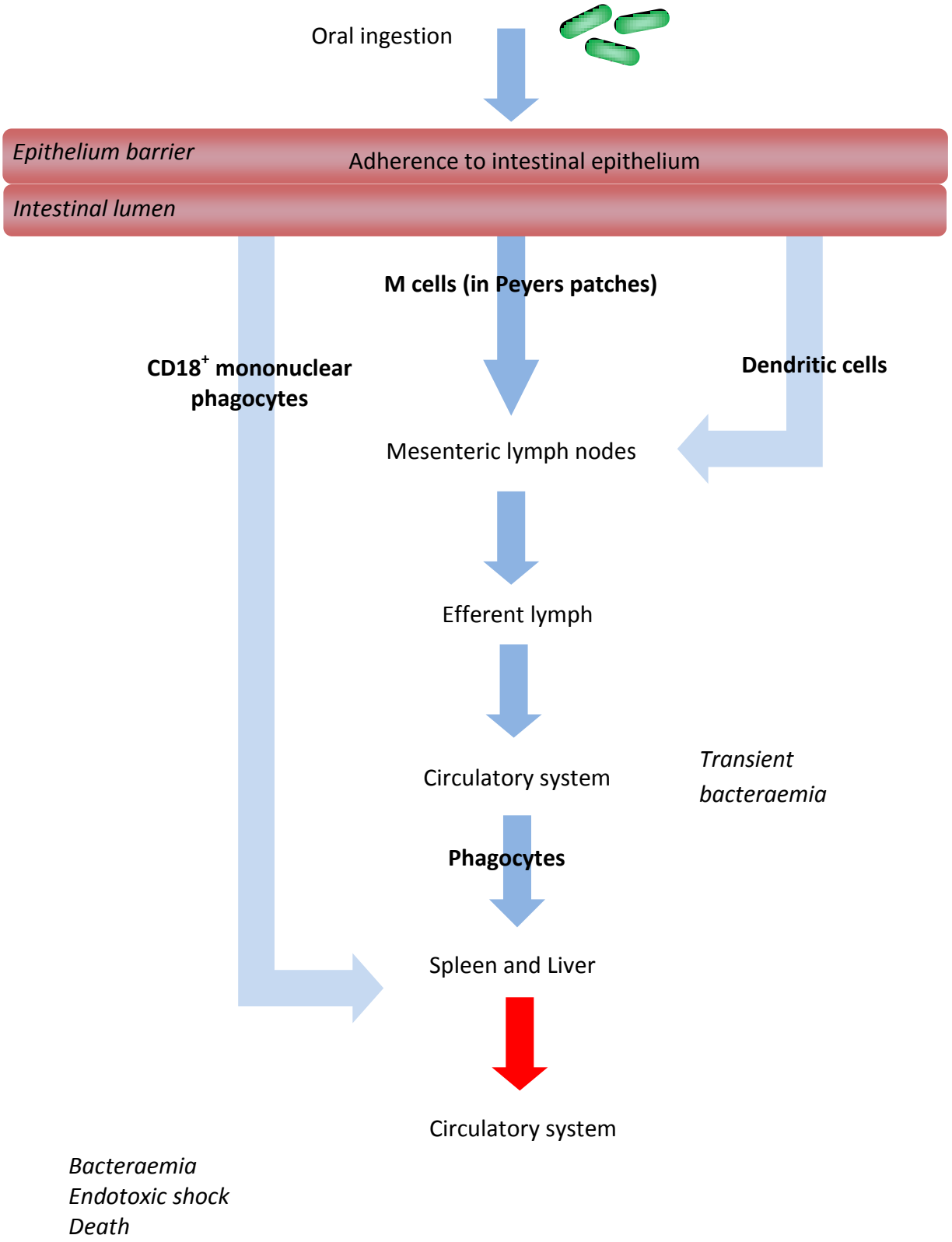
SPI T3SS effector proteins SipA, SopA, SopB, SopD, and SopE2 are required for eliciting inflammation and infiltration of neutrophils in the calf model of enterocolitis (Zhang et al., 2003) and this ability seems crucial to cause human intestinal disease

(McCormick et al., 1995). Just as in the development of systemic disease, invasion of epithelial cells is important in establishing an infection and entry into these cells is facilitated by T3SS-1 (Coburn et al., 2007). Full activation of T3SS-1 requires contact of the host-cell and bacterium. Critical saccharide residues of the outer core play different roles in the early interactions of serovars *S*. Typhi and *S*. Typhimurium with epithelial cells (Bravo et al., 2011). Inflammation occurs at the site of disease, namely the ileum, though often also the colon and occasionally in the stomach (Boyd, 1985, Coburn et al., 2005). Neutrophil recruitment begins within 1-3 hours of infection, and is associated with necrosis of the ileal mucosa (Zhang et al., 2003). Around 7 hours later, the injury to the intestinal epithelium leads to leakage of high protein exudates and massive transmigration of neutrophils into the intestinal lumen, a process normally prevented by the epithelial permeability barrier. Diarrhoea starts around 8-72 hours after bacterial colonisation (Tsolis et al., 1999) and typically resolves within 5-7 days (Coburn et al., 2005).

Figure 1.3 Multiple infection routes of *Salmonella* in mouse.

Following ingestion, *Salmonellae* adhere to the epithelium surface before crossing through, M cells, or through dendritic cells, or directly through enterocytes. From here they enter the mesenteric lymph nodes and then the efferent lymph, gaining access to the circulatory system. Phagocytes clear the bacteria from the blood and the bacteria colonises the spleen and liver. Some *Salmonellae* are carried directly from the intestinal lumen to the liver and spleen by $CD18^{+}$ phagocytes via the bloodstream. After a period of control by phagocytes, the infection overwhelms the host defences and gives rise to a secondary bacteraemia, endotoxic shock and leads to subsequent death of the animal.

Figure 1.3 Potential infection routes of fatal systemic *Salmonella* infection in mice



1.6 *Salmonella* virulence and resistance to killing

Salmonellae have approximately 4500 genes, and over 100 of these genes have been implicated in virulence (McClelland et al., 2001, Parkhill et al., 2001). A wide range of genes are required to establish an infection within a host, for example those involved in metabolism and biosynthesis (Hindle et al., 2002). During infection, *Salmonellae* deploy numerous mechanisms to evade the host immune system and they also can manipulate immune cell functions. They possess resistance against innate immune mechanisms and can avoid killing by macrophages and dendritic cells (DCs) (Jantsch et al., 2011). In addition, *Salmonellae* can prevent the formation of an efficient adaptive immune response by interfering with antigen presentation by DCs (Wick, 2004, Wick et al., 1995) and by directly inhibiting the proliferation of naive T cells by down-modulating T cell receptor expression on T cells (van der Velden et al., 2008).

1.6.1 *Salmonella* pathogenicity islands

The *Salmonella* pathogenicity islands (SPIs) encode a large number of important virulence genes, including virulence factors and the machinery for their secretion. Pathogenicity islands are products of horizontal transmission from other bacteria, indicated by their differing G+C content (Hensel, 2004). SPIs carry one or more virulence-associated genes and can occupy relatively large regions of the chromosome, ranging from 10 kb to more than 100 kb. They are common features of Gram-negative pathogens, but are notably lacking from related non-pathogenic species (Gal-Mor and Finlay, 2006). *Salmonellae* have numerous recognised SPIs, currently it is thought there are at least 17 (Vernikos and Parkhill, 2006). SPI-1 and 2 are the most comprehensively studied and have strong links to virulence, whereas little is known about the other SPIs.

SPI-1 is present in both *Salmonella* species (Ochman and Groisman, 1995) and its main role is in the initial steps of active epithelial cell invasion following oral infection. It

encodes a TTSS that transports bacterial effector proteins into the cytosol of host cells, resulting in cytoskeletal rearrangements that mediate uptake of the *Salmonella* into a membrane bound vesicle (Galan, 1999). In the mouse, SPI-1 mutants are attenuated for oral but not systemically administered infection (Galan and Curtiss, 1989).

SPI-2 is present in *S*. enterica but not *S*. *bongori,* placing its likely acquisition date after that of SPI-1 (Hensel, 2004). SPI-2 mutants are profoundly attenuated in mice, failing to proliferate in the spleen or liver (Hensel, 2000). SPI-2 also encodes TTSS apparatus and is involved in modifying the intracellular environment of host cells by impairing trafficking of important mediators of oxidative (Vazquez-Torres et al., 2000) and nitrosative burst (Chakravortty et al., 2002) and as such enhances the survival of *Salmonella* within phagocytes. SPI-2 further aids survival within macrophages by directing the formation and maturation of the *Salmonella* containing vacuole (SCV). The SPI-2 encoded protein SpiC inhibits the fusion of SCV with lysosomes and endosomes (Uchiya et al., 1999) and another SPI-2 gene product, SifA, is required for the SCV membrane integrity (Waterman and Holden, 2003). As well as offering protection from innate immunity, SPI-2 helps *Salmonellae* to evade aspects of the adaptive immune system by inhibiting antigen presentation and T cell activation by dendritic cells (Bueno et al., 2008, Tobar et al., 2006).

1.6.2 Lipopolysaccharide

Despite the focus on cell-mediated immunity to *Salmonella*, resistance to complement-mediated killing is a recognised virulence trait of *Salmonella* (Taylor, 1983) and LPS is an important determinant of this virulence. LPS can activate the classical and alternative pathways, and in some cases also the lectin pathway (Rautemaa and Meri, 1999). The length of LPS O-antigen (OAg) can influence interaction with complement and macrophages (Murray et al., 2006). Regulation of LPS OAg is required for virulence

(Murray et al., 2003) and growth-phase dependent regulation of LPS OAg influences serum resistance (Bravo et al., 2008). Size and distribution of LPS OAg can determine serum resistance (Grossman et al., 1987). Rough O-Ag deficient mutants are attenuated in mice (Ilg et al., 2009) and such strains show more bound complement than wild type strains which possess OAg (Rautemaa and Meri, 1999). Deposition of MACs in a unique location that doesn't lead to killing has been suggested as a mechanism by which *Salmonellae* confers resistance to serum-killing (Joiner et al., 1989). It has been also been hypothesised that lesions formed on the bacterial surface might not directly induce killing unless they are formed at precise targets, such as where the inner membrane is bound tightly with the outer membrane (Taylor, 1983). It is possible that LPS OAg prevents access of complement to these unique locations. Alternatively antibodies specific to LPS OAg might form an 'umbrella' which prevents binding of bactericidal antibody to the outer membrane (Maclennan, 2010).

1.6.3 Plasmid encoded outer membrane proteins

In addition to LPS, many *Salmonellae* have membrane proteins, mostly encoded by the virulence plasmid (pSLT), that serve as virulence factors by resisting complement killing and other antimicrobial defence systems. TraT increases resistance to complement-mediated killing, likely by interfering with MAC assembly and insertion (Rhen and Sukupolvi, 1988); outer membrane protein Rck prevents formation of fully polymerised tubular MACs (Heffernan et al., 1992); and the surface protease PgtE proteolytically cleaves C3b, C4b and C5, enhancing bacterial resistance to human serum (Ramu et al., 2007).

1.7 Regulation of virulence factors during infection

Changes from an extracellular to intracellular environment, such as after phagocytosis, can also effect widespread changes in *Salmonella* gene expression. Microarray analysis

has reported that almost 1000 genes are altered in regulation in response to a phagosomal environment inside phagocytes. Driven by these changes is an extensive surface remodelling of the bacteria (Prost et al., 2008). Factors that are recognisable by the host immune system such as the SPI-1- T3SS and flagellin, are down-regulated by the bacteria (Trent et al., 2001). The LPS structure is also altered, OAg repeat length is reduced and the number of acyl chains in lipid-A structures is changed. Changes are made to the protein content of both the outer membrane and inner membranes. As well as an alteration of structure, there is also up-regulation of the synthesis of enzymes that allow the bacteria to cope with oxidative and nitrogen-intermediates, which prepare the bacterium for survival in an intracellular environment (Jantsch et al., 2011).

Intracellular *Salmonellae* use a needle complex, the T3SS, consisting of more than 20 proteins to secrete effector proteins from their own cytosolic compartment across three membranes and directly into the cytosol of the host cell (Galan and Wolf-Watz, 2006). To prevent production of this intracellular tool in inappropriate environments whih would waste the bacterium's resources, the system is regulated by pH. By sensing sequential changes in pH the bacterium can coordinate its production to be most efficient. Upon entry into a host cell, the acid pH prompts the assembly of the T3SS needle complex, however, three regulatory proteins: SsaL, SsaM, and SpiC form a complex and prevent secretion of effector proteins. Pore-forming translocon proteins are released which allow the *Salmonellae* to create a holes in the vacuolar membrane of the cell and sense the neutral pH of the cytosol. This prompts disassembly of the regulatory protein complex and allows the secretion of bacterial effector cells into the cytosol (Yu et al., 2010).

1.7.1 Transcriptional regulation

In response to environmental factors, transcriptional regulation can change gene expression by altering when transcription occurs and how much RNA is created. By

tuning gene expression in response to environmental factors, transcriptional regulation determines the molecular machinery to maintain homeostasis and aid adaptation (Reviewed in (Balleza et al., 2009)). There are numerous mechanisms by which this is achieved, including protein molecules that either stimulate or inhibit gene expression by binding to specific operator or enhancer sequences. The *lac* repressor is an example of this and ensures that the bacteria only invest energy into metabolism of lactose when lactose is available (Gilbert and Muller-Hill, 1966).

1.7.2 Two-component systems

Two-component systems are the most common form of signal transduction enabling bacteria to regulate cellular behaviour in response to their environment. In a two-component system a sensor histidine kinase responds to specific signals by altering the phosphorylation of a response regulator protein. Binding of a specific ligand promotes conformational changes in the sensor protein that can potentially alter three enzymatic activities: autophosphorylation from ATP, transfer of the phosphoryl group to its cognate regulator, and dephosphorylation of the phosphorylated cognate regulator (Stock et al., 2000, Casino et al., 2009). Most response regulators are DNA-binding transcription factors whose affinities for their target promoters are altered by phosphorylation. Through changing the phosphorylated state of a response regulator, a signal can gene expression profile of an organism (Kato and Groisman, 2004). The PmrA/PmrB and PhoP/PhoQ are two prominent examples of two-component systems in *Salmonella.*

1.7.3 PhoP/Q regulation

A major regulator of *Salmonella* gene expression, particularly genes that may be involved in virulence, is the PhoP/PhoQ two component regulatory system. It regulates expression of at least 40 genes, including 15 genes encoding outer membrane components. It is involved in the formation of SCVs (Alpuche-Aranda et al., 1994) and

so is important for survival inside macrophages (Fields et al., 1986). Furthermore, it has a role in inducing macrophage apoptosis (Detweiler et al., 2001) and interferes with the presentation of antigens by phagocytes (Wick et al., 1995). As well as being involved in *Salmonella*:phagocyte interactions, PhoP/Q is implicated in active invasion of enterocytes (Behlau and Miller, 1993). Further to this, it has a role in LPS regulation; an NTS mutant constitutively expressing PhoP had reduced quantities of OAg (Guo et al., 1997). The enzymes that make lipid substitutions on the core galactosamines of *Salmonella* are also regulated by PhoP/Q (Guo et al., 1997). Finally, its involvement has been reported in regulation of resistance to acidic pH (Foster and Hall, 1990) and antimicrobial peptides (Fields et al., 1989).

1.7.4 Phase and antigen variation

Phase variation was first described in *Salmonella* flagellar antigens (Andrewes, 1922) and involves the variation of protein expression in an on-off fashion by genetic or epigenetic alterations at specific loci (Henderson et al., 1999). Phase variation is generally a random event and switches in expression (of certain phenotypes) occur at higher frequencies (>10^{-5} per cell per generation) (Stocker, 1949) than spontaneous mutation (10^{-8} to 10^{-6} per cell per generation) (Wisniewski-Dye and Vial, 2008). Antigen variation is the expression of one of a number of alternative surface phenotypes. Phase variation and antigen variation share certain features at the molecular level. Both result in phenotypically heterogeneous populations and are used by numerous bacterial species to increases bacterial fitness. In the context of infection, it can facilitate evasion of specific antibodies and immune responses and contribute to virulence (Ikeda et al., 2001) (Reviewed in (van der Woude and Baumler, 2004)). Phase-variable expression mainly occurs in surface-exposed proteins or proteins that modify or regulate surface proteins. In addition to flagellar phase variation (Bonifield and Hughes, 2003),

Salmonellae also show variation in expression of fimbriae (Nicholson and Low, 2000, Clegg et al., 1996) and pill (Morris et al., 2003).

Noise in gene expression can result in differences in expression in genetically identical cells (Raser and O'Shea, 2005). The resultant population of different phenotypes can offer an overall advantage to bacteria. For *Salmonella* infection, this effect could promote the formation of cooperative sub-populations that die while preparing the ground for a successful infection; indeed such a model has been experimentally shown to capture key features of *S.* Typhimurium enterocolitis pathogenesis. Normally the presence of intestinal microflora hinders the colonisation by *S*. Typhimurium, however invasion into gut tissue by a subset of *Salmonella* expressing invasion mechanisms produces an inflammatory response which kills off competing microflora and so allows the *Salmonellae*, which are more resistant, to survive and colonise (Stecher et al., 2007). Invasiveness in the bacteria is controlled by flagella and T3SS, which are differently expressed throughout the population of *Salmonella*, allowing survival of a percentage and the development of an infection (Ackermann et al., 2008).

1.8 Immunity against nontyphoidal *Salmonella*

Control, clearance and protection of NTS infection involves the collaboration of many aspects of the innate and adaptive immune systems, with both cell mediated and humoral immunity involved (Uppington et al., 2006, Mittrucker and Kaufmann, 2000).

It has long been appreciated from mouse studies (Blanden et al., 1966, Mackaness et al., 1966) and more recently from studies of patients with rare primary immunodeficiencies (MacLennan et al., 2004, Bustamante et al., 2008) that cell-mediated immunity is important for protection against invasive *Salmonella* disease. Effective immunity requires both T and B cells (Mastroeni and Menager, 2003).

Adoptive transfer studies have found that optimal protection against *Salmonella* in mice is conferred by antibody and T cells (Mastroeni et al., 1993). From studying NTS disease in Malawi, it has recently been shown that NTS bacteraemia particularly affects African children between four months and two years, the period in which antibody levels to NTS are low or absent (MacLennan et al., 2008). *In vitro* human work has further suggested at the importance of both bactericidal and opsonising antibody (Lindow et al., 2011, Gondwe et al., 2010, MacLennan et al., 2008). Studies of *Salmonella* infections in mice have shown that blood antibody titres correlate with protection against *Salmonella* (Xu et al., 1993). Antibody has been shown to be important for protecting innately-susceptible *Nramp*^{s} (*SLC11A1*) mouse strains (Hormaeche, 1979b, Hormaeche, 1979a), such as C57BL/6 and BALB/c, both in adoptive transfer studies (Mastroeni et al., 1993, McSorley and Jenkins, 2000) and studies in T-cell deficient mice (Cunningham et al., 2007, Gil-Cruz et al., 2009). Despite rapid uptake of *Salmonella* by the spleen and liver during murine systemic salmonellosis, there is a chronic low-grade bacteraemia that can become uncontrolled and cause death (Collins, 1969). In C57BL/6 mice, antibody against *Salmonella* has been shown to markedly reduce bacteraemia as well as to prevent primary infection and impede haematogenous spread of NTS (Cunningham et al., 2007).

1.8.1 Innate recognition

Pattern recognition receptors (PRRs) that recognise pathogen-associated molecular patterns (PAMPs) are innate mechanisms that are key to recognising and controlling *Salmonella* infection in its initial stages. The most important PAMPs that PRRs detect on *Salmonellae* are LPS and flagellin. LPS is recognised mainly by toll-like receptor 4 (TLR4) (Royle et al., 2003) and flagellin is detected by TLR5, though detection without TLR involvement also occurs (Hayashi et al., 2001). These responses are key in the initiation of inflammation which both hinders and helps the progress of infection. TLR4

knockout mice show greatly increased susceptibility to infection, demonstrating that overall the response is advantageous to the host (Vazquez-Torres et al., 2004, O'Brien et al., 1980).

1.8.2 Phagocytes

Phagocytes are central to control of *Salmonella* infection in the initial stages of infection. Understanding the role of distinct phagocyte cell populations *in vivo* is complicated because they share many of the same surface molecules and their roles in directing immune responses overlap (Wick, 2007).

Dendritic cells, macrophages and neutrophils are recruited early after *Salmonella* infection (Rydstrom and Wick, 2007). A rapid expansion occurs in the Payer's patches and spleens of orally infected mice (Wick, 2004). Phagocytes are responsible for clearing the initial bacteraemia that results when *Salmonellae* cross the epithelial barrier. They also help to control early growth in the liver and spleen. This is evidenced by the fact that macrophages and neutrophils are critical for the survival of mice during NTS infection (Friedman and Moon, 1977, O'Brien et al., 1979, Vassiloyanakopoulos et al., 1998).

The outcome of a *Salmonell*a-phagocyte interaction is dependent on a number of factors, most notably opsonisation and activation state. These elements determine whether killing, or alternatively survival and subsequent replication of the bacterium results. Prior opsonisation of the bacteria by antibodies and complement increases phagocytosis and respiratory burst activity of macrophages, monocytes and neutrophils in mice and man (Gondwe et al., 2010, Uppington et al., 2006). Experiments with C1q-deficient mice demonstrate the role of C1q in successful phagocytosis and killing of *Salmonellae*. When infected with *Salmonella,* these mice had increased susceptibility to

infection and failed to control bacterial growth within macrophages (Warren et al., 2002). Knockout mice have also been used to provide evidence to the role of antibody in enhancing phagocytosis. Mice lacking FcγRI, II and III show impaired immunity to virulent *S. enterica* (Menager et al., 2007).

1.8.3 Cytokines

Activation state of phagocytes, such as macrophages, is largely mediated by cytokines. Cytokines are a diverse group of secreted signalling polypeptides. They function as key communication molecules between host cells in the defence against many microorganisms. *Salmonella* infection induces expression of numerous chemokines and proinflammatory cytokines in both cultured intestinal epithelial cells and macrophages. In animal models, protective roles have been shown for IL-1alpha, TNFalpha, IFNγ, IL-12, IL-18 and IL-15, whereas IL-4 and IL-10 inhibit host defences (Eckmann and Kagnoff, 2001). IFNγ and TNFα are two particularly important cytokines that induce bactericidal mechanisms such as reactive oxygen and nitrogen-intermediates, as well as improving handling of bacteria-containing phagosomes (Gulig et al., 1997, Nauciel and Espinasse-Maes, 1992). Macrophage activation by IFNγ enhances killing of intracellular *Salmonella* (Eckmann and Kagnoff, 2001). Production of such inflammatory cytokines is a hallmark of *Salmonella* infection (Ramarathinam et al., 1991) and has an important role in protection (Nauciel and Espinasse-Maes, 1992), although the effect may only be present during the early stages on infection (Muotiala and Makela, 1990). Natural killer (NK) cells, an important component of the immune system, are thought to be a major source of IFNγ in this early infection stage of NTS infection. Cytokines' importance is demonstrated in a clinical setting by the vulnerability of persons with defects in macrophage activation systems to invasive NTS disease (MacLennan et al., 2004).

1.8.4 T cells

Overall, the immunology of the initial stages of *Salmonella* infection is characterised by recruitment and activation of phagocytes and IFNγ-mediated inflammation. While these innate defences help to control infection, the involvement of adaptive immunity and a specific lymphocyte response is required to clear infection. This response is likely part initiated by the stimulation of *Salmonella*-specific T cells by antigen presenting dendritic cells (Valdez et al., 2009).

The significance of T cells in both primary and secondary responses is well established (Mastroeni et al., 1992, Mastroeni et al., 1993, Nauciel and Espinasse-Maes, 1992, Hochadel and Keller, 1977), although the role of different T cell subsets and their effects during different phases of infection is less clear. CD4+ T cells seem to be the key αβ T cell subset, but CD8+ T cells may also be important, particularly in conferring resistance after vaccination (Mastroeni et al., 1992, Nauciel, 1990, Pie et al., 1997). Indeed, both are required for optimal resistance to infection (Mastroeni et al., 2009, Hess et al., 1996, Ugrinovic et al., 2003). The broad *Salmonella*-specific T-cell response is directed against *Salmonella* protein antigens such as the porins, flagellin, and pilli (Mastroeni, 2002, Bergman et al., 2005). CD8+ cells may help control *Salmonella* infection through antigen-specific target lysis. In addition to this mode of action, CD8+ T cells contain granulysin which has antimicrobial activity against *S.* Typhimurium (Stenger et al., 1998). T cells, specifically CD4 T-cells provide pathogen-specific help for activation and differentiation of B-cells (Cosgrove et al., 1991). This help is required for the production of antibody responses to *Salmonella* protein antigens and isotype switching of antibodies (Sinha et al., 1997). A study in T-cell deficient mice showed no production of IgG1, IgG2a and IgG2b, and low production levels of IgG3 and IgM (Mittrucker et al.,

1999). By modulating antibody production, T-cells are known to help establish a long lasting humoral response.

γδ T cells may also contribute to defence against *Salmonella*. Despite being a minority subset of total T cells, they represent a large fraction of T cells in the gut mucosa (Mixter et al., 1994) and so may have increased importance against enteric pathogens, such as *Salmonella*. Mice depleted of γδ T cells were more susceptible to oral infection with *Salmonella*, although the importance of this subset seems less than that of αβ T cells (Weintraub et al., 1997). T cells seem to play only a minor role in combating early infection in mice, as αβ T cell knockouts are able to suppress early growth of *Salmonella* (Hess et al., 1996). Yet, upon re-infection prompt production of phagocyte-activating IFNγ by memory T cells is likely part responsible for the protective effect of T cell immunity (Mittrucker and Kaufmann, 2000).

NK cells are important immune effectors for preventing bacterial invasion and dissemination (Vivier et al., 2008). They appear to have an important role in controlling *Salmonella* infection in both mice (Harrington et al., 2007, Griggs and Smith, 1991) and humans (Lapaque et al., 2009). NK cells can exert a protective effect through two means: cytotoxicity and the secretion of cytokines such as IFNγ.

1.8.5 B cells

B cells function as antibody-producing, cytokine-secreting cells and are also involved in antigen presentation. B cells are essential for full immunity to oral challenge and contribute to early protection in primary challenge (Mittrucker et al., 2000). B cell deficient mice show impaired resistance to primary *Salmonella* infections due to a defect in antibody production (Mastroeni, 2002). In addition, production of Th-1 type cytokines, IL-12 and IFN-γ was impaired in B cell deficient mice, which resulted in the failure to

control growth of virulent *Salmonella* in secondary infections (Mastroeni et al., 2000a). The principal modality of immunity is mediated through antibody production, which contributes to opsonising and complement-mediated killing of *Salmonellae*. But, B cells may also have another role in protective immunity, because passive transfer of immune serum into immunised B cell knockout mice did not provide full resistance as seen in wild type mice (Mastroeni et al., 2000).

Marginal zone (MZ) and B1b B cells lack a requirement for T cell dependent germinal centre reaction which means that they can be rapidly recruited into the early adaptive immune responses (Weill et al., 2009, Berkowska et al., 2011). These B cell subsets are well positioned and adapted to mount a prompt T cell independent immunoglobulin responses as a first line of defence against systemic blood-borne antigens that enter the circulation and pass into the spleen (Balazs et al., 2002, Kearney, 2008). MZ B cells exist in a pre-activated state and respond rapidly certain antigens to secrete antibodies (Oliver et al., 1997) and B-1 B cells are a major source of natural IgM serum antibodies (Berland and Wortis, 2002). MZ and B1b B cells are particularly involved in type-2 T cell independent (TI) responses to LPS antigens, which further suggests their importance in *Salmonella* infection (Zouali and Richard, 2011).

Murine MZ B cells are non-circulating and segregate anatomically into the marginal zone of the spleen (Martin and Kearney, 2002). This area of the spleen contains multiple subtypes of macrophages, dendritic cells, as well as MZ B cells. It is not fully formed until 2 to 3 weeks after birth in mice and 1 to 2 years in humans (MacLennan et al., 1985). Human MZ B cells share many properties with their mouse equivalents. However, they also have key differences. In humans MZ B cells can recirculate and have somatic mutations in their B cell receptor (Weill et al., 2009). However, functionally, in terms of

responses to blood-borne pathogens, it is unlikely that there are major differences between man and mouse in these populations. Very little is understood as to why, in mice, the spleen is the only site of MZ B cell localisation, or why exactly the spleen is required for MZ B cell generation in both mice and men (Pillai et al., 2005).

1.8.6 Antigen responses

Antigens are classified as T cell dependent (TD) or T cell independent (TI), depending on whether T cells are required to produce an antibody response.

1.8.6.1 T-independent antigen responses

There are two types of TI antigens, classified based on their interaction with B cells: TI types 1 and 2 (TI-1 / 2) (Mosier et al., 1977). TI-1 antigens, such as LPS, are polyclonal B cell activators and can activate both naïve and mature B cells non-specifically (Mond et al., 1995). For example, LPS interacts with TLR4 on the surface of B cells and activates it, producing a diverse array of antibodies (Lesinski and Westerink, 2001). In contrast, TI-2 antigens, such as bacterial capsular polysaccharides, are large molecules with repeating epitopes that are flexible and able to simultaneously engage multiple B cell receptors. They activate mature B cells by cross-linking surface exposed immunoglobulin and induce production of antigen-specific antibodies (Mond et al., 1995, Lesinski and Westerink, 2001). Responses to TI antigens develop significantly more rapidly than TD responses and thus can play a critical role against invasive *Salmonella* infection. However, B cells in young children and the elderly respond poorly to TI-2 antigens (Adderson, 2001). Although, It now appears that TI-2 antigens, like TD antigens, are able to generate B cell memory (Defrance et al., 2011).

1.8.6.2 T-dependent antigen responses

Proteins and peptides are typically TD antigens. They are presented to T cells by the Major Histocompatibility Complex (MHC) molecules present on B cells, dendritic cells and macrophages. These T cells, specifically CD4 T-cells provide pathogen-specific help for activation and differentiation of B-cells (Cosgrove et al., 1991). This help is required for the production of antibody responses to *Salmonella* protein antigens and isotype switching of antibodies (Sinha et al., 1997). A study in T-cell deficient mice showed no production of IgG1, IgG2a and IgG2b, and low production levels of IgG3 and IgM (Mittrucker et al., 1999). TD antigens result in long-lasting immunity by inducing memory B and T lymphocytes and long-lived plasma cells. The antibodies they induce are of high affinity and cover a wide range isotypes (Reviewed in (Parker, 1993)).

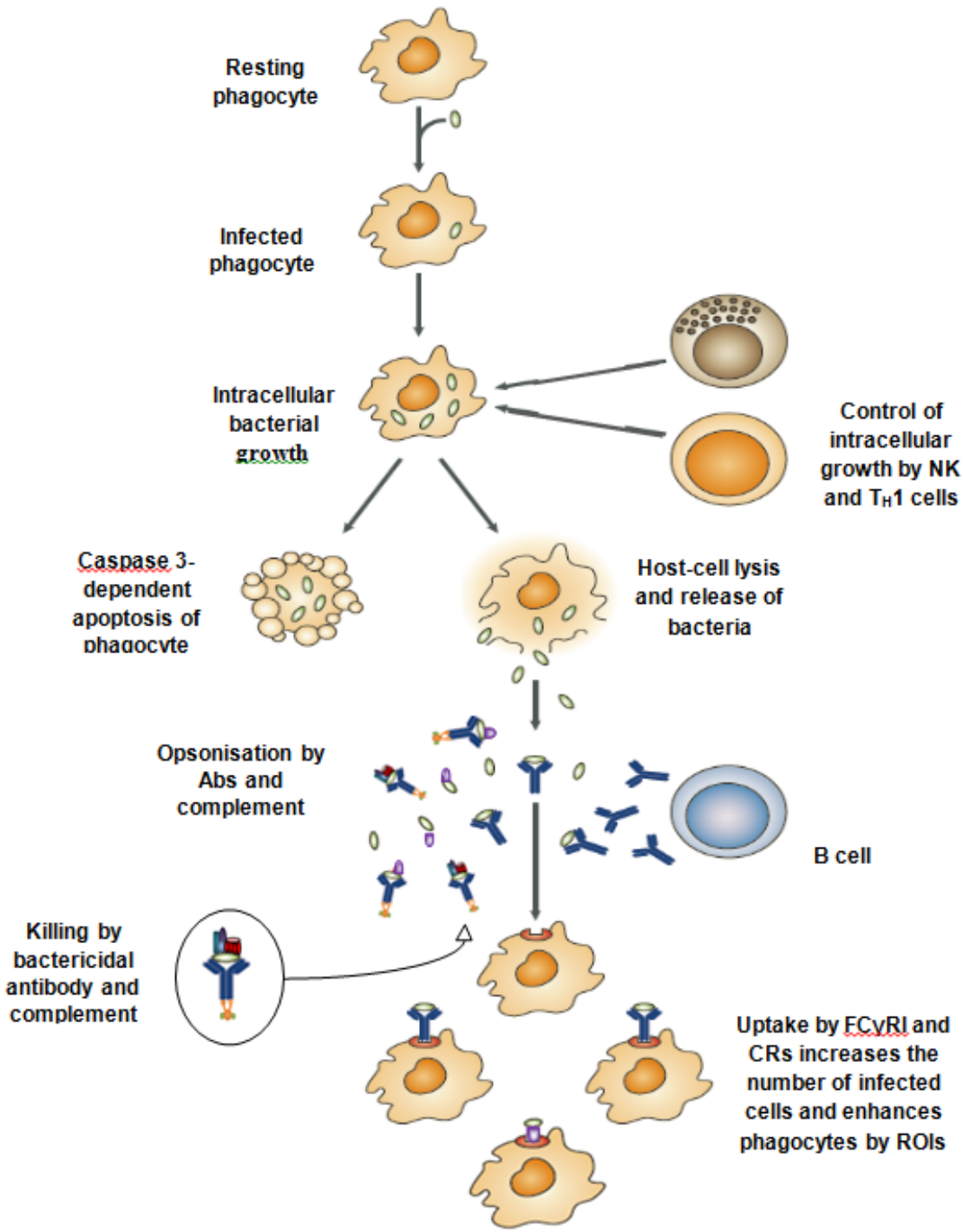
1.8.7 Context in infection spread

How *Salmonellae* spread from infected cell to a new cell during growth is not fully understand and is a topic of debate. *Salmonella*-infected mouse livers showed bacteria were only present in intracellular locations (Richter-Dahlfors et al., 1997). In addition there is evidence for *Salmonella*-induced apoptosis of host cells (Richter-Dahlfors et al., 1997, Lundberg et al., 1999, Paesold et al., 2002). Apoptotic pathways could allow spread of infection to new cells without exposure to extracellular components of immunity, such as antibodies. The inability of gentamicin, an antibiotic with poor eukaryotic cell penetration, to completely clear NTS infection in mice supports this, suggesting a fraction of *Salmonellae* are not exposed to the extracellular environment (Bonina et al., 1998). Yet, mathematical models and microscopy observations have indicated that cells housing *Salmonellae* undergo predominantly necrotic death and release the bacteria into extracellular space (Brown et al., 2006, Mastroeni et al., 2009). Consistent with this data, the majority of cell death observed in infected mouse livers

was due to caspase-3-independent mechanisms (Grant et al., 2008). Such necrotic cell death has been reported in *Salmonella* lesions in humans (Mert et al., 2004). It seems that different types of cell-death play a role in spread of *Salmonella* in a host. It is likely that cell mediated immunity controls infection within phagocytes and the humoral arm of immunity is central for controlling spread after release from infected cells (Mastroeni et al., 2009).

Figure 1.4: The key immunological factors that control the intracellular and extracellular phases of infection in humans. *S.* Typhimurium grows in phagocytic cells. Cell-mediated immunity enhances the antibacterial functions of phagocytes and may contribute to host-cell lysis. Mathematical modelling and microscopic observations indicate that S. *enterica* escapes from infected cells following density-independent, stochastic lysis of phagocytes (Mastroeni et al., 2009). This results in the formation of new clonal infection foci that start from a single founder bacterium (Mastroeni et al., 2009, Mastroeni and Sheppard, 2004). During their cell-to-cell spread, the bacteria are transiently present in the extracellular space, where they are opsonised by Abs and complement, then targeted to Fc and complement receptors. This process enhances bacterial internalisation by macrophages through FcγRI and activates cellular antibacterial functions that are dependent on the production of reactive oxygen intermediates (ROIs) (Mastroeni et al., 2009, Uppington et al., 2006). In humans significant killing is seen through the direct action of bactericidal antibody and complement (MacLennan et al., 2008). FcγRI, high affinity immunoglobulin-γ Fc receptor i; NK, natural killer; TH1, T-helper 1.

Figure 1.4: Key immunological factors that control the intracellular and extracellular phases of infection in humans.



Adapted from (Mastroeni et al., 2009)

Killing by bactericidal antibody (MacLennan et al., 2008)

1.9 Complement

1.9.1 Complement in host defence

The complement system has an essential role in host defence against pathogens, playing a part in both non-specific and specific immunity (Brown et al., 1983, Carroll, 2004, Fearon, 1998). It consists of more than 60 proteins that function as enzymes, binding proteins, activation products, regulators, inhibitors and cell-surface receptors. Cell-surface receptors exhibit specificity for sub components of complement proteins and are present on cells of the immune system (Zipfel and Skerka, 2009). There are several regulatory membrane and soluble proteins that function to prevent autologous complement activation and serve to limit host damage (Muller-Eberhard, 1988). Factor H is the key inhibitor of the alternative pathway and C1 inhibitor and C4bp are the classical pathway's equivalent (Ziccardi, 1982, Liszewski et al., 1991).

Complement can be activated by three different pathways: the classical pathway (Sakai et al., 1979), the lectin pathway (Fujita et al., 2004) and the alternative complement pathway (Pangburn and Muller-Eberhard, 1984). All the pathways serve the ultimate function of activating C3. However, the means of recognition by which they achieve this activation varies. The classical pathway is activated by the binding of antibody molecules (specifically IgM and IgG1, IgG2 and IgG3) to a foreign particle. The pathway is then initiated by binding of the antibody molecule to C1q (Schumaker et al., 1976). The capacity of the four human IgG subclasses to bind C1q decreases thus: IgG3>IgG1>IgG2. IgG4 does not activate complement (Flanagan and Rabbitts, 1982, van Loghem, 1986). The lectin pathway is activated after the recognition and binding of pathogen-associated molecular patterns (PAMPs) by lectin proteins, such as mannose-binding lectin protein (Carroll, 2004). The alternative pathway is constantly 'on', rather than being activated by a particular surface, it is activated by surfaces that aren't

protected by inhibitors (Carroll, 2004). The proteins C3, B, D, H, I, and P together perform the functions of initiation, recognition and activation (Schreiber et al., 1978). As well as possessing spontaneous initiation, the alternative pathway is unique in that it has a positive feedback amplification mechanism. C3 spontaneously forms C3b at a slow rate. If this C3b binds to an 'unprotected' surface it binds B to form C3 convertase which acts on more C3 to form C3b, which then binds to the surface too, and so on (Muller-Eberhard and Gotze, 1972). This can quickly turn an initial small deposit of C3b, placed by any pathway, into a massive production of C3b.

All activation pathways enzymatically catalyse the formation of the target cell bound C3 convertase, which promotes the binding of large amounts of C3b to the cell, as described above. C3b binds covalently to the bacterial cell membrane and opsonises the bacteria, enabling phagocytes to internalise them and kill more effectively (Uppington et al., 2006). C3a, which is created from the same reaction, is an anaphylaxtoxin and mediates inflammation. The C3 convertase can be 'activated' by more C3b to generate C5 convertase (Medicus et al., 1976), this results in the cleavage of C5a. C5a is a strong mediator of inflammation and also chemotactic, hence it promotes leukocyte migration to its site of production. C5b triggers the assembly of the terminal components of complement into a membrane attack complex (MAC). The MAC is assembled from five hydrophilic precursor proteins: C5, C6, C7, C8, and C9 (Muller-Eberhard, 1986). Hydrophobic interactions with the lipid bi-layer of target membranes allow the developing MAC to from a strong bind to its target. The final step in formation of a MAC is polymerisation of C9, causing weakening of the membrane structure and the formation of trans-membrane channels and events that lead to cell death. MAC assembly is regulated by the fluid phase complement regulator S-protein of plasma, which binds to the MAC, rendering it cytologically inactive. Homologous restriction factors of host cell

membranes also contribute to MAC regulation. Lysis can occur in many cells including Gram negative bacteria (Muller-Eberhard, 1986).

1.9.2 Mouse complement

Mouse serum, from both wild and inbred species, has long been known to lack bactericidal activity against a wide range of bacteria (Marcus et al., 1954). Furthermore, it lacks haemolytic activity against sensitised sheep cells (Brown, 1943). Mouse complement activity has since been demonstrated using modifications to standard haemolytic assay conditions, such as the use of rabbit erythrocytes and large quantities of sensitising antibody (Van Dijk et al., 1980, Rosenberg and Tachibana, 1962). The format of a haemolytic assay can greatly impact upon the results, for example Ish and co-workers showed a great variety in the specificity of alternative complement pathway-mediated lysis of erythrocytes using both complement and erythrocytes from 25 different species (Ish et al., 1993). The current view is that mouse complement, in comparison to human complement, has weak alternative pathway activated activity and even weaker still classical pathway activated activity (Morgan, 2009).

In addition to this generally weaker complement action, mouse complement is sexually dimorphic, with male mice having significantly higher titres than females (Baba et al., 1984). There are also substantial differences in C4 levels between strains (Hansen et al., 1974). Some inbred species have been noted to lack certain complement components. For example A/J mice are deficient in C5 (Nakano et al., 1995). BuB/BnJ have been reported to possess elevated complement activity in haemolytic assays. However, a recent investigation using a bacteria based assay showed no increase in levels compared to other mouse strains (Osmers et al., 2006), highlighting the importance of assay format in determining complement activity.

1.9.3 Complement-mediated Killing of Gram-negative bacteria

Serum-susceptible Gram-negative bacteria are killed by deposition and insertion of the terminal complement components in their membrane and the subsequent formation of a membrane-attack complex (MAC / C5b-9) (Joiner et al., 1984, Joiner et al., 1985b). The disruption of the lipid bilayer leads to the loss of cellular homeostasis, disturbance of the proton gradient across the membrane, the penetration of enzymes such as lysozyme into the cell, and the rapid destruction of the pathogen (Taylor, 1983). Although many of the molecular details of this cell death are not currently understood, it is thought that dissipation of cellular energy is the fatal event for the cell (Dankert and Esser, 1986).

1.9.4 Molecular details of complement-mediated bactericidal activity

Killing requires formation of a stable MAC complex on the outer membrane (OM) (Inoue et al., 1968, Joiner et al., 1982b, Joiner et al., 1982a), though cell death is dependent on damage to the inner membrane (IM) (Wright and Levine, 1981, Feingold et al., 1968). While formation of poly-C9 (a tubular polymerisation product of C9, containing around 20 subunits (Podack, 1984)) is not required for bacterial killing (Dankert and Esser, 1986, Dankert and Esser, 1985), multimeric C9 is required. Three or four C9 subunits are necessary per C5b-8 complex for the MAC to exert bactericidal activity, regardless of the amount of MAC inserted into the bacterium (Bhakdi et al., 1987, Bloch et al., 1987, Joiner et al., 1985b). Incorporation of additional C9 molecules does not increase killing (MacKay and Dankert, 1990). Addition of pre-formed C5b-8 or C5b-9 complexes to bacterial OMs, by liposome fusion (Tomlinson et al., 1990) or a bacterial cell donor system (Blanchard and Dankert, 1994), fails to kill bacteria. Illustratively, subsequent killing ensues upon incubation of purified C9 with cells baring the C5b-8 complex, but not the C5b-9 complex, or a control.

Bacteria naturally contain channels in their OM, known as porins, (Lugtenberg and Van Alphen, 1983). Preformed C5b-9 MAC complement complexes transferred into the OM of *Salmonella* functioned as water-filled channels but did not affect viability (Tomlinson et al., 1990). In addition most of the OM can be removed, without greatly impairing cell viability (Neu and Heppel, 1964). As such, it is unlikely that pore formation by MAC alone would lead to cell death and it is probable that an additional process or factor is required to kill the cell. Nevertheless, insertion of MAC into the OM does significantly alter the membrane. Cell permeability is increased as a result of this pore formation (Born and Bhakdi, 1986) and/or a restructuring of lipids in the immediate vicinity of the MAC leading to a so called 'leaky patch' (McCloskey et al., 1989, Bhakdi and Tranum-Jensen, 1991). Indeed, freeze-fracture electron microscopy (EM) studies have demonstrated that the lipids surrounding MACs in bilayers are severely distorted (McCloskey et al., 1989).

Past studies using bactericidal lysozyme-free serum have shown MAC to only be deposited on the OM (Taylor and Kroll, 1984), which presents a puzzle as to how MAC can exert a bactericidal effect at a relatively distant position from the IM. The length of the MAC is estimated to be around 30nm (Podack, 1984). These dimensions pose physical problems for the generation of a transmural pore that could simultaneously damage the OM and IM, even in the absence of long chain OAg (and especially in the presence)/ Yet evidence shows anti-OAg antibody to be bactericidal and very long STm OAg can contain over 100 repeat units (Bravo et al., 2008) modelled at around 1 nm each (Kastowsky et al., 1992). It is possible that translocation of the entire complex or merely an aspect of it could effect killing which would allow causation of cell death with a more distant initial MAC binding (Blanchard and Dankert, 1994). There is support for an individual component of MAC being able to translocate. Dankert showed that when C9 was introduced into the periplasmic space by means of an osmotic shock procedure, cell

killing occurred. Other proteins, such as C8 or serum albumin, were not toxic. Insertion of C9 directly into the periplasm was ineffective against two serum-resistant strains. This indicates an additional non-LPS linked mechanism of resistance, especially considering that rough strains made serum-resistant by genetic addition of LPS remained susceptible to killing by C9 inserted into their periplasm (Dankert and Esser, 1987). Another group have established that C9 is converted from a protoxin to a toxin by periplasmic conditions in *Escherichia coli* and this toxic form was responsible for direct killing of the bacteria (Wang et al., 2000). An essential role for C9 is supported by the weak and slow or absent killing by C9-deficient sera (Pramoonjago et al., 1992), as well as increased Neisserial disease in those with absent or reduced C9 (Lassiter et al., 1992, Botto et al., 2009, Morgan and Walport, 1991).

One investigation by Goldman and Miller did show evidence of IM bound complement components (Goldman and Miller, 1989) though the presence of large amounts of OM proteins in the isolated IM fraction indicated by SDS PAGE analysis of membrane fractions was criticised by Tomlinson et al as a potential indication of contamination (Tomlinson et al., 1990). Studies showing a lack of complement on the IM have typically used lysozyme free serum. While this can still effect killing, it is unknown whether the mechanism of killing is the same as the mechanism naturally occurring *in vivo.* Analysis of MAC killing of erythrocytes revealed penetration of C9 in the C5b-9 complex across the membrane into the cytoplasmic space (Whitlow et al., 1985), though, the gram-negative cell is a more complex target owing to its more complex envelope structure, and ability to respond rapidly to changes in the environment.

Bacterial bio-adhesion zones, which connect the OM and IM (Bayer, 1991), have been suggested as points that may allow formation of an OM bound MAC to effect near-

simultaneous damage against the IM and so kill the bacterial cell (Wright and Levine, 1981, Joiner et al., 1983, Born and Bhakdi, 1986, Bhakdi et al., 1987). As the relative amount of adhesion zone fractions decreases under conditions of limited or slow cell growth (Bayer, 1991, Blanchard and Dankert, 1994) this hypothesis could help explain the increased serum-resistance of bacteria in stationary phase or subjected to energy poisons (Dankert and Esser, 1987, Taylor, 1983, Davis and Wedgwood, 1965). Supporting this, Wright found that the kinetics of release of periplasmic enzymes and intracellular cations after exposure to lysozyme free serum are virtually identical, indicating that IM damage and OM damage are closely coupled events (Wright and Levine, 1981). In addition, plasmolysis of bacteria in hypertonic media protected the cells from serum killing, potentially by limiting OM-IM adhesion sites due to the osmotic contraction of the IM (Feingold et al., 1968). Periseptal annuli have been suggested as alternative but similar sites of deadly MAC insertion (Tomlinson et al., 1990).

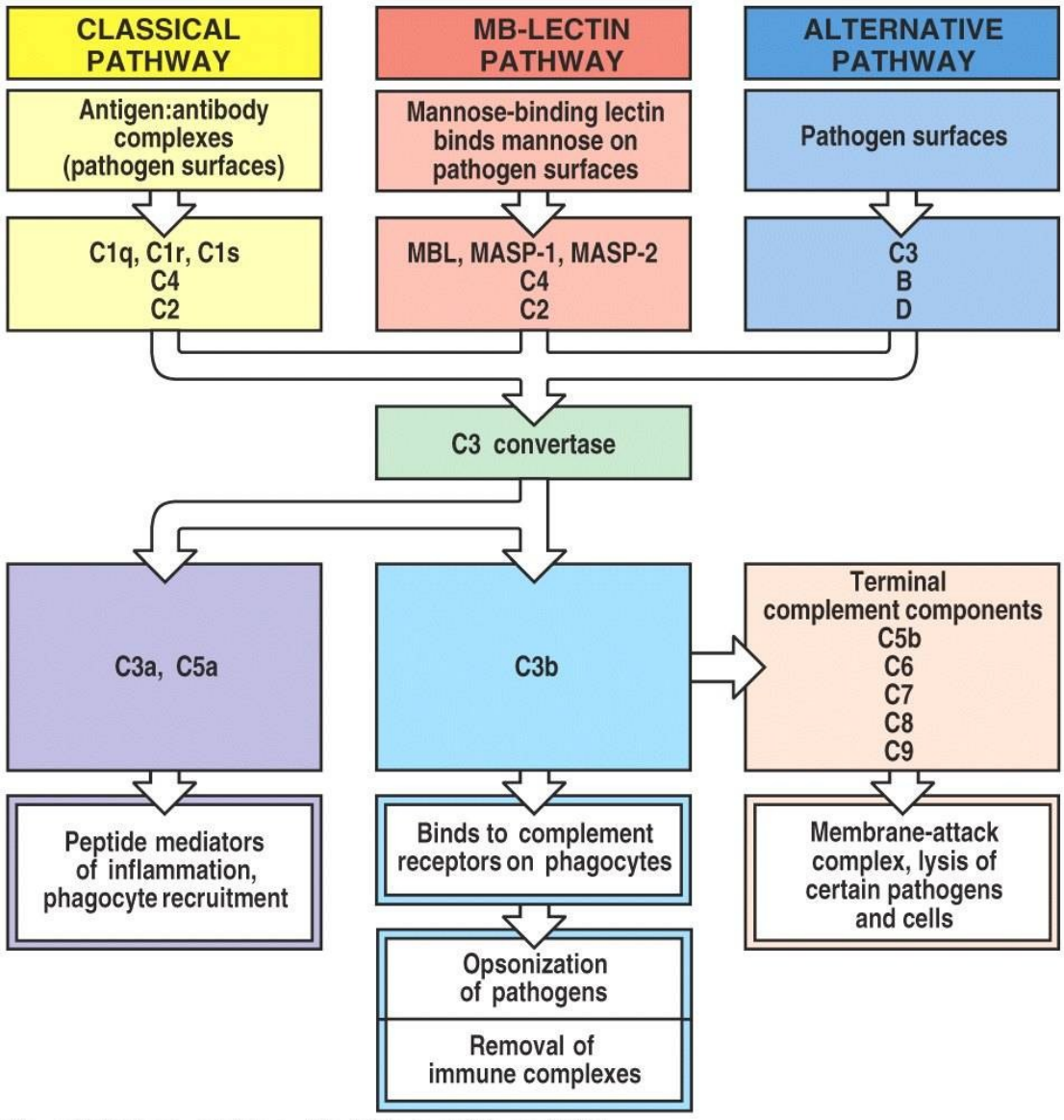


Figure 1.5: Overview of the main components and effector actions of complement. The early events of all three pathways of complement activation involve a series of cleavage reactions that culminate in the formation of C3 convertase, which cleaves complement component C3 into C3b and C3a. The production of the C3 convertase is the point at which the three pathways converge and the main effector functions of complement are generated. C3b binds covalently to the bacterial cell membrane and opsonises the bacteria, enabling phagocytes to internalise them. C3a is a peptide mediator of local inflammation. C5a is also a powerful peptide mediator of inflammation. C5b triggers the late events in which the terminal components of complement assemble into a MAC that can damage the membrane of certain pathogens. MASP - mannan-binding lectin-associated serine protease; B- factor B; D – Factor D. (Charles A. Janeway Jr, 2001)

1.10 Antibodies in host defence

Antibodies can effect anti-microbial action in several ways. They can neutralise pathogens to stop them entering or damaging host cells (Michetti et al., 1994). They can opsonise pathogens and bind to specific Fcγ receptors (FcγR) on phagocyte surfaces thereby increasing phagocytosis and killing rates. Finally, dependent on the pathogen, antibodies can be bactericidal either by activating complement through the classical pathway and inducing complement-mediated lysis (as mentioned in section 1.7), or without complement by inducing antibody-dependent cellular cytotoxicity (Casadevall, 1998).

Distribution of human IgG subclass in specific antibody responses varies based on the number and nature of the epitopes, and physicochemical properties of the antigen. Dosage and route of entry, as well as host factors are also significant. Polysaccharide antigens are typically T cell-independent and develop mainly in the form of IgG2 with some IgG1 production. In contrast, protein antigens are thymus-dependent, require interaction with T cells, and mainly produce IgG1 and IgG3. Immunisation with several encapsulated bacteria leads to an almost exclusive IgG2 anti-polysaccharide response (Siber et al., 1980). Yet, typically the IgG subclass distribution in an anti-bacterial response is heterogeneous, due to the variety of antigenic epitopes that thy bacteria possess. Antibodies develop against a variety of antigens in mice immunised with NTS. A large proportion of the antibody response is directed against LPS, but antibodies against Omps, flagella and fimbriae are also present (Brown and Hormaeche, 1989, Mastroeni and Menager, 2003). Murine studies have revealed that while the initial antibody production is of the IgM class, as would be expected, switching to IgG subclasses can depend on the antigen and mouse strain (Cunningham et al., 2007, Matsiota-Bernard et al., 1993).

The effector functions of IgG subclasses and their ability to carry out different function varies due to structural differences, particularly the length and flexibility of the hinge region. IgG subclasses differ in their affinity to FcγR. Broadly speaking, IgG3 and IgG1 are the most effective opsonising sub classes of IgG, though IgG2 has a strong affinity for FcγRIIa-H131 (Nimmerjahn and Ravetch, 2008). Binding of the Fc region of IgG to an FcγR is key in activation of a cell's effector functions (van de Winkel and Capel, 1993) and interaction between FcγRs and IgG antibodies can be pivotal in the immune response against some infectious agents (Bredius et al., 1994). Mice lacking FcγRI, II and III can control and clear primary infection with S. enterica of low virulence but have impaired immunity when challenged with virulent bacteria (Menager et al., 2007).

1.11 Vaccines

With increasing recognition of the problem of NTS in Africa, difficulty in diagnosis, and widespread antibiotic resistance, there is a need for a cheap and effective vaccine against this disease. Currently, there are no vaccines against NTS licensed for human use.

In experimental models, live vaccines confer better protection than killed vaccines as they tend to induce mucosal, cellular and humoral immunity (Hormaeche et al., 1990). Whilst killed vaccines produce strong antibody responses and confer a moderate degree of protection (Levine et al., 1989), they are often reactogenic and typically induce poor cell-mediated immunity (Harrison et al., 1997). Live attenuated vaccines also have other advantages over nonviable vaccines because of their ease of administration and ability to carry heterologous antigens (Mastroeni et al., 2001). Several live attenuated *S.* Typhimurium vaccine strains have been licensed for use against *Salmonella* infections in poultry (Holt et al., 2003, Curtiss and Hassan, 1996, Babu et al., 2003) and attenuated

strains show success in vaccinating mice. While phase 1 clinical trials have been performed in humans using attenuated human diarrhoeal *S*. Typhimurium strains as live oral vaccines or as live vectors which express foreign antigens, none of these trials have advanced in development due to negative effects of reactogenicity or inadequate immunogenicity (Hindle et al., 2002, Angelakopoulos and Hohmann, 2000). Ideally, live attenuated *Salmonella* vaccine strain candidates should harbour multiple independent attenuating mutations to limit the chances of a reversion to virulence. Live attenuated *Salmonellae* have great potential as single-dose oral vaccines against salmonellosis and as delivery systems for the construction of multivalent vaccines (Mas 2001)

Despite this promise, safety is a concerning factor for live attenuated vaccines. Some live attenuated *Salmonella* potential vaccines strains, such as aromatic dependent (aroA) mutants or SPI-2 mutants can cause fatal infections in animals with immunodeficiencies (Hess et al., 1996, Vazquez-Torres et al., 2000). This is problematic as immune-suppression is common in Africa, which is a major intended area for use of a NTS vaccine. As immunodeficiency may not be diagnosed in the individual at the time of vaccination, it is important that any live vaccine strains have no potential to cause disease. Promising new attenuated vaccine strains are being developed (Tennant et al., 2011), although it will be difficult to generate a mutant that creates sufficient immunity in healthy individuals but cases no harm to those with immunodeficiency.

Several vaccines have been developed and licensed for use against *S.* Typhi in humans: acetone or heat-phenol killed whole cell vaccines (Warren and Hornick, 1979), the Ty21a live attenuated vaccine (Levine et al., 1987) and purified Vi surface antigen (Acharya et al., 1987). Of these only the latter two remain in usage. While meta-analysis indicates that the currently used Ty21a live and Vi vaccines are both safe and

efficacious for preventing typhoid fever, their effectiveness is somewhat limited, with a 3-year cumulative efficacy of around 50% (Fraser et al., 2007). To maintain this level of protection boosters are required every 5 or 2 years, respectively. In addition, only those older than six (Ty21a) or two (Vi) years of age can be vaccinated, as Typhoid can affect infants this leaves a demand for a suitable vaccine in young children. The heat killed vaccines have significantly greater efficacy (73% at 3 years), but because of frequent side effects (20% of vaccinees fell ill and 10% missed school or work) their use has been discontinued (Engels et al., 1998). The Vi vaccine is a rare example of an efficacious *Salmonella* subunit vaccine. However, Vi is only expressed by serovars *S*. Typhi, *S*. Paratyphi C and some strains of *S*. Dublin (Grimont, 2007) and so it is unsuitable for use against NTS. Other subunit based vaccines, such as those based on flagella, porins and polysaccharide fractions have proved ineffective (Mastroeni and Menager, 2003), though major NTS porin OmpD appears to be a strong vaccine candidate (Gil-Cruz et al., 2009).

Efficacy of subunit vaccines is poor when administered without adjuvant or a delivery system. An alternative system, vaccine-conjugation has developed partly to avoid this problem. Conjugation of protective antigens to carrier proteins can be an effective way to generate a thymus-dependent response from what is a thymus-independent antigen when unconjugated. This gives immunogenic benefits in terms of a better response in infants and production of high levels of long-lasting antibody. There are conjugate vaccines in development such as Vi-rEPA - a conjugate of the capsular polysaccharide of *S.* Typhi and Vi bound to a recombinant *Pseudomonas aeruginosa* exotoxin A [rEPA]) which show good efficacy and mild side effects but as yet are not licensed for use in man (Fraser, 2007). Other potential vaccines using Vi in conjunction with carrier proteins such as, VI-CRM (CRM is a non-toxic mutant of diphtheria toxin) are also in early development (Rondini et al., 2011, Micoli et al., 2011). If suitable antigen targets can be

identified, these types of conjugated vaccines will also likely be a successful avenue of vaccine development for NTS strains.

1.12 Aims

The fundamental aim of the project is to further our understanding of complement and antibody-dependent immunity against NTS, with a focus on bactericidal antibody, in order to help in the creation of an effective vaccine. In this study we have achieved this in five ways:

- Firstly, by determining whether antibody acts in the same way in mouse models of infection as in humans.
- Secondly, analysing how growth phase and growth conditions can affect the resistance of NTS to serum-killing.
- Thirdly, creating an *in vitro* model of the time course of infection following entry of NTS into the bloodstream.
- Fourthly, by absorbing normal serum with different strains, serovars and genotypes of NTS
- Finally, using electron microscopy analysis to image the location of antibody binding and complement deposition and to determine the potential roles of inhibitory antibodies, as well as identify the target of bactericidal antibody.

1.13 Hypotheses

To accomplish these aims (1.12) we set key falsifiable hypotheses for each chapter of work:

Chapter 3: Mice serum lacks antibody-dependent complement-mediated bactericidal activity against an invasive African *S*. Typhimurium isolate.

Mice serum also lacks complement-mediated bactericidal activity against an OAg-lacking mutant strain that is exquisitely sensitive to human serum.

Chapter 4: Growth conditions and phase of *S*. Typhimurium affect its serum resistance.

In vitro phagocytosis of *S*. Typhimurium by neutrophils and monocytes in blood occurs before all *Salmonellae* are killed by cell-free serum-killing in a comparable *in vitro* system.

Chapter 5: Antibodies specific against *S*. Typhimurium can be removed from serum in a manner that preserves complement activity.

Antibody-mediated cell-free complement-killing of NTS is serovar specific.

Antibodies against *Salmonell*a LPS and specifically OAg are not bactericidal.

Chapter 6: HIV-infected inhibitory serum does not damage *S*. Typhimurium

HIV-infected inhibitory serum shows differences in location and quantity of deposition of antibodies and complement factors on *S*. Typhimurium compared to healthy bactericidal serum.

2. Materials and Methods

2.1 Bacteria and sera

2.1.1 Bacteria

This study principally used strains of *Salmonella enterica* enterica serovars Typhimurium and Enteritidis (*S*. Typhimurium and *S*. Enteritidis). These two serovars represent the most common causes of systemic nontyphoidal *Salmonella* (NTS) infection in Africa (Morpeth et al., 2009). A total of seven isolates taken from blood cultures from child patients in Malawi, Blantyre, were used, as well as a number of 'laboratory' strains (summarised in Table 2.1).

The main clinical isolate strain used was *S*. Typhimurium D23580, a clinical invasive isolate from a 26-month-old female bacteraemic Malawian who was admitted to Queen Elizabeth Central Hospital in Blantyre, Malawi in 2004 (MacLennan et al., 2008, Kingsley et al., 2009). This strain is sensitive to killing by human adult serum, undergoing a one to three $\log_{10}$ kill over a three hour time course (SBA: section 2.2). It is representative of over 90% of NTS strains isolated from bacteraemic individuals in Malawi since 2002 and has been sequenced by the Sanger Core Sequencing Facility at the Wellcome Trust Sanger Institute (Kingsley et al., 2009). The rough *galE* knockout of this D23580 is exquisitely sensitive to killing of human serum in the absence of antibody (MacLennan et al., 2008).

The main laboratory strain used was SL1344. Its *aroA* attenuated mutant, SL3261, kindly provided by B. A. D. Stocker was used for mouse immunisations. Various LPS knockouts (Table 2.2) and LPS chimera strains (Hormaeche et al., 1996) (Table 2.3) were also utilised. The LPS chimera strains expressed either their own LPS or that of

another serovar (*S*. Typhimurium C5 O:4 or O:9, and *S*. Enteritidis Thirsk O:9 or O:4).The phenotypic effects on LPS expression are shown in Figure 2.2.

2.1.2 Preparation of stationary phase cultures

A few single colonies were picked from a growth of *Salmonella* on a lysogeny broth (commonly known as Luria Bertani) (LB) agar (Sigma) plate and inoculated into 10mls of LB broth. This was grown in a loose capped universal tube overnight at 37^{o}C in an incubator (New Brunswick Scientific) to stationary phase. M9 minimal media (5 × M9 salts 200 ml, D-glucose (20g/100ml) 20ml, 1M $MgSO_4$ 2ml, 1M $CaCl_2$ 0.1ml, L Histidine 50µg/ml) (all Sigma) was used in place of LB for galE^{-} growths, as the phenotypic expression of the mutation depends on absence of galactose.

2.1.3 Preparation of logarithmic phase cultures

An overnight stationary phase culture was diluted 1 in 100 into pre-warmed (37^{o}C) LB broth. Dependent on the volume required for the application, tight capped universal tubes or baffled flasks were used. For work carried out in Birmingham (Chapters 3, 4, parts of 5 and 6) this was incubated at 37^{o}C for 2 hours in a tight capped universal tube on a rocker plate (Stewart Scientific) at 20rpm. Work undertaken in Siena (parts of chapter 5 indicated in text) used a shaking incubator (New Brunswick Scientific) at 180rpm for the same time at the same temperature. To produce different phase growths, such as early, mid or late log phase, the inoculum was incubated for a different time period as determined by a growth curve. Viable *Salmonellae* were prepared by first pelleting the appropriate quantity (dependent on the desired concentration) of inoculum by spinning at 3,300 x g in an Eppendorff micro-centrifuge (Hamburg, Germany), then washing the bacteria by removal of the supernatant, addition of 1 ml of PBS and spinning as described above. The wash step was repeated and the bacteria

resuspended in an appropriate amount of PBS to reach the desired concentration. The concentration of the final inoculum was checked by serial dilutions. As per section 2.1.2, M9 minimal media was used in place of LB for galE$^-$ bacteria.

2.1.4 Mice

C57BL/6, BALBc and C3H mice obtained from HO Harlan OLAC mice were age and sex matched, housed in pathogen-free conditions, and used between 6 and 12 weeks of age. For immunisations, *Salmonellae* were grown in LB media to log phase (as described in section 2.1.3) and heat-killed as required at 72°C for one hour. No exogenous adjuvants were used for immunisations. Mice immunised with heat killed *Salmonellae* were given 10^7 *Salmonellae*/animal via the intraperitoneal (i.p.) route at day 0, boosted with the same dose of heat-killed bacteria at day 14, and exsanguinated at day 28 by cardiac puncture. Those immunised with live *Salmonellae* were given $5x10^5$ bacteria i.p. at day 0 and exsanguinated at day 35. The schedule is summarised in Table 2.4.

2.1.5 Ethical approval

HIV-infected sera and clinical African *Salmonella* isolates were collected under ethical approval from The College of Medicine Research and Ethics Committee (COMREC), University of Malawi. Animal studies had ethical and home office approval.

2.1.6 Serum preparation

Human blood was obtained from volunteers and stood at room temperature until sufficient clotting had occurred. The blood was centrifuged at 3,300xg in a Megafuge 1.0R (Hereus, Germany) or a microfuge (dependent on quantity) at 4^{o}C for 20 minutes and then the serum supernatant aliquoted. Mouse blood was obtained by cardiac

puncture. As it is more heat-labile, to maintain optimum complement activity it was clotted for one hour on ice and then separated and frozen. Serum samples were stored at -80^{o}C and not subjected to freeze-thaw cycles.

Table 2.1: Wild type *Salmonella* strains and isolates used in study

Serovar	Strain name	Isolation
Enteritidis	ATCC4931	Human gastroenteritis, Copenhagen, Denmark
Enteritidis	D24359	Human child blood culture, Malawi
Enteritidis	D24953	Human child blood culture, Malawi
Enteritidis	D24954	Human child blood culture, Malawi
Enteritidis	P125109	Human gastroenteritis isolate
Typhi	D21685	Human gastroenteritis isolate
Typhimurium	D22477	Human child blood culture, Malawi
Typhimurium	D23005	Human child blood culture, Malawi
Typhimurium	D23580	Human child blood culture, Malawi
Typhimurium	LT2 (ATCC 700720)	Unknown natural source (1948)
Typhimurium	SL1344	Calf isolate (1978)

Table 2.2: LPS knock-out *Salmonella* strains used in study

Serovar	Strain Name	Knockout Gene
Enteritidis	P125109	*galE*$^-$
Enteritidis	P125109	*wbaP*$^-$
Typhimurium	D23580	*galE*$^-$
Typhimurium	D23580	*waaC*$^-$
Typhimurium	D23580	*waaF*$^-$
Typhimurium	D23580	*waaG*$^-$
Typhimurium	D23580	*wbaP*$^-$
Typhimurium	SL1344	*wbaP*$^-$

Table 2.3: *Salmonella* LPS chimera strains

Serovar	Parent Strain	Strain number	O-Antigen
Enteritidis	Thirsk	SL7488	4
Enteritidis	Thirsk	SL7490	9
Typhimurium	C5	SL5559	4
Typhimurium	C5	SL5560	9

2.2 Serum bactericidal assay (SBA)

5µl of, 2 hour log phase *Salmonella* culture (prepared as described in sections 2.1.3-4) diluted to a concentration of ~2x10^7 cfu/ml was added to 45µl of serum. Samples were incubated at 37^{o}C either in microfuge tubes with slight agitation on a rocker plate (20rpm), or without agitation in 96 well plates (Sarstedt). At desired time points, 5µl was removed from each sample and diluted as per the Miles and Misra method in order to

allow calculation of cfu/ml. Viable bacteria concentrations were recorded after overnight incubation at 37^{o}C on LB agar plates. Comparison of these values with the starting inoculum gave values for $\log_{10}$ change in bacteria. An increase in bacterial counts corresponded to a positive $\log_{10}$ change and therefore growth with the opposite applying for killing. The range of detection of the assay is a 3 $\log_{10}$ kill and a 2 $\log_{10}$ growth.

For supplementation of mouse serum or adsorbed serum with either anti-*Salmonella* antibodies or complement, the serum was mixed at a 1:1 ratio with heat-inactivated immune serum or serum lacking anti-*Salmonella* antibodies.

2.3 Flow cytometry assays

2.3.1 Quantification of antibody binding on the surface of *salmonella*

A 1-in-10 dilution of sample serum to PBS was prepared. 5µl of fixed and concentration adjusted (to 2x10^{9} cfu/ml) or live *Salmonella* inoculum was added to 45µl of diluted serum. PBS was used as a negative control. Samples were gently mixed and then incubated at room temperature for 20 minutes. 1ml PBS was added to each tube and samples were mixed by inversion and spun for 5 minutes at 3,300xg in an Eppendorf micro-centrifuge (Hamburg, Germany) to wash the bacteria. The supernatant was discarded and the samples washed again. An anti-antibody FITC conjugated antibody (Human: total IgG and total IgM; and mouse: total IgG and total IgM) (Table 2.5) was added to each sample and incubated at room temperature for 20 minutes, protected from light. Two more wash steps were performed before fixing the samples by adding 200µl 1% Formaldehyde PBS. Samples were mixed and analysed by flow cytometry. A FACSCalibur (Becton Dickinson) or FACSCANTO (BD Biosciences) machine was used in combination with CellQuest (Becton Dickinson) or FACS DIVA (BD Biosciences) software respectively to acquire and analyse flow cytometric data. Further analysis and

presentation of data was performed using FlowJo software (Tree Star Inc). The *Salmonella* population was gated by forward and side scatter light characteristics (Figure 2.1) and the geometric mean fluorescence intensity (GMFI) of the fluorescence channel 1 (FL1), FITC signal was used as the relative anti-*Salmonella* antibody titre. Internal quality control of the cytometer was determined using CaliBRITE beads (Becton Dickinson) on each day of use.

2.3.2 Quantification of complement components on the surface of *Salmonella* by flow cytometry

Assessment of the binding of complement was performed as described in section 2.3.1 with two slight modifications. Namely, serum was used undiluted and the appropriate anti-complement antibodies were used in place of anti-immunoglobulin antibodies. Anti-C3c FITC conjugated antibody was used for C3 measurement and MAC binding was analysed used an anti-C5b-9 neo-epitope antibody in combination with a rabbit-anti-mouse FITC conjugated antibody (Table 2.5).

2.4 Imaging of antibody and complement binding by confocal microscopy

The protocols for complement and/or antibody assays (2.3.1 and 2.3.2) were followed up until the second wash after incubation with the relevant FITC labelled antibody. At this point fixative, either 1% Formaldehyde or various mixtures of paraformaldehyde (Sigma) and glutaraldehyde (Agar Scientific) were added. The sample was vortexed and then left overnight at 4°C. The following day the sample was again vortexed prior to spinning at 3.300xg in an Eppendorf micro-centrifuge (Hamburg, Germany), for 5 minutes. The supernatant was aspirated off to a volume of 50µl and the pellet re-suspended. 5µl was pipetted onto a Superfrost Plus charged microscope side (Leica Microsystems) and the dried at 37°C for around 20 minutes. A drop of acetone (Sigma) was pipetted onto the sample and left to dry. Once dry, a drop of DAPI ProLong Gold mountant (Invitrogen)

was added on top of the sample. A coverslip was applied and the slides stored protected from light at 4°C until viewing under an oil immersion 1003 objective lens with an Axiovert100M confocal microscope (Zeiss). Analysis and presentation of images was performed using LSM Image Browser (Zeiss). Where reference is made to a wet sample the drying and acetone steps were omitted. I.e. the sample was applied to the slide and DAPI ProLong Gold mountant was added directly.

Day	Mice Inoculation	Bacteria/mouse	Bacteria prep (200ul/mouse)	Mice exsanguination
0	**C57BL/6** HI D23580 x 6 HI SL1344 x 6 SL3261 x 6 (Control) x 6 **BALB/c** HI D23580 x 6 (Control) x 6 **C3H** HI D23580 x 6 (Control) x 6	**HI D23580** $1x10^7$ **HI SL1344** $1x10^7$ **SL3261** $5x10^5$	**HI D23580** $5x10^7$ cfu/ml **HI SL1344** $5x10^7$ cfu/ml **SL3261** $2.5x10^6$ cfu/ml	N/A
14	**HI bacteria BOOST** **C57BL/6** HI D23580 BOOST x6 HI SL1344 BOOST x6 **BALB/c** HI D23580 BOOST x6 **C3H** HI D23580 BOOST x 6	**HI D23580** $1x10^7$ **HI SL1344** $1x10^7$	**HI D23580** $5x10^7$ cfu/ml **HI SL1344** $5x10^7$ cfu/ml	N/A
28	N/A	N/A	N/A	**C57BL/6** (HI D23580) x 6 **C57BL/6** (HI SL1344) x 6 **C57BL/6** (Control) x 6 **BALB/c** (HI D23580) x 6 **BALB/c** (Control) x 6 **C3H** (HI D23580) x 6 **C3H** (Control) x 6
35	N/A	N/A	N/A	**C57BL/6** (SL3261) x 6

Table 2.4: Mice immunisation schedule

Table 2.5: Antibodies used for antibody binding and complement deposition FACS and confocal format assays

Specificity	Clonality	Target Species	Host species	Conjugate	Supplier
C3c	Polyclonal	Human/Mouse	Rabbit	FITC	DakoCytomation
C5b-9	Monoclonal	Mouse	Mouse	N/A	DakoCytomation
Ig	Polyclonal	Mouse	Rabbit	FITC	Sigma
IgA	Polyclonal	Human	Rabbit	FITC	Sigma
IgG	Polyclonal	Human	Rabbit	FITC	Sigma
IgG	Polyclonal	Mouse	Goat	FITC	Sigma
IgM	Polyclonal	Human	Rabbit	FITC	Sigma
IgM	Polyclonal	Mouse	Goat	FITC	Sigma

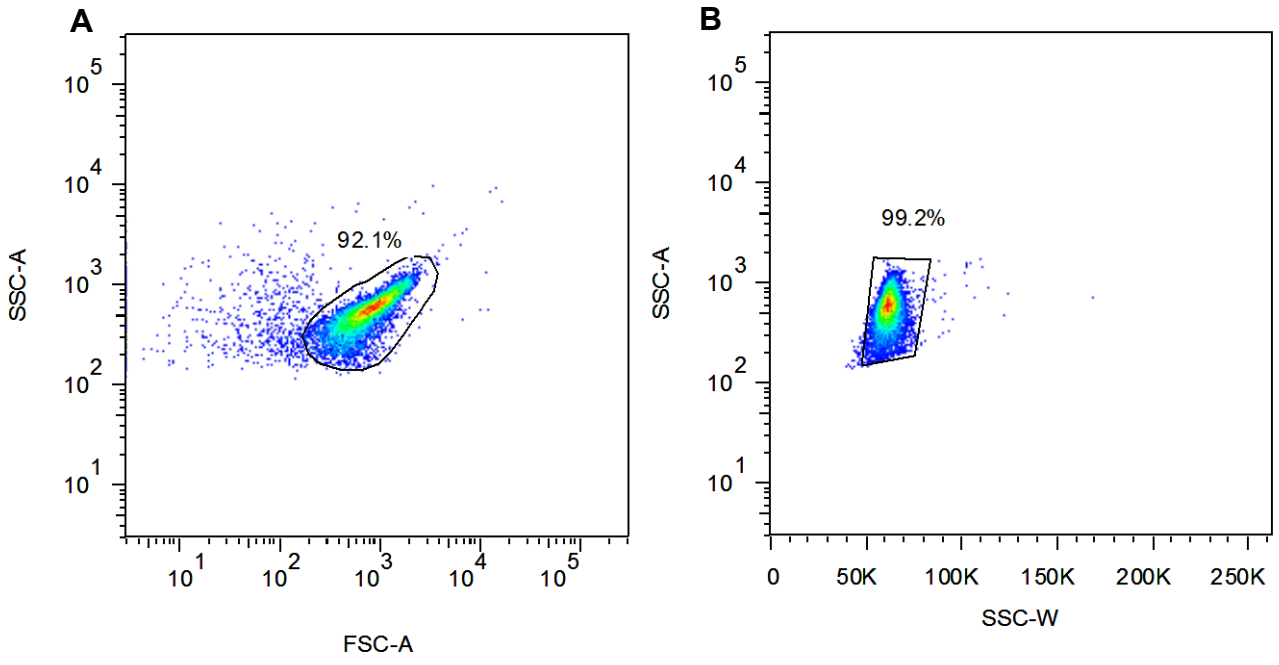


Figure 2.1: Gating of *Salmonella* population by flow cytometry

A pseudo-colour density dot plot of ***A*** forward scattered against side scattered light using FlowJo software shows the *Salmonella* population for antibody and complement FACS assays. ***B*** gating for single bacteria using information for the side scattered area and width of light.

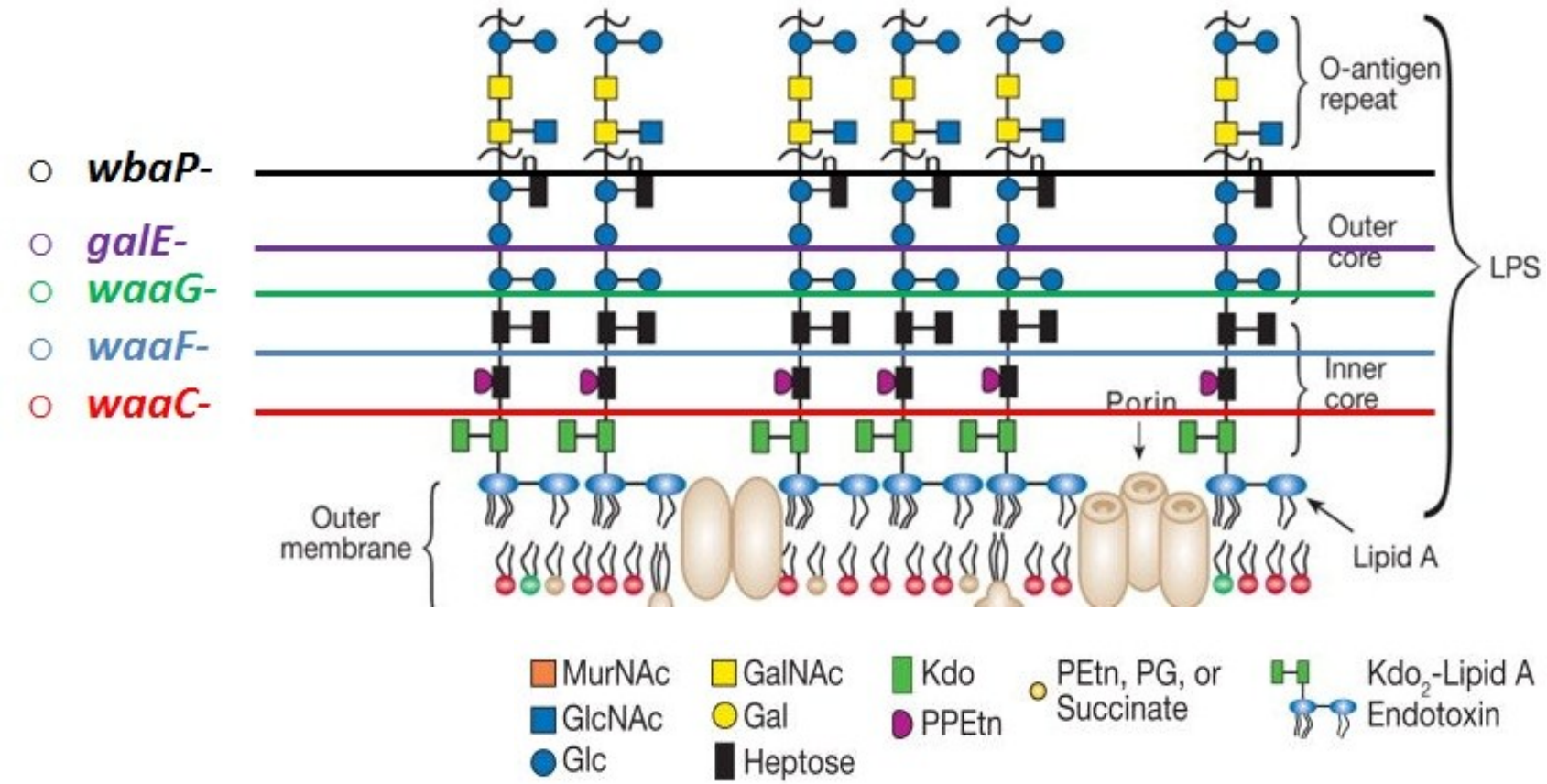


Figure 2.2: A cartoon representation of a generalised *Salmonella* LPS structure annotated with lines demonstrating the sites of truncations resulting from various LPS gene knockouts. Gene knockouts responsible for LPS truncation listed alongside line in corresponding colour to line.

2.5 Imaging of antibody and complement binding by electron microscopy

2.5.1 Assay

The protocols for complement and/or antibody assays (2.3 and 2.4) were essentially followed up until the incubation of *Salmonellae* with serum. However, in order to produce a bacterial pellet large enough for sectioning, both serum and bacteria volumes were increased 20 fold. So that 100µl of *Salmonellae* ($2x10^9$ cfu/ml) was added to 900µl serum.

2.5.2 Pre resin-embedding staining technique

2.5.2.1 Staining

Following incubation with serum, the samples were washed in PBS for 5 minutes 3 times. The bacteria were pelleted after each wash at 3,000xg and re-suspended in PBS containing 5% fetal calf serum (FCS) (Sigma). The samples were again pelleted and re-suspended in antibody-gold (1:20) in PBS with 5% FCS and incubated for 30 minutes before pelleting (Table 2.6). Samples were re-suspended and rinsed in PBS 3 times for 5 minutes a time, then pelleted.

2.5.2.2 Fixation

The samples were fixed for 30 minutes at 4°C in a solution of 2% glutaraldehyde (Agar Scientific), 5% paraformaldehyde (Sigma) and 0.1% ruthenium red (Sigma) in PBS. Samples were then washed in PBS before the addition of sonicated 1% osmium tetraoxide (Agar Scientific) in pH 7.4 0.1M sodum cacodylate (Sigma). After 30 minutes of fixation at 4°C, the samples were washed 3 times with sodium cacodylate and kept at 4°C before transport to the electron microscopy facility.

2.5.2.3 Dehydration processing

Once at the facility, the samples were pelleted as described previously before addition of 20% ethanol in dH_2O in order to cover the whole surface of the pellet. At this stage of processing it is desirable to keep the pellet intact and so care was taken to avoid re-suspension, additional centrifuging and thus re/pelleting was carried out only when needed. After 15 minutes incubation the solution was removed and 30% ethanol was added for 15 minutes. Following removal, a solution 1 part ethanol, 1 part uranyl acetate (Agar Scientific) and 1 part dH_2O was added and incubated in the dark, again for 15 minutes. The next few incubations were performed out of direct light to prevent any residue of uranyl acetate reacting. 15 minute incubations with 50%, then 70% and finally 90% ethanol solutions followed, before a series of three 20 minute incubations with 100% ethanol. When working with high concentrations of ethanol care was taken not to allow the pellet to remain uncovered by solution, as evaporation could lead to excessive drying. After the ethanol dehydration steps propylene oxide (Agar Scientific) was added, immediately changed with a new propylene oxide solution and incubated for 30 minutes. During this time 50ml of resin was made using an Agar 100 resin kit (Agar Scientific) and mixed by slow inversion in a 50ml falcon tube. After removal of the propylene oxide a solution of 50% propylene oxide and 50% resin was added to samples and allowed to incubate for one hour. Finally 1ml of 100% resin was added to each sample and allowed to set overnight at room temperature.

2.5.2.4 Sectioning and analysis

The following sections (2.5.2.4, and 2.5.3) were performed by Dr David Goulding.

The next morning the resin embedded specimens were cured in an oven at 65°C on a flat moulded tray. Thin sections were cut on a Leica EM UCS and collected onto copper grids, contrasted with uranyl acetate and lead citrate (Agar Scientific) before viewing on

an FEI 120kV Spirit Biotwin transmission electron microscope. Images were recording using an F4.15 Tietz camera.

2.5.3 Post-embedding cryo-section technique

2.5.3.1 Fixation and preparation

Cells were fixed with 2% paraformaldehyde and 0.2% glutaraldehyde in PBS for 1 hour on ice. They were then washed with 0.1% glycine in PBS x2 at 37°C, pelleted at 3000rpm. 1ml of 1% gelatin (Sigma) in PBS at 37°C was added and the samples were re-pelleted by centrifuging for 5 minutes at 37°C. The procedure was repeated but using 10% gelatin before being stored at 4°C. The gelatin was chopped into pieces and infiltrated with 2.3M sucrose on a roller in the cold room overnight. Finally, the samples were mounted and frozen onto pins and cut into ultrathin sections using a Leica EM FC6.

2.5.3.2 Immunocytochemistry for cryosections

Sections were blocked, first with 0.02M glycine (Sigma) in PBS for 20 minutes and then with 10% FCS in PBS for 1 hour. Following blocking, the primary antibody was applied and incubated for 1 hour. The samples were washed in PBS for 30 minutes three times, centrifuging at 3,300xg after each wash. 10nm gold conjugated protein A was applied and incubated for 1 hour. Next, samples were washed 6 times, as described above, before fixation in 2.5% glutaraldehyde in PBS for 30 minutes. Thorough washing, 10 times in ddH2O using capillary forceps followed. Uranyl acetate in methyl cellulose (Sigma) (1.8%/0.4%) was added to the samples and incubated for 10 minutes on ice. Afterwards, they were collected in loops, blot dried on Whatman paper and finally air dried. Samples were viewed on a FEI Spirit Biotwin 120kV TEM.

Table 2.6: Antibodies used for electron microscopy

Detection	Specificity	Clonal	Target Species	Host	Conjugate	Supplier
C3	C3c	Polyclonal	Human	Rabbit	-	Dako
-	IgG		Rabbit	Goat	10nm Gold	Agar Scientific
C5b-9	C5b-9	Monoclonal	Human	Mouse	-	Dako
-	IgG		Mouse	Goat	10nm Gold	Agar Scientific
IgG	IgG		Human	Goat	10nm Gold	Agar Scientific
IgM	IgM		Human	Goat	10nm Gold	Agar Scientific

2.6 Haemolytic assays

2.6.1 Human serum

Haemolytic kits, based on an a traditional radial immunodiffusion (RID) assay, were obtained from The Binding Site (Birmingham) and used to determine the alternative and total haemolytic activity of human serum. The alternative pathway haemolytic complement assay used is an adaptation of a classic method, using the principle that chicken erythrocytes will bind C3b, allowing the formation of C3 convertase. This activates the alternative complement pathway leading to the haemolysis of the erythrocyte. The total haemolytic complement assay uses the principle that sheep erythrocytes coated with anti-sheep-erythrocyte antibody (haemolysin) will, in the presence of normal human serum, activate the classical complement pathway leading to haemolysis of the erythrocytes. The kit features RID plates which house erythrocytes supported in agarose. To determine the degree of alternative and total haemolytic pathway activity, samples were added to the wells in the plate and incubated at 4^{o}C overnight. This first incubation allows the complement components in the sample to

diffuse through the gel. The temperature was then raised to 37^{o}C for around 2 hours, which allows activation of the complement cascade that was previously being inhibited by the lower temperature. A clear zone of haemolysis is produced around wells with samples which have functional complement. The diameters of any zones were measured and, using a standard curve of known haemolytic activity, the activity of the sample was calculated.

2.6.2 Mouse complement haemolytic assay

2.6.2.1 Preparation of mouse anti-rabbit erythrocyte (sensitising) antibody

Three female BALB/c mice (6-8 weeks of age) were subcutaneously immunised with 100µl of packed rabbit erythrocytes (RE) washed in PBS, with an identical inoculation boost was given in the second week. A final inoculation was administered intraperitoneally (i.p.) in the third week. Two days post i.p. boost the mice were exsanguinated and serum was prepared and pooled before storage at -80°C.

2.6.2.2 Complement assay

Washed rabbit erythrocytes were re-suspended in complement fixation diluent (CFD) (Oxoid, UK) at 2% (vol/vol) and sensitised by incubation for 30 minutes at room temperature with a 1:500 final dilution in CFD of mouse anti-rabbit erythrocyte antiserum. Sensitised erythrocytes were washed, re-suspended at 1% in CFD, aliquoted into the wells of a 96-well plate (50µl/well), and incubated at 37°C for 30 minutes with 50µl dilutions of mouse serum in CFD. Zero, 50µl of CFD, and 100%, 50µl of distilled water, lysis controls were included in all assays. Plates were centrifuged, and complement activity was assessed by hemoglobin release (absorbance at 415 nm). Percentage lysis for individual wells was calculated from the following formula: % lysis = (A_{415}(test) –

A_{415}(min))/(A_{415}(max)-A_{415}(min)) x 100. Corresponding serum was heat inactivated at 56°C for 30min and used to determine background, due to the colouring of the serum.

2.7 Quantification of phagocytic activity of monocytes and neutrophils in whole blood

2.7.1 FITC labelling of *Salmonella*

1mg/ml FITC (Sigma) was made up in PBS and filtered. 1 part of this FITC solution was added to 40 parts (7.5µl to 300µl) of 3 hour grown $2x10^{10}$ cfu/ml heat killed nontyphoidal *Salmonella* (heat killed by incubation at 56°C for 30 minutes). The mixture was protected from light by foil and incubated for 20 minutes at 37°C on a rocker shaking at 20rpm. The labelled NTS was washed by adding 1ml PBS and spinning at 3,300xg for 5 minutes. The supernatant was tipped off and the NTS washed again, this time the supernatant was aspirated off by pipette and re-suspended in 100µl (or the required volume) of PBS. Aspiration at this step is vital for consistent labelling.

2.7.2 Phagocytosis assay

50µl heparinised whole blood (1µl heparin per ml of blood) was transferred to a 5ml FACS tube. Negative control blood samples were placed in an ice bath. The other blood samples were pre-heated to 37°C in an incubator for 30 minutes. The FITC-labelled NTS was vortexed hard and 10µl was added to each blood sample and mixed. The labelled NTS was added at time intervals that allowed phagocytosis to be stopped at the same time in all samples. After the determined length of time incubation was stopped by placing the samples in an ice bath filled with 'ice water' and adding 50µl ice-cold trypan blue 0.16% (Sigma) which served as a fluorescence quenching solution. 1.5ml of ice cold PBS was then added per tube, before vortexing and spinning at 250xg for 5 minutes at 4°C. Supernatant was tipped off and the pellet re-suspended. The cells were washed and the pellet re-suspended a second time prior to the addition of 1ml of pre

warmed (at room temperature) 1x lysing solution (FACS Lysis (BD Biosciences) diluted 1 in 10 in dH_2O), samples were mixed and incubated for 20 minutes at room temperature, protected from light. After incubation, samples were spun at 250xg, 4^{o}C for 5 minutes. Supernatant was discarded and the pellet re-suspended by racking. A final wash step was performed before tipping away the supernatant, re-suspending the pellet and adding 100µl of 20µg/ml propidium Iodide. Propdium iodine binds to DNA and is detectable in the FL2 channel of a FACSCalibur flow cytometer, as it is excluded from viable cells it can be used to eliminate dead cells from analysis. Samples were mixed and incubated for 10 minutes on ice, protected from light. Samples were read within 60 minutes on a FACSCalibur instrument. Analysis was performed using CellQuest software as shown in figure 2.3.

Table 2.7: Monoclonal antibodies used in ELISA experiments

Specificity	Target species	Conjugate	Source
IgG	Human	Alkaline Phospatase (AP	Southern Biotechnology Associates
IgM	Human	Alkaline Phospatase (AP)	Southern Biotechnology Associates
IgA	Human	Alkaline Phospatase (AP)	Southern Biotechnology Associates

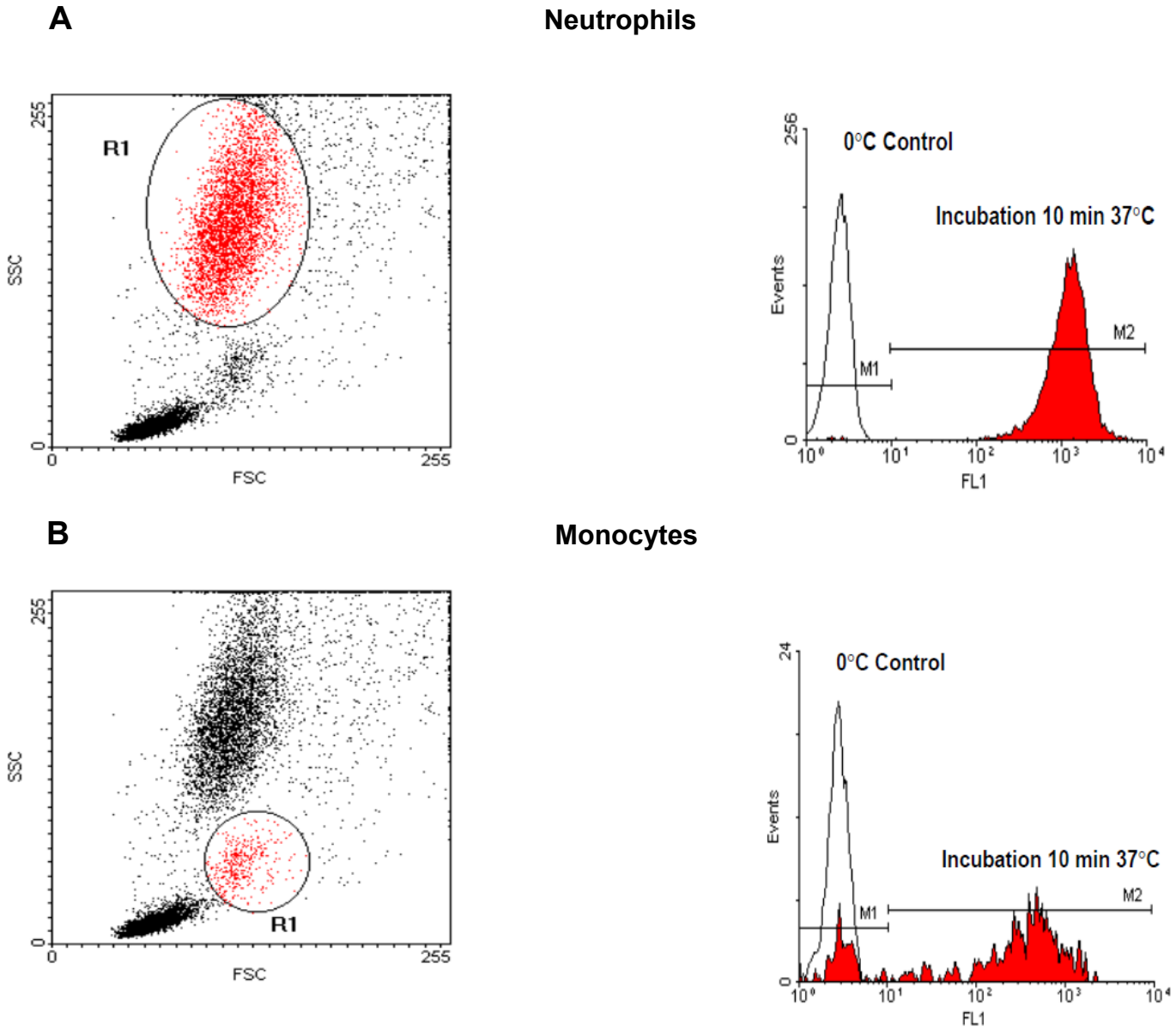


Figure 2.3: *Left Panel* Typical dot plots FSC/SSC showing recommended gating and *Right Panel* FL1 histograms of the phagocytosis test (incubation time of 10 min with, kit provided, *E.coli* at 37°C). (**A**) Neutrophils, (**B)** Monocytes. (PhagoTest Operator's manual Version 7/97)

2.8 ELISA

2.8.1 Lipopolysaccharide capture

50µl of 1mg/ml *S*. Typhimurium lipopolysaccharide (LPS) (Alexa) was mixed with 10ml carbonate coating buffer (Sodium carbonate(Na_2CO_3) and Sodium bicarbonate ($NaHCO_3$) diluted in 1 litre of distilled water) giving a 5µg/ml final concentration of LPS. Flat bottom 96-well plates (Nunc) were coated with LPS by filling each well with 100µl of 5µg/ml LPS and incubating overnight at 4^0C. The coated plates were then washed 3 times with PBS 0.05% Tween (500 µl Tween (Sigma) in 1 litre PBS). The blocking buffer (1% bovine serum albumin (BSA) in PBS) was made by mixing 160ml PBS with 1.6g BSA (Sigma). 100µl of 1% blocking buffer was added to each well, and incubated for 1 hour at 37^0C. Plates were washed three times with 0.05% PBS Tween. 150µl of a 1:10 dilution of each serum in 1% BSA was added to the first row of the 96-well plate and 100µl of 1% BSA was added to the rest of the wells. Three-fold dilutions of the each were made by transferring 50µl from the first well of each column in the 96-well plate to the second well, and serially diluting into each subsequent well (Fig 2.3). The plates were incubated at 37^0C for one hour and washed four times with PBS 0.05% Tween. 100µl of 1:2000 dilute AP-conjugated-secondary antibodies (IgG, IgM or IgA) were added to each well and incubated for 1 hour at 37^0C. The plates were washed three times in PBS 0.05% Tween and 100µl Sigma-fast alkaline phosphate (AP) substrate was added as the AP chromogenic substrate. This was prepared by reconstituting 2 tablets of p-nitrophenyl phosphate (Sigma) with 20ml distilled water. The results were read using ELISA plate reader set at 405nm using SoftMax Pro software (Greiner).

2.8.2 Outer membrane protein preparation for ELISA

Outer membrane protein (Omp) preparations of *S*. Typhimurium D23580 Wild type, *galE*$^-$ and *waaC*$^-$ were prepared by Dr Denisse Leyton from the School of Biosciences,

Department of Immunity and Infection, University of Birmingham. Overnight culture of *S.* Typhimurium D23580 was grown by inoculating a few single colonies of the bacteria in 5ml LB broth. 100ml LB broth was inoculated with a 1/100 dilution of the overnight bacterial culture and grown to a late log phase (OD_{600} 1.0). For galE$^-$ growths M9 minimal media was used in place of LB. The cells were harvested by centrifugation at 10,000 x g at 4°C for 10 minutes and re-suspended in 20ml 10mN Tris Buffer (pH 7.4). The bacteria were passed through a French pressure cell at 20,000 psi twice to disrupt the outer membrane of the bacteria. The cells were then centrifuged at 6,000 x g at 4°C for 10 minutes to remove unbroken cells. Separation of the cell envelope and cytoplasmic fractions was performed by centrifuging the cells at 50,000 x g at 4°C for 90 minutes. The pelleted envelopes were re-suspended in 10ml 10mM Tris Buffer pH 7.4-2% (v/v) TritonX-100 and incubated at 25°C for 15 minutes to allow solubilisation of the inner membrane. The TritonX-100-extracted envelopes were harvested by centrifugation at 50,000 x g at 4°C for 90 minutes. The pellet was washed three times in 1ml 10mM Tris Buffer pH 7.4 and re-suspended in 1ml 10mM Tris Buffer pH 7.4. The cell envelope suspension was aliquoted into 250 µl volumes and stored at -20°C. ELISA assay used for assessing levels of antibody against the outer membrane protein was performed as described in section 2.8. A concentration of 5 µg/ml of the outer membrane protein and 1:2000 dilutions of AP-conjugated secondary antibodies (IgG, IgM and IgA) were used. Preparations were tested for contamination using a monoclonal anti-O:4 antibody as the primary antibody in the ELISA format described in section 2.7.1.

2.9 Mutagenesis: LPS gene knockouts

LPS gene knockout was performed using the lambda red recombinase methodology outlined in Figure 2.4 (Datsenko and Wanner, 2000). This protocol replaces the target gene with an antibiotic resistance cassette. The study used mutants created by

ourselves as well as some kindly gifted by Rob Kingsley, Sara Sa Silva and Francesca Necchi. All mutants were created using the protocol outlined here.

2.9.1 Primer design

Oligonucleotide primer pairs designed to amplify an antibiotic resistance cassette were designed using Artemis and the annotated genome of *S*. Enteritidis PT4 strain P125109 (EMBL accession no. AM933172). Primers were constructed by Primm Biotech. The 5´ ends consisted of ~50bp homologous to the flanking sequence of the gene being targeted for deletion. The 3´ ends were specific to primer sites flanking the resistance cassette. Thus the PCR amplification product produced was a resistance gene flanked by sequence that defined the desired deletion site.

2.9.2 PCR of antibiotic resistance cassette

Ten 50µl PCR reactions were prepared in 0.2 ml thin walled PCR tubes using the designed primers. Each reaction mixture contained, 1µl template DNA, 1µl primer A (Forward), 1µl primer B (Reverse), 1µl 100mM dNTP`s (dNTP), 1.5ul MgCl (MgCl), 5µl 10x PCR buffer (10x), 39.3µl PCR grade water (dH_2O) and 0.2µl Taq polymerase (Taq) using a thermal cycler a PCR Cycle similar to shown in Figure 2.5.

2.9.3 Concentration and de-salting of PCR product

PCR reactions were pooled in a sterile 2ml microfuge tube. In order to concentrate and purify the PCR product, 2.5 x volume of 100% ethanol, 60µl of 3M sodium acetate (pH 5.2) and 1µl of glycogen were added and mixed well then incubated on ice for 10 minutes. Next, samples were centrifuged at 16,000x g for 15 minutes in a bench-top microfuge. The supernatant was aspirated off and 70% ethanol used to wash the pellet before another centrifuge spin at 16,000xg for 5 minutes. Once again supernatant was

aspirated followed by air drying for around 10 minutes until no liquid was visible. 20μl of sterile dH_2O was added and mix by pipetting. The DNA samples were stored on ice.

2.9.4 Analysis of PCR product size and concentration

The size and concentration of purified PCR products was checked by running on a 1% agarose gel containing SYBR safe DNA dye (Invitrogen). 1μl of PCR product was added to 39μl of sterile dH_2O and 10μl of the mixture was loaded. Samples were run at 90v for 40 minutes and the fragments sized by comparison with a Hyperladder 1 marker (Invitrogen).

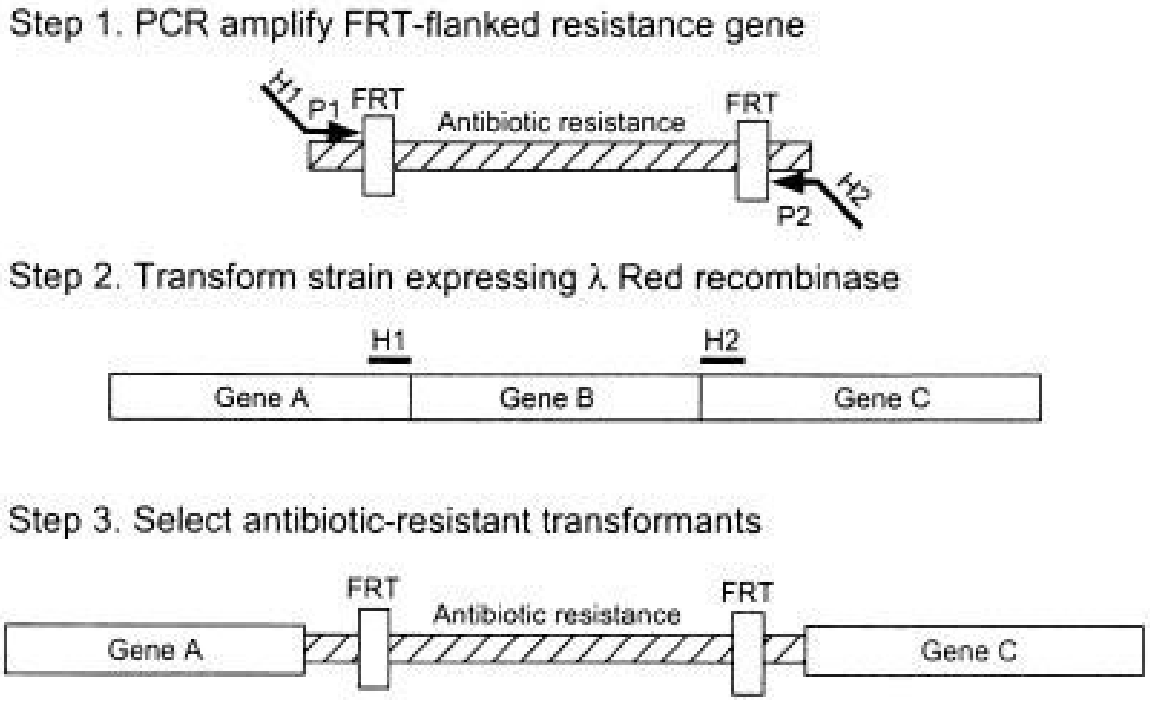


Figure 2.4: Gene disruption strategy. H1 and H2 refer to the homology extensions or regions. P1 and P2 refer to priming sites. (Datsenko and Wanner, 2000)

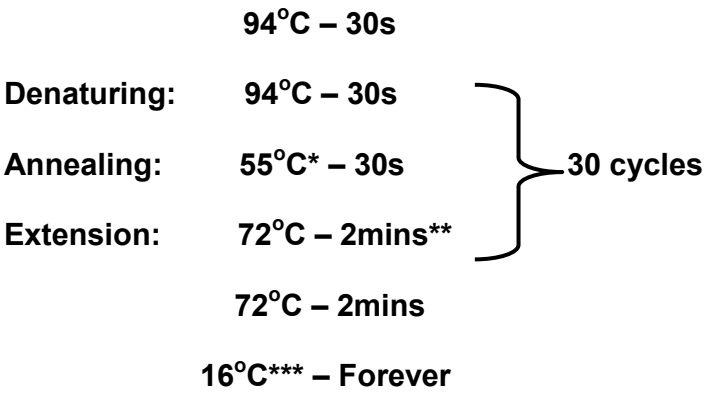


Figure 2.5: PCR cycle for amplification of primers for replacing gene targets with antibiotic resistance casettes.* Dependent on melting temperatures of primers. ** Dependent on size of fragment. *** Storage at 4°C also used

2.9.5 Preparation of electro-competent cells

An overnight culture of *Salmonella* that had been previously transformed with the plasmid pSIM18 (Figure 2.6) was grown as section 2.1.2 and the next morning a 1-in-100 dilution was made into 100ml of LB-hygromycin. The bacteria were incubated at 30°C with shaking at 180rpm until an OD at 600nm of around 0.4. The pSIM18 plasmid encodes hygromycin resistance and a heat inducible (42°C) lambda red recombinase gene, as well as a temperature sensitive replicon to facilitate subsequent curing of the plasmid. The growth was split into two 50ml falcon tubes and incubated in a water bath at 42°C for 15 minutes. The tubes were then immediately transferred to ice water and left for 8 minutes. Cells were pelleted at 4°C by centrifuging at 3,300xg for 10 minutes. The supernantant was removed and the pellets pooled into one 50ml falcon in 45ml of ice-cold dH_2O. The bacteria were again pelleted as above and re-suspended in 1.5ml of ice cold dH_2O. The samples were washed through pelleting in a microfuge, at 4°C for 2 minutes at 10,000rpm, and changing of the dH_2O a further three times. After completion of the washing the cells were re-suspended in final volume of 100µl of ice-cold dH_2O and placed on ice until required

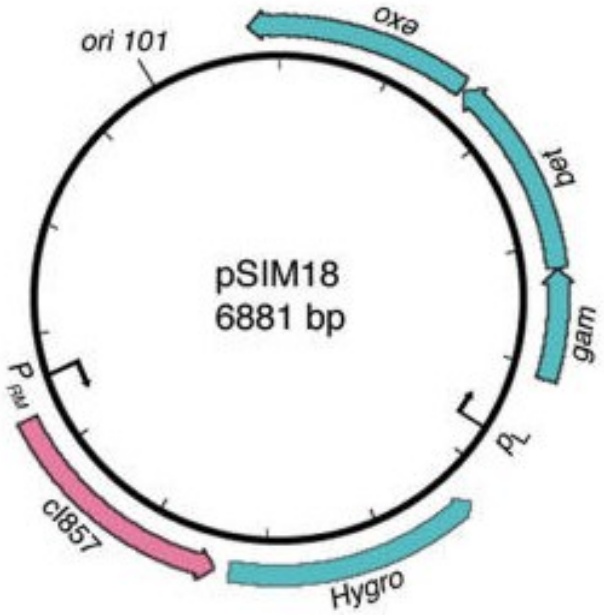


Figure 2.6: pSIM18 plasmid pSim plasmids provide an alternative convenient route for recombineering. Hygromycin (pSim18)-resistant gene-coding sequence is driven by PRM promoter. All pSim plasmids have the pSC101 temperature sensitive, low-copy replication origin.

2.9.6 Transformation of cells with PCR product

Two 0.2cm electroporation cuvettes were chilled on ice for 5 minutes. 4µl of PCR product was added to one cuvette and the same volume of ultra-pure water to the other. 40µl of electro-competent cells prepared in section 2.9.5 were added to each cuvette and mix gently by tapping the cuvette. The metal contacts of the electroporation cuvettes were dried and then electroporated using a Bio-Rad Gene Pulser set at 2.4kv, 25µF, 600 ohms. An audible pop signals an unsuccessful electroporation and the cells should be discarded and the procedure repeated. Immediately after electroporation, 400µl of pre-warmed SOC medium was added to each cuvette and mixed gently by tapping the cuvette. They were incubated at 37^{o}C without shaking for 2 hours**.** After incubation the suspension was mixed by pipetting and spread on LB plates containing the homologous antibiotic transformed to the bacteria and incubated overnight at 37^{o}C. The cuvettes

were also incubated overnight, though at room temperature on bench at room temperature. The next morning plates were checked for growth. The negative control should not have any colonies. The remaining contents of the electroporation cuvettes were plated onto the appropriate selective media as above.

2.9.7 Checking colonies for successful gene knockout

Insertion of the antibiotic cassette and thus removal of the targeted gene was checked through PCR of genomic DNA from bacterial colonies and also, where needed, sequencing.

2.9.7.1 Purification of bacterial genomic DNA

DNA was purified using a GenElute Bacterial Genomic Kit (Sigma). Briefly, cells were harvested from an overnight growth, re-suspended in a lysis solution with RNase added. Following incubation at room temperature the cells were lysed by addition of proteinase K, incubation at 55°C and then addition of a second lysis solution and further incubation at 55°C. The lysed bacterial solution was spun through a column in order to bind DNA, before washing of the column and finally elution and collection of DNA. Concentration and purity of DNA was checked on a NanoDrop spectrophotometer (Thermo Sceintific).

2.9.7.2 PCR of bacterial genomic DNA

PCR and running of the products on an agarose gel were carried out as in section 2.9.2 using the same primers, where appropriate primers with internal sites within the resistance cassette were also used. The fragment sizes obtained for knockout and wild type colonies were compared using a marker ladder.

2.9.7.3 DNA Sequencing

For cases in which the antibacterial cassette was similar in size to the gene that was being knocked out DNA sequencing was employed using the same primers as used for PCR. Prior to sequencing the DNA generated by PCR was purified using a Wizard® SV Gel and PCR Clean-Up System (Promega). DNA sequencing data was analysed using FinchTV software (Geospiza Inc) and the Nucleotide BLAST web-tool (NCBI).

2.10 O:Antigen assays

2.10.1 Agglutination assay

A drop of monovalent anti-Salmonella O4, 9 or 12 antiserum (BioRad) was spotted onto a glass microscope slide (Menzel-glaser). A section of a single colony was picked from an agar plate using an inoculating needle (Nunc) and mixed with the antiserum on the slide. Agglutination was determined by eye.

2.10.2 Flow cytometry O-antigen surface staining assay

The flow cytometry assay as described in section 2.3 was used with modifications. Namely, instead of incubation with serum, an anti-O4/9/12 antigen mouse monoclonal antibody (AbCam) was used as a primary antibody source. A FITC conjugated rabbit anti-mouse Ig antibody (Dako) allowed relative quantification of O-antigen using analysis described in section 2.3.

2.11 Antibody adsorption

An inoculum of OD 0.1 at 600nm of the desired *Salmonella* was grown (as per section 2.1.2-3) (around 4 hour growth dependent on the strain). It was washed in PBS before a 100x concentration to a final inoculum concentration of ~1x10(11) cfu/ml. For every 1ml of sera, 100µl of the concentrated bacterial solution was required for each adsorption cycle. To limit the dilution of serum, the *Salmonella* suspension was spun at 3300x g in a

micro centrifuge and the supernatant (around 50-70µl dependent on *Salmonella* strain) removed. The bacteria and serum were both pre-cooled to 4°C, before addition of serum direct to the *Salmonella* pellet, ensuring thorough re-suspension. This mixture was incubated for ~1 hour at 4°C per cycle. *Salmonellae* were removed between cycles by centrifuging at 4° at 3300 x g and the final cycle contained an additional filtration step through a 0.2µm syringe filter to ensure the sterility of the sera. Sera were stored at -80°C until use. Sera for use in experiments requiring functional complement were always freshly defrosted. Adsorptions ranged from a single cycle to five cycles and the conditions used are listed in the relevant sections. A minimum of two cycles were required in most circumstances to remove bactericidal activity.

Chapter 3

Work in this chapter has been published in the Journal of Immunology. The full manuscript is included in the appendix.

Serum bactericidal activity against invasive nontyphoidal *Salmonella* in the mouse

3.1 Introduction

An improved knowledge of the relevant protective mechanisms of immunity against *Salmonella* is required for the development of an effective vaccine against NTS. Much of the assessment of vaccine candidates needs to be performed *in vivo* and so animal models of infection are required to complement *in vitro* studies with human tissues. Since *S*. Typhimurium causes invasive disease in the mouse, this animal provides a useful model for the study of NTS infections *in vivo*. As such, it is important to understand the similarities and differences between the immune response to *Salmonella* in mice and men.

Antibodies are protective in the mouse model of salmonellosis (Cunningham et al., 2007, Gil-Cruz et al., 2009, McSorley and Jenkins, 2000, Xu et al., 1993), and in African children *Salmonella*-specific antibodies are associated with resistance to invasive NTS disease (MacLennan et al., 2008). It is likely that cell-mediated immunity controls infection within phagocytes and the humoral arm of immunity is important for controlling spread after release from infected cells (Mastroeni et al., 2009). This potential role of antibodies in limiting NTS spread within the body may mean that a vaccine stimulating an antibody response could offer protection, especially in preventing more serious systemic NTS infections such as bacteraemia. Good cell-mediated responses have proved difficult to elicit through vaccination and so this vaccine strategy could prove a successful approach.

Antibodies are thought to protect against *Salmonella* disease in two main ways: either in a cell-independent manner through complement-mediated bactericidal activity or through a cell-dependent mechanism involving the oxidative burst by opsonising bacteria for uptake and killing by phagocytic cells, increasing internalisation and killing (Menager et al., 2007). Antibodies have previously been shown to have an important role in opsonising *Salmonella* for phagocytosis and cellular killing in both C57BL/6 mice (Uppington et al., 2006) and humans (Gondwe et al., 2010). However, the ability of mouse serum to kill *Salmonella* using the cell-free complement-mediated mechanism described in humans is uncertain, particularly in view of the known poor function of mouse complement (Brown, 1943, Marcus et al., 1954). Clarification of this issue is important in harmonising *in vitro* and *in vivo* work and clinical evidence in order to assist with vaccine creation.

To test the hypothesis that mouse serum lacks cell-free complement-mediated killing activity against African NTS, we studied the bactericidal capacity of serum, antibodies and complement from C57BL/6, BALB/c, and C3H/HeNHsd (C3H) mice immunised with heat killed *S*. Typhimurium strains D23580 and SL1344 and live-attenuated strain SL3261.

3.2 Statistical Analysis

For SBA data, which were normally distributed, comparison of two groups was performed using Student's t test. One-way analysis of variance tests were used for comparisons of multiple groups. Haemolysis data displayed non-Gaussian distribution and thus statistical significance was tested using a Kruskal Wallis test. The D'Agostino-Pearson omnibus K2 normality test was used to help make an informed decision about the distribution of data.

3.3 Results

3.3.1 Mouse serum has preserved complement function

C57BL/6, BALB/c and C3H mice were found to have intact lytic activity against sensitised rabbit erythrocytes at a 1:20 dilution using the haemoglobin release assay to assess classical pathway complement activity (Fig. 3.1 A). There was no statistical difference in the degree of lysis between any groups including freshly-exsanguinated C57BL/6 serum (p=0.7799 Kruskal-Wallis) demonstrating that the processing and storage of the mouse sera had not adversely affected the quality of the complement. Heat inactivation absolved the activity of all sera, consistent with the assay being a determinant of haemolytic activity.

3.3.2 Mice mount an antibody response to heat-killed African *S.* Typhimurium strain D23580

Groups of six BALB/c, C3H or C57BL/6 mice were primed and boosted, after 14 days, with heat-killed African *S*. Typhimurium D23580 at a dose of 10^7 bacteria/ml or PBS (negative control group) via the i.p. route. An additional two groups of C57BL/6 mice were either immunised with heat killed laboratory strain *S*. Typhimurium SL1344 (as described above) or with one immunisation of $5x10^5$ cfu/ml of live attenuated vaccine strain SL1344, also via i.p. After 28 days, for immunisations with heat-killed bacteria, or 35 days for immunisations with live bacteria, mice were exsanguinated by cardiac puncture.

Serum was assessed for antibody response to *Salmonella*. While no anti-*Salmonella* antibodies were detected in serum from the control group, all mice immunised except two had produced IgG and IgM antibodies to *Salmonella* (Fig. 3.1 *B*). The levels were approximately 10-100 times the level found in bactericidal control volunteer human serum. Sera from mice producing no response were discarded.

Mouse serum bactericidal activity against African Salmonella

The lack of anti-*Salmonella* antibodies in control mouse serum demonstrated both that the mice had an absence of natural and cross-reactive antibodies to *Salmonella* and indicted that the assay was specific to anti-*Salmonella* antibodies. Antibody titres were similar regardless of whether the assay was performed using *S*. Typhimurium D23580, SL1344 or SL3261 (Fig. 3.2). However, the low statistical power of using three samples might produce a false negative. A significant difference between capture stains may be illustrative of antigenic differences between the strains and so increasing the number of samples could lead to a potentially important observation.

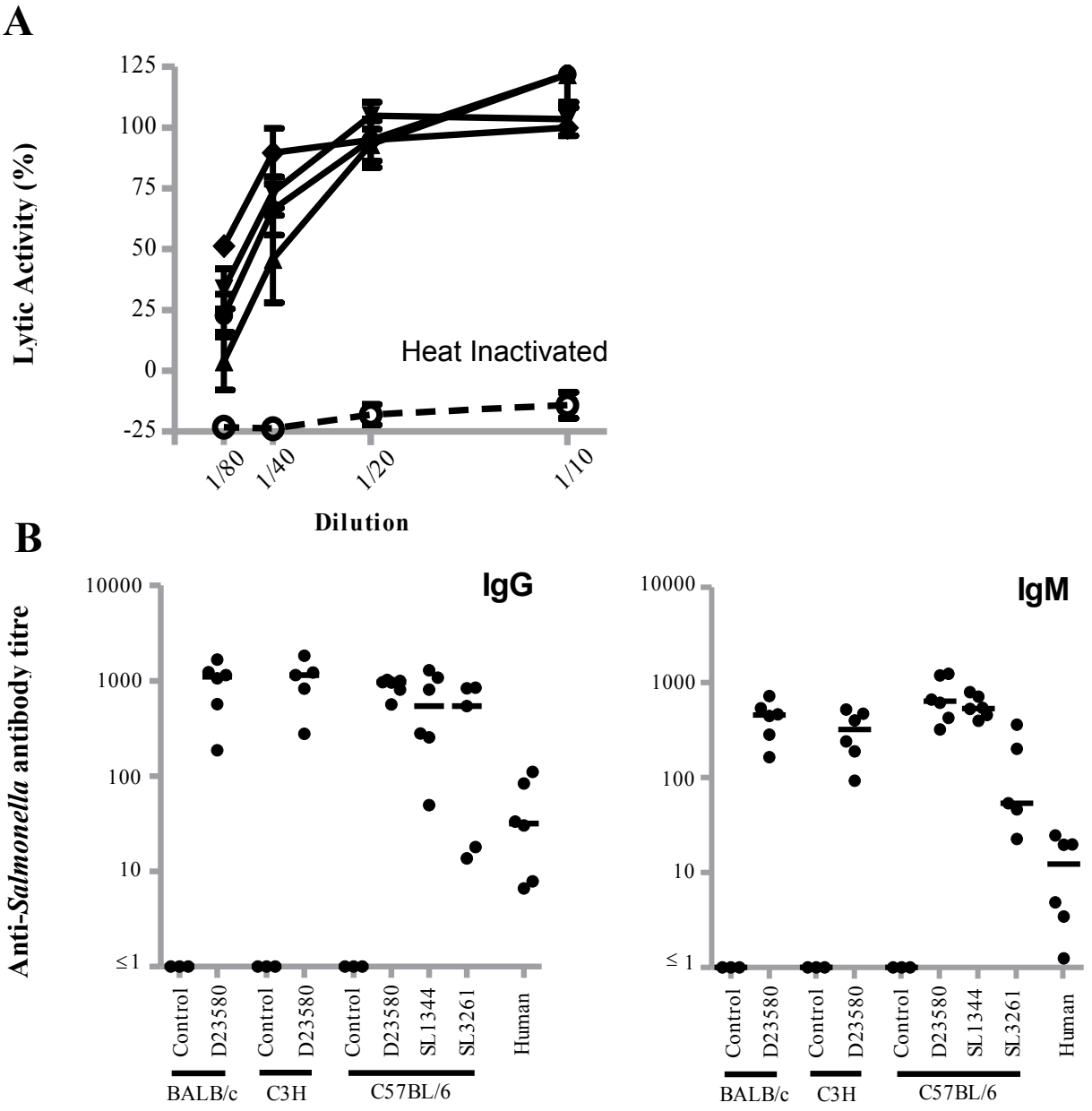


Figure 3.1: Antibody response and classical pathway complement activity in mouse sera following immunisation with African and laboratory strains of *S*. Typhimurium.

A, Lytic activity of mouse sera against rabbit erythrocytes sensitised with mouse anti-rabbit erythrocyte antiserum, measured using the haemoglobin-release assay. Fresh C57BL/6 mice sera (diamonds), frozen freshly-thawed mice sera: BALB/c (inverted triangles); C3H (triangles); C57BL/6 (filled circles), heat-inactivated C57BL/6 sera (empty circles). Data are means of experiments with sera from six mice ± SD.**B**, IgG and IgM titres of anti-*Salmonella* antibody at 28 days after i.p. immunisation with two doses of heat-killed *Salmonella,* immunisation with live-attenuated *Salmonella* and unimmunised. Immunisations for heat-killed *Salmonella* were with 10^7 heat-killed *S*. Typhimurium D23580 (African strain), SL1344 (laboratory strain). Immunisations for live-attenuated SL3261 were with $5x10^5$ *Salmonellae*. Groups of BALB/c, C3H and C57BL/6 mice received D23580 immunisations with groups of C57BL/6 mice also receiving SL1344 and SL3261. Antibody measured by flow cytometric analysis of antibody-binding to fixed *S*. Typhimurium D23580. Each point represents serum from one mouse. Horizontal bars indicate median values.

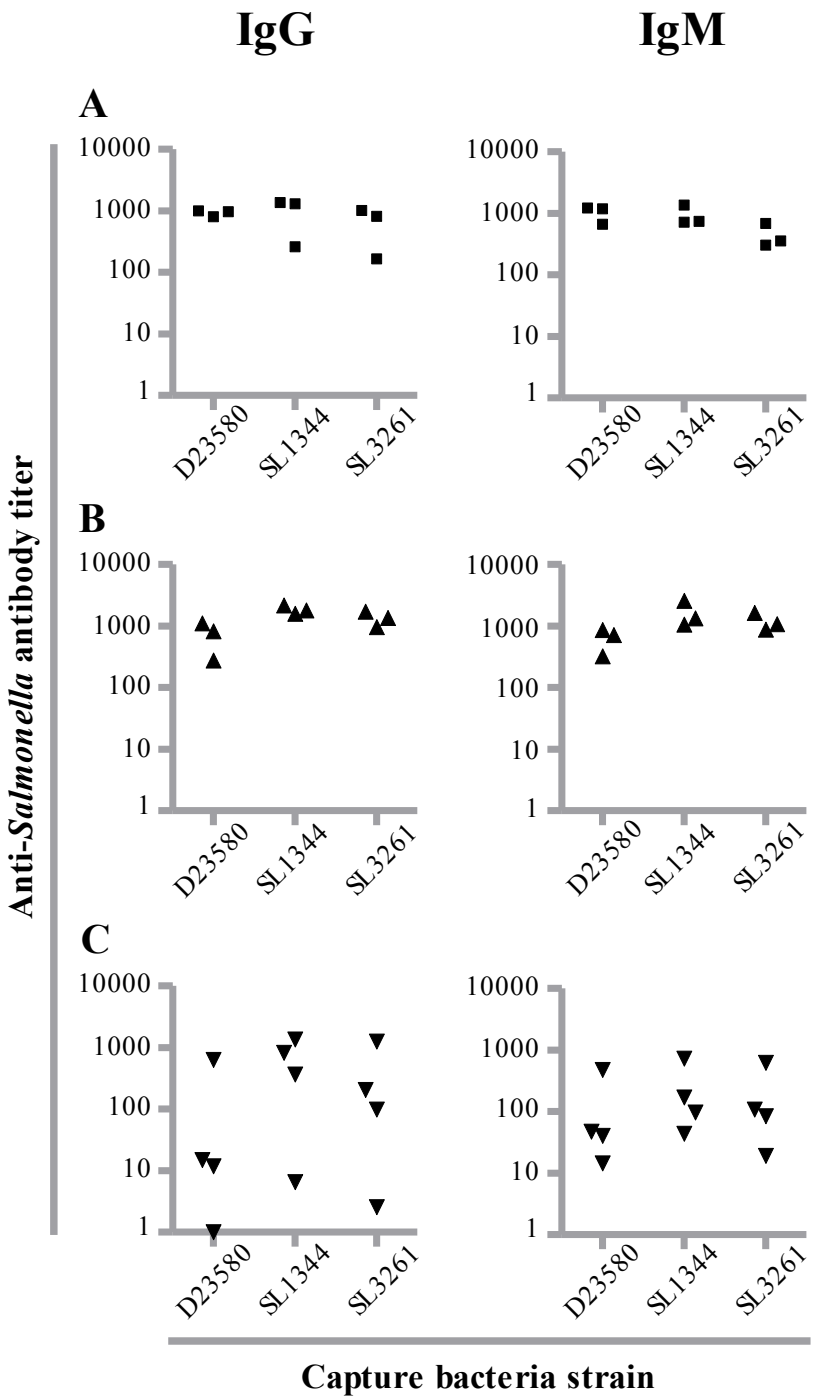


Figure 3.2: Antibody characterisation of immune mouse sera by flow cytometry using heterologous and homologous strains of S. Typhimurium.

Titres of anti-*Salmonella* antibody in diluted serum (1in10) from C57BL/6 mice immunised, as described in Fig 3.1, were measured using whole bacteria homologous and heterologous to the immunising strain. Sera from mice immunised with: **A** D23580 (squares), **B** SL1344 (triangles), or **C** SL3261 (inverted triangles). IgG is shown in the left panels and IgM in the right panels. Each point represents serum from one mouse.

3.3.3 Sera from *S.* Typhimurium-immunised mice lack bactericidal activity against Wild Type D23580

We next tested serum from immunised mice for ability to kill viable *S.* Typhimurium D23580 in log-growth phase. It has been reported that serum from African children, in the presence of specific IgG or IgM antibodies, is capable of antibody-dependent complement-mediated killing of D23580 (MacLennan et al., 2008).

Despite the presence of anti-*Salmonella* antibodies, sera from immunised C57BL/6 (Fig. 3.3A), BALB/c (Fig 3.3B) and C3H (Fig 3.3C) mice were unable to kill wild type D23580 *in vitro* over a three hour time course, regardless of the immunisation strategy. Unimmunised sera were also unable to effect a kill. The concentration of viable *Salmonella* increased by over one $\log_{10}$ in this period and the same result was obtained using serum from unimmunised mice. In contrast, African adult serum effected a two $\log_{10}$ kill over three hours (Fig 3.3D). The lack of killing of *Salmonella* by sera from all groups of mice compared with the killing of *Salmonella* by human serum was highly significant (Student's t test $P<0.0001$).

These findings could result from the antibodies, induced following immunisation of mice with D23580, lacking inherent bactericidal activity. The antibodies may be unable to facilitate the deposition of complement membrane attack complex on the *Salmonella* outer-membrane. Alternatively, mouse complement *per se* may lack the necessary bactericidal activity to kill *Salmonella*, as suggested by early experiments on mouse complement (Brown, 1943, Marcus et al., 1954).

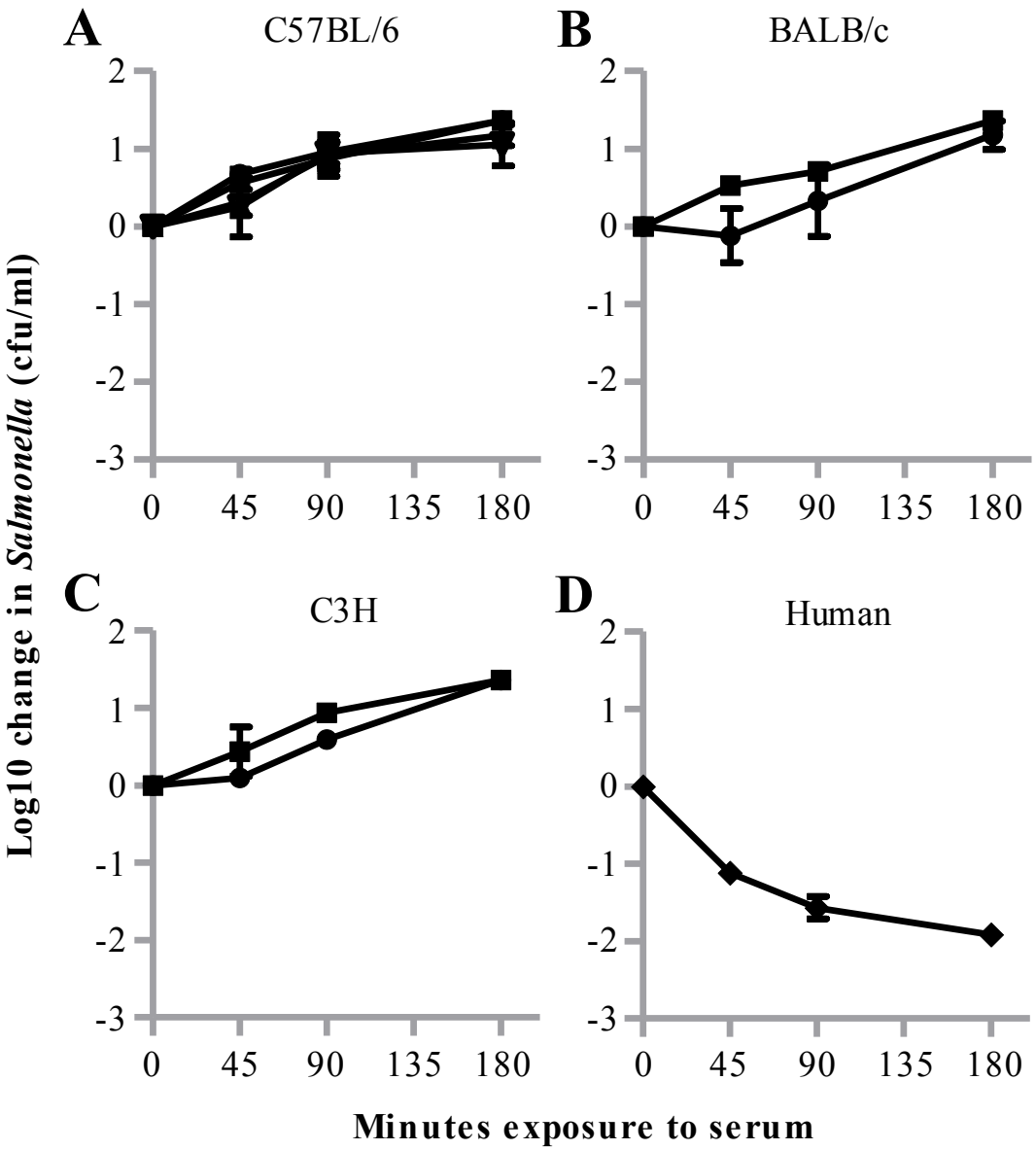


Figure 3.3: Absence of killing of African wild-type S. Typhimurium D23580 by neat mouse sera compared with human sera.

In vitro serum bactericidal assay with 10^6 *Salmonellae*/ml at 45, 90 and 180 minute time points using sera from **A** C57BL/6, **B** BALB/c, or **C** C3H mice immunised with D23580 (squares) and unimmunised mice (circles). **A** also shows data from C57BL/6 mice immunised with SL1344 (triangles) and SL3261 (inverted triangles). Data are means of experiments with sera from three mice ± SD. **D** Killing of wild type D23580 by control serum from an African adult. Significant difference in $\log_{10}$ change of *Salmonella* at 180 minutes between all mice and immunisations versus human control all $P<0.0001$(Student's t tests). No significant difference between mice species and immunisations $P<0.0855$ (One way ANOVA).

3.3.4 Sera from C57BL/6 mice immunised with *S.* Typhimurium strain D23580 also lack bactericidal activity against a *galE*⁻ knock-out rough mutant of D23580.

To explore the two concluding possibilities of section 3.4.3, we repeated the serum killing assays using a *galE*⁻ knock-out mutant of D23580 that is unable to synthesise the O-antigen polysaccharide of LPS (MacLennan et al., 2008). It has previously been shown that this rough mutant is susceptible to killing by serum from African children lacking *Salmonella*-specific antibodies by alternative pathway complement activity (MacLennan et al., 2008).

There was no difference between the results from these experiments with those using wild-type D23580. No *Salmonella*-killing occurred with sera from C57BL/6, BALB/c and C3H mice immunised with D23580, SL1344, and SL3261 or from unimmunised mice (Fig. 3.4 A-C). By contrast, African child serum lacking anti-*Salmonella* antibodies was able to effect a three $\log_{10}$ kill of *S*. Typhimurium D23580 (Fig. 3.4D). Again, the lack of killing of *Salmonella* by sera from all three groups of mice compared with the killing by human serum was highly significant (Student's t test $P<0.0001$). These findings strongly suggest that the lack of bactericidal activity is the result of poor mouse complement function, regardless of mouse strains, the deficiency affecting both classical (antibody-dependent) and alternative pathways.

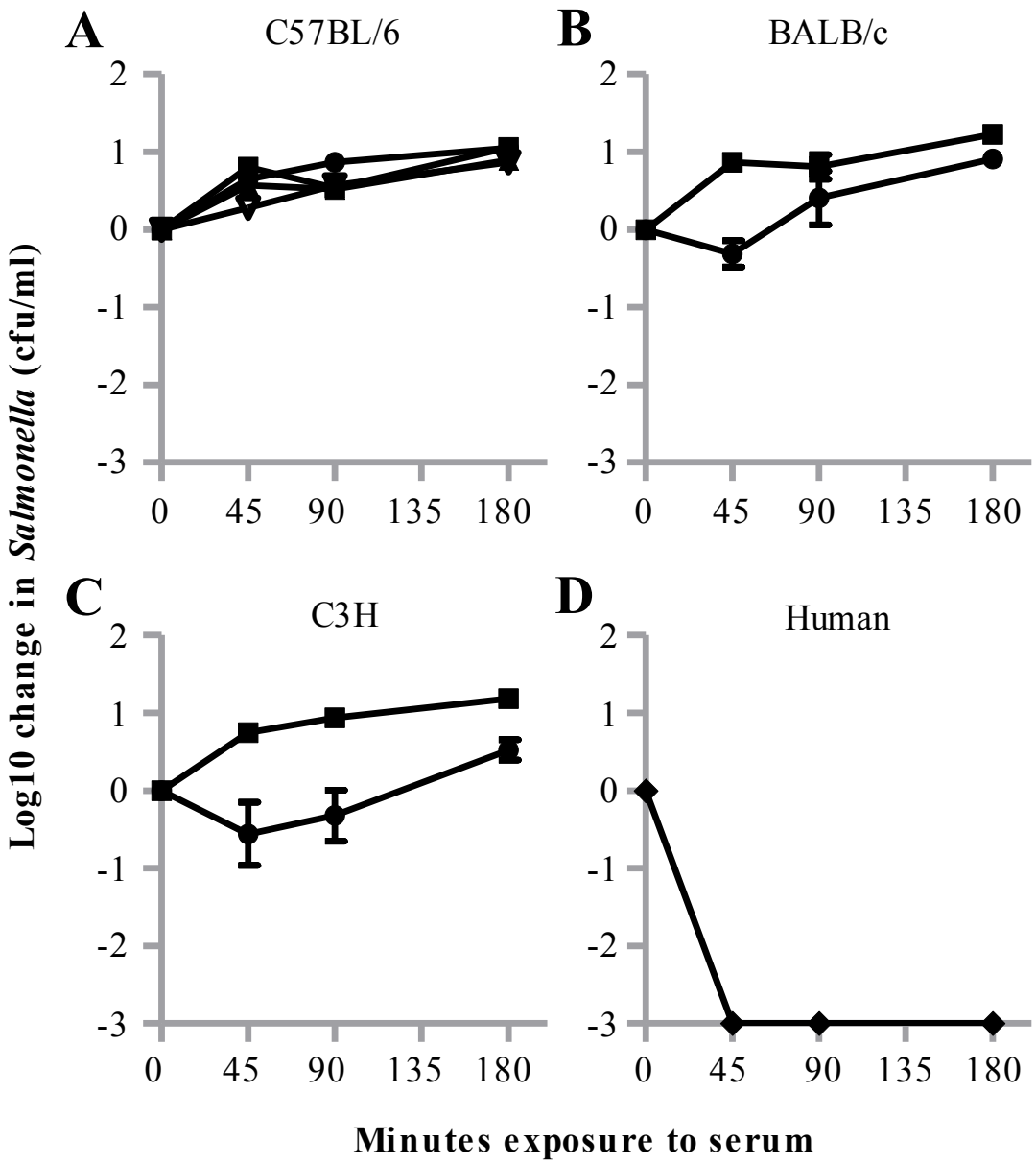


Figure 3.4: Absence of killing of African rough galE- mutant of S. Typhimurium D23580 by neat mouse sera compared with human sera.

In vitro serum bactericidal assay with 10^6 Salmonellae/ml at 45, 90 and 180 minute time points using sera from **A** C57BL/6, **B** BALB/c, or **C** C3H mice immunised with D23580 (squares) and unimmunised mice (circles). **A** also shows data from C57BL/6 mice immunised with SL1344 (triangles) and SL3261 (inverted triangles). Data are means of experiments with sera from three mice ± SD. **D** Killing of wild type D23580 by control serum from an African adult. Significant difference in $\log_{10}$ change of *Salmonella* at 180 minutes between all mice and immunisations versus human control all $P<0.0001$(Student's t tests). No significant difference between mice species and immunisations $P<0.1973$ (One way ANOVA).

3.3.5 Sera from *S.* Typhimurium immunised mice deposit reduced levels of C3 complement on *Salmonella* compared with human sera.

Since cleavage of C3 and deposition of C3b is the central event in all complement activating pathways, we next looked at deposition of C3 on the surface of wild type *S.* Typhimurium D23580 by sera from mice immunised with *Salmonella* using flow cytometry. While sera from unimmunised mice were unable to deposit C3 on *Salmonella*, sera from C57BL/6, BALB/c and C3H mice immunised with D23580, SL1344 and SL3261 were able to deposit C3 on the surface of D23580, though approximately one $\log_{10}$ less than the amount of C3 deposited by immune African serum (Student's t test $P<0.0001$) (Fig. 3.5).

Using confocal microscopy, qualitatively less C3 deposition was visualised on *Salmonella* incubated with mouse serum compared with human serum (Fig 3.6). These data therefore indicate deficiency in C3 deposition on *Salmonella* by mouse serum via the classical pathway relative to human immune serum. It has previously been shown that a minimum threshold level of C3 must be deposited on viable *Salmonellae* by serum from African children for antibody-dependent complement-mediated killing of *Salmonella* to proceed (MacLennan et al., 2008). Although the numerical value is likely to be dependent on assay conditions, it demonstrates the kinetics that might allow mice sera to deposit C3, but not kill the bacteria. The results also do not exclude the possibility of poor activity in the terminal pathway of complement.

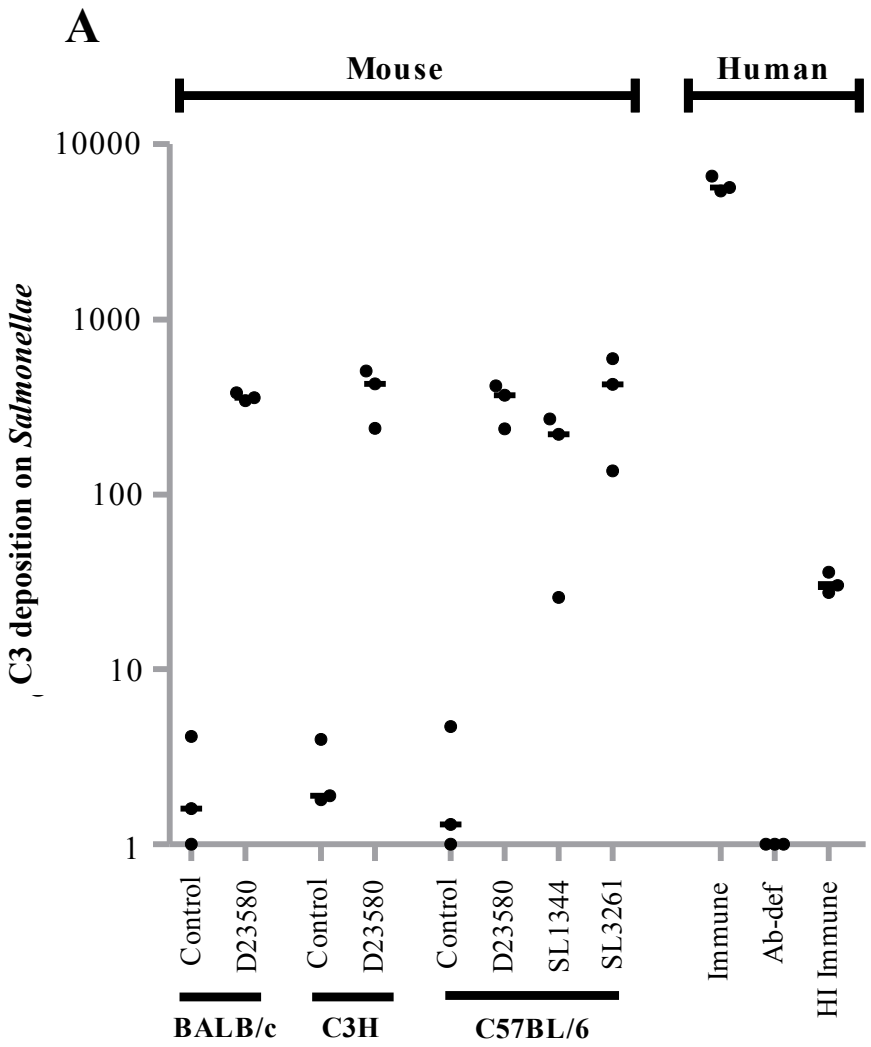


Figure 3.5: Deposition of complement C3 on *S.* Typhimurium D23580 by mouse sera compared with human sera measured by flow cytometry.

Titres of C3 deposited on *S.* Typhimurium D23580 following incubation in neat sera from BALB/c, C3H and C57BL/6 mice immunised with *S.* Typhimurium D23580, or unimmunised mice (control), C57BL/6 mice were also immunised with SL1344 and SL3261. Human panel shows serum ± heat inactivation (Immune/ HI Immune) from a healthy African adult as well as antibody deficient human serum (Ab-def). Each point corresponds to one experiment. Significant difference between C3 deposition by immunised mice sera and control human sera: all $P<0.0001$(Student's t tests). No significant difference between groups of immunised mice $P = 0.3496$ (One way ANOVA).

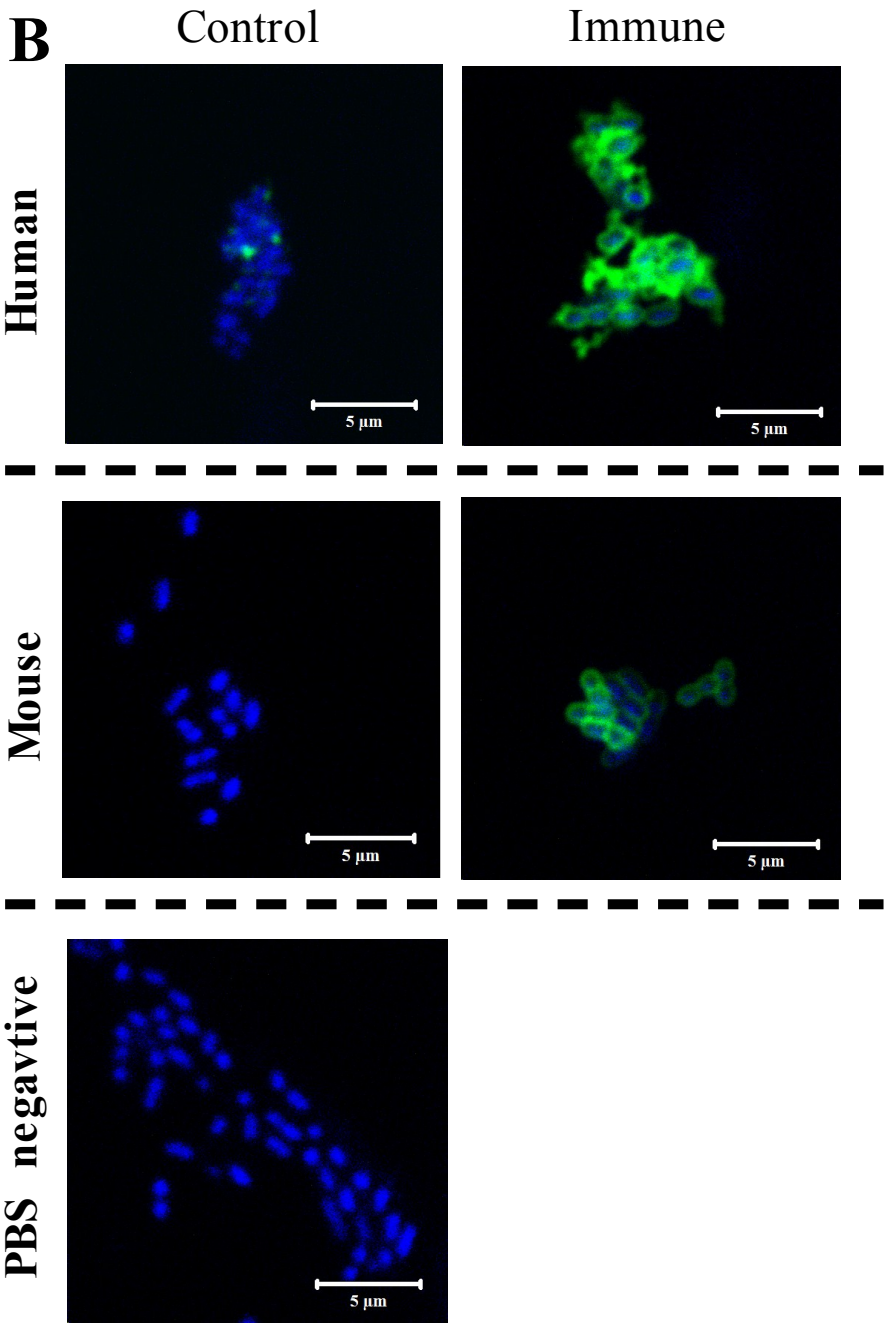


Figure 3.6: Confocal microscopy images of deposition of C3 component of complement on *S.* Typhimurium D23580 by mouse sera compared with human sera.

Confocal imaging of C3 deposited on *Salmonellae* using FITC conjugated anti-C3 antibody following incubation in various neat mice and human sera. Human top panel shows normal immune serum (left) and heat-inactivated serum (right). Mouse middle panel shows C57BL/6 D23580 immunised (left) and unimmunised (right). Bottom panel shows a negative control incubated with PBS instead of serum. All images acquired with identical settings.

3.3.6 Immune mouse sera supplemented with human bactericidal anti-*Salmonella* antibodies do not kill *S.* Typhimurium D23580

To check whether the absence of killing of *Salmonella* by mouse serum was due to a lack of bactericidal antibodies, we supplemented sera from unimmunised BALB/c, C3H and C57BL/6 mice with heat-inactivated serum from African adults containing antibodies to *Salmonella*. It has previously been shown that sera from African children that lack antibodies to *Salmonella* become bactericidal when exogenous anti-*Salmonella* antibodies are added (MacLennan et al., 2008). Addition of human anti-*Salmonella* antibodies did not correct the inability of mouse sera to kill D23580, the absence of killing of *Salmonella* being highly significant compared with the killing by fresh-frozen immune human serum (t test $P<0.0001$) (Fig. 3.7). This finding suggests that lack of killing of *Salmonella* by immune mouse serum is not due to a lack of antibody function, further implicating limited mouse complement function as the reason for absent bactericidal activity.

3.3.7 Immune mouse sera supplemented with human complement can kill *S.* Typhimurium D23580

To confirm that impaired mouse complement function was the reason for absent killing of *Salmonellae*, we supplemented C57BL/6, BALB/c and C3H mouse serum with human complement by mixing mouse serum with human serum lacking antibodies to *Salmonella*.

Sera from all mouse immunisation groups could now kill and/or prevent the growth of *Salmonella* at 10- or 100-fold dilutions with PBS (Fig. 3.8). This killing was statistically significant compared with both the lack of killing by the anti-*Salmonella* antibody-deficient human serum alone and immunised mouse serum alone (Student's t test $p<0.0001$ for serum from mice immunised with heat-killed bacteria and $p<0.05$ for serum from mice immunised with live-attenuated bacteria). Killing was greater with serum from

mice immunised with heat-killed *Salmonellae* compared with serum from mice immunised with live-attenuated bacteria. This experiment confirms that absent killing of *Salmonella* by mouse sera is the result of limited mouse complement function and that the mouse antibodies against *Salmonella* have potential bactericidal activity leading to complement-mediated killing of *Salmonella* provided a suitable source of exogenous complement is available.

Though the sera from live attenuated immunisation (Table 3.1) could not achieve killing comparable to that observed with heat killed bacteria when given a functional complement source, we were later able to demonstrate such a level of killing when live attenuated immunised sera from an earlier time point than day 35 was supplemented with exogenous complement. C57BL/6 mice were subjected to the same single dose of D23580, SL1344 or live attenuated SL3261 bacteria as before, but exsanguinated on day 14 (as opposed to day 28 or 35).

The 14-day sera were also unable to kill *Salmonella* (Fig 3.9C). However, when given a source of functional complement, all sera (including the live-attenuated immunised) effected a ~2 $\log_{10}$ kill (Fig 3.9B). This killing was statistically significant compared with both the lack of killing by the anti-*Salmonella* antibody-deficient human serum alone and immunised mouse serum alone (Student's t test $P<0.001$). Importantly, it demonstrated that immunisation with live-attenuated *Salmonella* could generate bactericidal antibodies of the same potency as heat-killed immunisation. Antibody levels were tested and revealed that the day 14 sera had much lower levels of anti-*Salmonella* IgG (Fig 3.9A) and IgM (Fig 3.9B) than day 28/35, (Fig 3.1B). This was reflected by the fact these sera killed without dilution in comparison to the day 28/35 sera which required a prior dilution of 1in10 or 1 in100. Whilst a prozone-like inhibitory effect resulting, at least in part, from an excess in antibody levels is well known in *in vitro* serum bactericidal systems

(Muschel et al., 1969, MacLennan et al., 2010) the effect we encountered likely involves more factors than quantity alone and the reasoning is covered in the discussion (section 3.6) of this chapter.

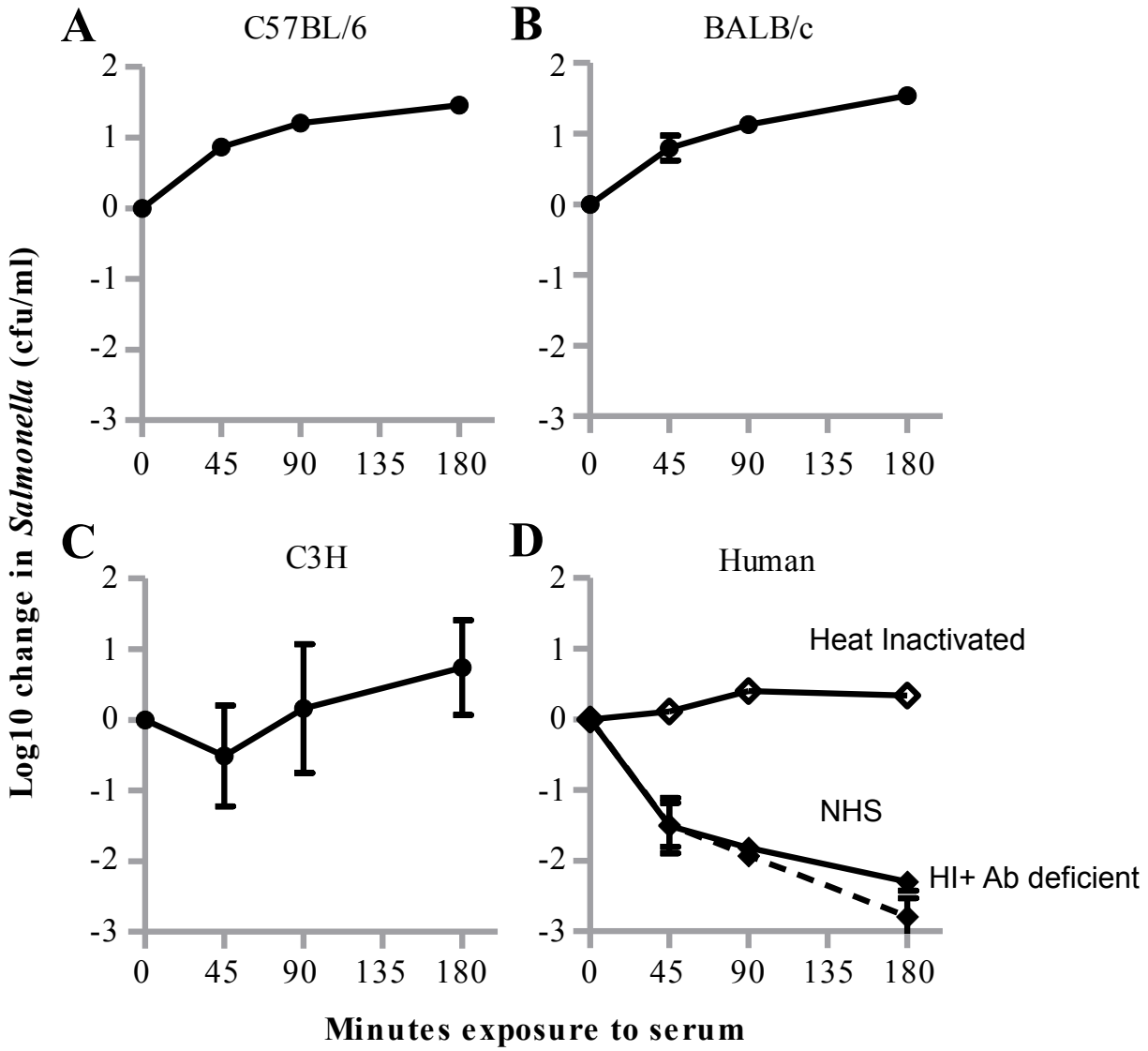


Figure 3.7: Absence of killing of *S.* Typhimurium D23580 following supplementation of mouse sera with human anti-*Salmonella* antibody.

In vitro serum bactericidal assay with 10^6 *Salmonellae*/ml at 45, 90 and 180 minute time points using sera from A C57BL/6, B BALB/c, or C C3H unimmunised mice all supplemented with heat-inactivated immune human serum containing both anti-*Salmonella* IgG and IgM. Data are mean of experiments with sera from three mice ± SD. D Lack of killing of wild type D23580 by heat-inactivated human control serum (hollow diamonds). Killing by control human serum (diamonds) and heat-inactivated human control serum with and without human complement added (diamonds with dashed line and open triangles). Significant difference in $\log_{10}$ change of *Salmonella* at 180 minutes between all mice and immunisations and heat-inactivated human serum versus normal human serum control all $P<0.0005$ (Student's t tests). No significant difference between mice groups $P=0.0857$ (One way ANOVA).

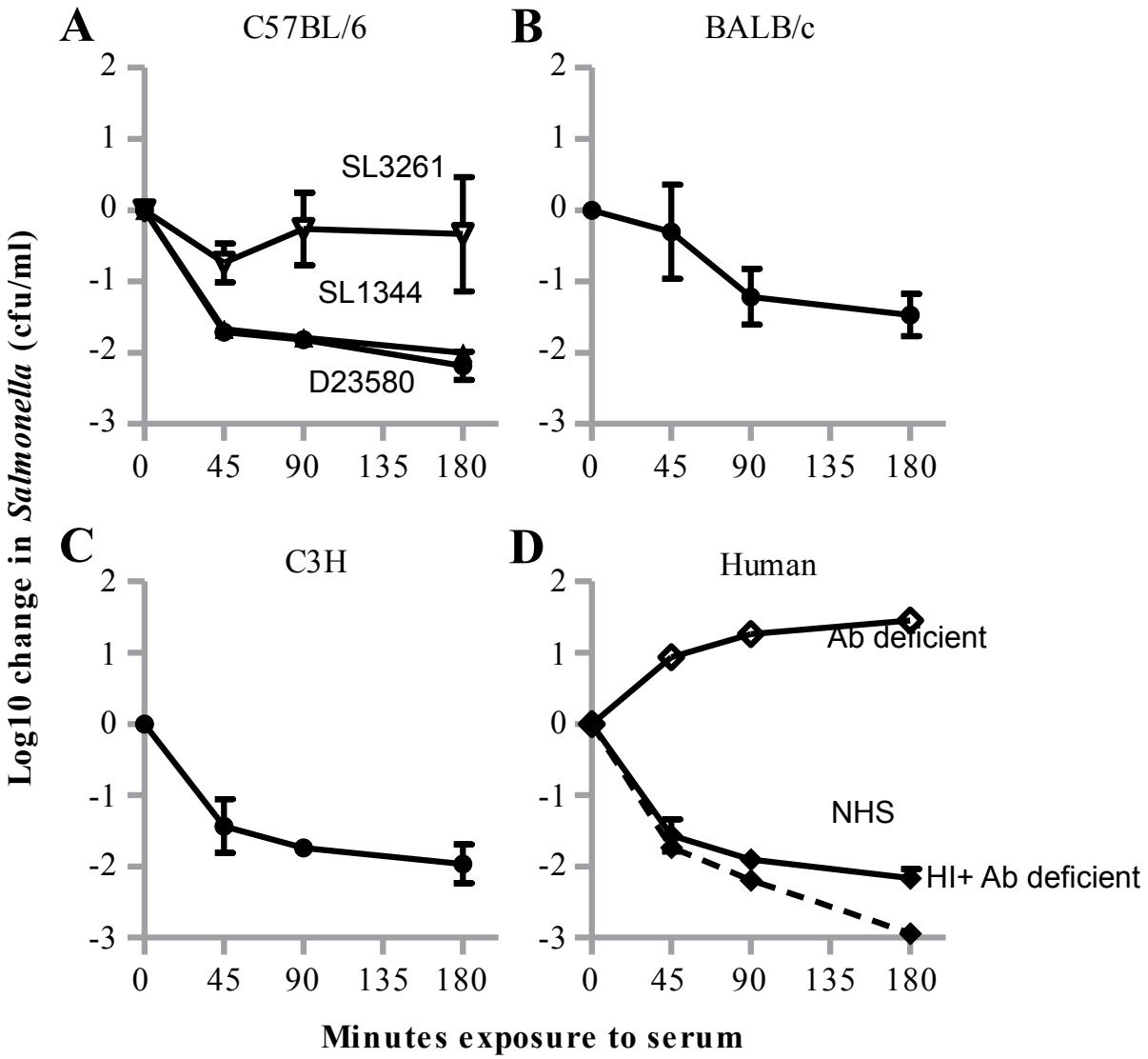


Figure 3.8: Killing of *S.* Typhimurium D23580 following supplementation of mouse sera with human complement.

In vitro serum bactericidal assay with 10^6 *Salmonellae*/ml at 45, 90 and 180 minute time points using sera from **A** C57BL/6, **B** BALB/c, or **C** C3H mice immunised with D23580 (squares) **A** also shows data from C57BL/6 mice immunised with SL1344 (triangles) and SL3261 (inverted triangles) all supplemented with human complement. Data are mean of experiments with sera from three mice ± SD. **D** Lack of killing of wild type D23580 by antibody-deficient human serum (hollow diamonds), killing by control human serum (diamonds) and antibody-deficient human serum supplemented with heat-inactivated immune human serum. Significant difference in $\log_{10}$ change of *Salmonella* at 180 minutes between all mice and immunisations and equivalent mouse sera without supplemented human complement (shown in figure 3.3) C3H D23580 and C57BL/6 D23590 and SL1344 $P<0.0001$, BALB/c D23580 $P<0.005$ and C57BL/6 $P<0.05$ (Student's t tests).

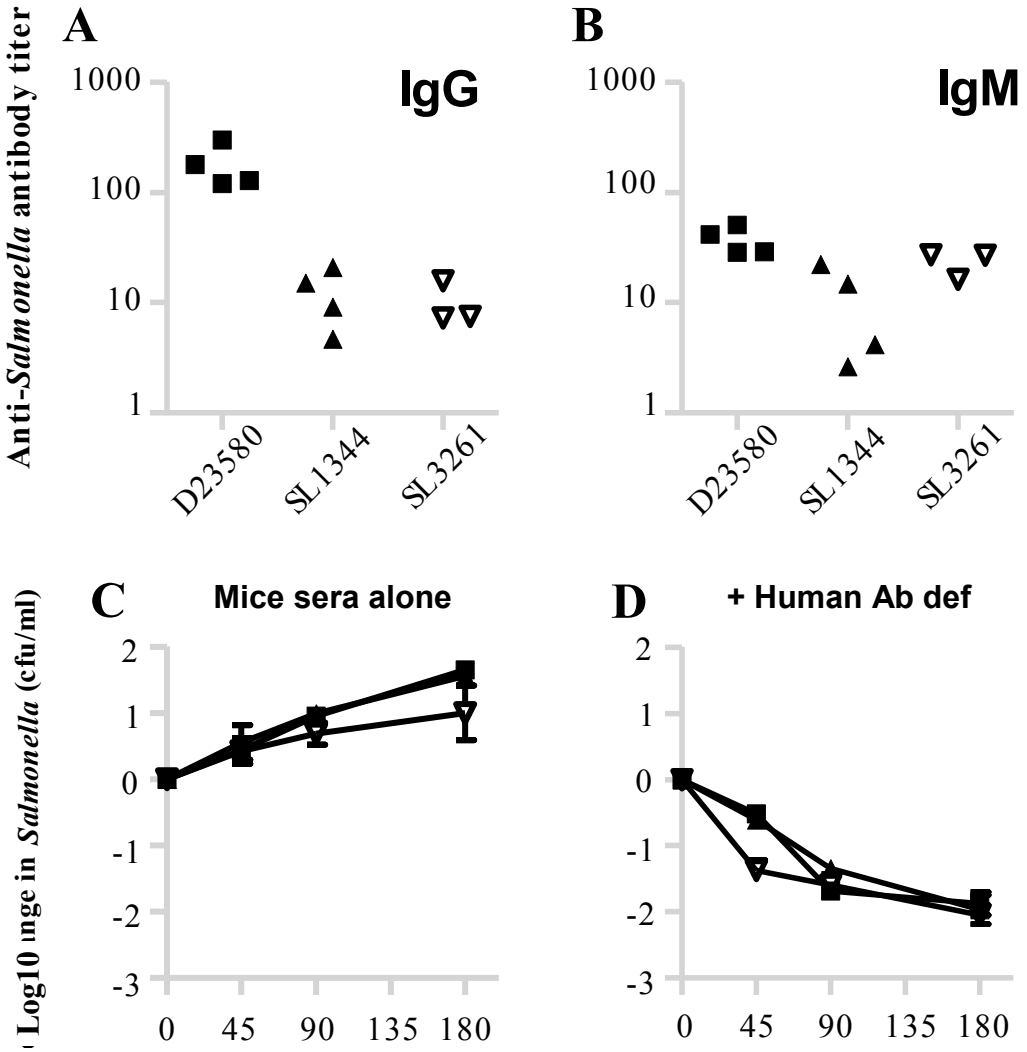


Figure 3.9: **Antibody assay and wild type SBA experiments repeated with sera from mice immunised once and exsanguinated on day 14**

A, IgG and **B**, IgM titres of anti-*Salmonella* antibody at 14 days after i.p. immunisation with heat-killed or live-attenuated *Salmonella* and unimmunised. Immunisations for heat-killed *Salmonella* were with 10^7 heat-killed *S*. Typhimurium D23580 (African strain), SL1344 (laboratory strain). Immunisations for live-attenuated SL3261 were with $5x10^5$ *Salmonellae*. Antibody measured by flow cytometric analysis of antibody-binding to fixed *S*. Typhimurium D23580. Each point represents serum from one mouse. **C**, *In vitro* serum bactericidal assay with 10^6 *Salmonellae*/ml at 45, 90 and 180 minute time points using neat sera from C57BL/6 mice immunised with D23580 (squares), SL1344 (triangles) or SL3261 (inverted triangles), D shows the same sera supplemented with human complement. Data are mean of experiments with sera from four mice ± SD. Significant difference in $\log_{10}$ change of *Salmonella* at 180 minutes between all mice and immunisations plus complement versus homologous sera without additional complement, all $P<0.0001$(Student's t tests).

3.4 Discussion

The main finding from this study is that although BALB/c, C3H and C57BL/6 mice mount both an IgG and IgM antibody response to African invasive *S*. Typhimurium strain D23580, serum from immunised mice lacks bactericidal activity against *Salmonella*. This contrasts with the one to three $\log_{10}$ killing of D23580 which is effected by African adult and child serum containing anti-*Salmonella* antibodies. Serum from African children does not kill *Salmonella* when it lacks specific antibodies and this was the basis for previously describing bactericidal antibodies in Africans as an effector of humoral immunity against NTS (MacLennan et al., 2008).

S. Typhimurium D23580 was chosen for this study because it is a typical invasive strain of NTS from Africa, representative of over 90% of NTS strains isolated from bacteraemic individuals in Malawi since 2002, is sensitive to killing by bactericidal antibodies and has been sequenced at the Wellcome Trust Sanger Institute (Kingsley et al., 2009, Institute, 2009, MacLennan et al., 2008). Experiments using S. Typhimurium laboratory diarrhogenic strains SL1344 and, its aromatic amino acid auxotrophic mutant, SL3261 were included to demonstrate that the lack of bactericidal activity was not restricted to African invasive NTS and was independent of whether the immunisation was with heat-killed wild-type or live-attenuated *Salmonellae.* Our data also confirmed an absence of bactericidal activity in these immunised sera.

C57BL/6 mice were chosen because mechanisms of immunity to *Salmonella* have been studied extensively in these mice (Cunningham et al., 2007, Gil-Cruz et al., 2009, Uppington et al., 2006) and the antibody response to vaccination with *S*. Typhimurium in C57BL/6 mice has been shown to protect against subsequent infection with *S*. Typhimurium (Cunningham et al., 2007, Gil-Cruz et al., 2009). The experiments were also performed with BALB/c and C3H mice to show that deficiency in serum bactericidal

activity against *Salmonellae* is not restricted to one inbred strain of mice. Our findings suggest that the protective effect of mouse anti-*Salmonella* antibodies in mice is not mediated by cell-independent bactericidal activity in any of these strains.

Of the mouse strains used, C57BL/6 and BALB/c are broadly classed as 'extremely susceptible' to *Salmonella* infection (Lam-Yuk-Tseung and Gros, 2003) and C3H/HeN is classed as 'resistant' (Roy and Malo, 2002). These classifications are somewhat arbitrary, being based on resistance to specific dosage and inoculation routes, but they represent relative resistance. This variation is likely to largely be due genetic factors such *SLC11A1* alleles (formerly *Nramp1*), which controls replication of intracellular *Salmonella* by altering the intravacuolar environment of the phagosome (Cuellar-Mata et al., 2002). Natural resistance-associated macrophage proteins (Nramp) are multispecific symporters facilitating proton-dependent import of divalent metals. SLC11A1 restricts microbial access to essential micro-nutrients such as iron and manganese within professional phagosomes. SlC11A1 has many pleiotropic effects on macrophage activation, including regulation of the CXC chemokine KC, interleukin-1β (IL-1β), inducible nitric oxide synthase (iNOS), major histocompatibility complex (MHC) class II molecules, tumour necrosis factor α (TNFα), and nitric oxide (NO) release, L-arginine flux, oxidative burst and tumoricidal as well as antimicrobial activity (Blackwell et al., 2001). The overall effect of these functions is that SLC11A1 is crucial to immunity against *Salmonella*. BALB/c and C57BL/6 are $Nramp^s$ and C3H/HeN is $Nramp^r$ (Malo et al., 1994). The C3H strain used, C3H/HeNHsd like C57BL/6 and BALB/c, has a normal LPS response (HarlanLabs, 2011) and is not to be confused with the LPS-hyporesponsive C3H/HeJ strain (Sultzer, 1968, Roy and Malo, 2002). The mice also vary in major histocompatibility complex haplotypes, which have been shown to alter response to *Salmonella* infection (Hormaeche et al., 1985, Nauciel et al., 1988). We found that these differences did not affect either the IgG or IgM responses to

immunisation with heat-killed D23580 as there was no statistically significant difference between either antibody titre for the three mouse groups (IgG and IgM both p= 0.3679 Kruskal Wallis test).

The *galE*⁻ rough mutant of *S*. Typhimurium D23580 is sensitive to killing via the alternative pathway of complement by human serum lacking anti-*Salmonella* antibodies (MacLennan et al., 2008). Absent killing of the *galE*⁻ mutant by all mouse sera therefore implicates limited complement function rather than absence of bactericidal antibodies themselves as the reason for lack of killing with wild type D23580. Impaired complement function in mouse serum was first described over sixty years ago (Brown, 1943). Indeed, our finding that serum from immunised mice supplemented with human complement can effect cell-free killing indicates that the antibodies generated following immunisation with D23580, SL1344 or SL3261 is capable of directing complement deposition to the *Salmonella* surface at locations that enable membrane attack complex to kill the bacteria (MacLennan et al., 2008).

Our data suggest that complement from BALB/c, C3H and C57BL/6 mice is defective in killing D23580 at multiple points in the complement pathway. Because it has been previously shown that deposition of complement and killing of wild type D23580 is antibody-dependent and does not proceed via alternative or mannose-binding lectin pathways (MacLennan et al., 2008), the low titres of C3 deposition on *Salmonella* seen with serum from immunised mice compared with human serum indicate that classical pathway activity is reduced compared to human serum. The lack of killing of the D23580 *galE*⁻ mutant also indicates defective alternative pathway activity and indicates that the terminal pathway has limited activity.

Nevertheless, functional classical pathway complement activity was detected against sensitised rabbit erythrocytes in the haemoglobin release assay. We employed this assay to ensure that lack of complement function against *Salmonella* was not merely an artefact of sub-optimal preparation and storage of sera. These finding suggest that differences exist between the susceptibility of red blood cells and *Salmonella* to the lytic/bactericidal effects of complement with *Salmonella* being more resistant than red cells. Complement that does not insert into the *Salmonella* outer membrane can be shed from the surface of *S*. Minnesota (Joiner et al., 1982a, Joiner et al., 1982b) and various molecules on the *Salmonella* surface including those encoded by the *rck* (Heffernan et al., 1992), *traT* (Rhen and Sukupolvi, 1988) and *pgtE* (Ramu et al., 2007) genes confer some degree of resistance to complement-mediated killing. Such mechanisms are likely to raise the threshold of complement-deposition required for bactericidal activity against *Salmonella* to proceed. It has been previously shown that the *rck* gene is encoded in the virulence plasmid of D23580 and that C9 is necessary for serum bactericidal activity against D23580 (MacLennan et al., 2008). In contrast, several studies on complement-mediated haemolysis have demonstrated that this can proceed in the absence of C9 by limited perturbation of the membrane by C8 (Stolfi, 1968, Biesecker and Muller-Eberhard, 1980, Dankert and Esser, 1985).

With mouse complement lacking bactericidal activity against *Salmonella*, it is likely that the protective effect of anti-*Salmonella* antibodies in BALB/c, C3H and C57BL/6 mice is mediated by opsonisation facilitating phagocytosis, respiratory burst and intracellular killing of *Salmonella* by neutrophils, monocytes and macrophages. The importance of anti-*Salmonella* antibodies for these modalities of cell-mediated immunity has been previously demonstrated using serum and cells from C57BL/6 mice (Uppington et al., 2006). Other work in men (*in vitro*) and mice has indicated that opsonisation of *Salmonella* with C3 is required for optimal intracellular killing (Gondwe et al., 2010,

Liang-Takasaki et al., 1983, Saxen et al., 1987, Nakano et al., 1995) and work with C1q-deficient mice has shown a role for the classical pathway of complement in protecting against *S*. Typhimurium (Warren et al., 2002). It has been found that a minimum threshold level of C3 must be deposited on viable *Salmonellae* by serum from African children for antibody-dependent complement-mediated killing of *Salmonella* to proceed (MacLennan et al., 2008). Although the numerical value is likely to be dependent on assay conditions it demonstrates the kinetics that might allow mice sera to deposit C3 (Fig 3.5), but not kill the bacteria (Fig 3.3).

Though *Salmonellae* are facultative intracellular bacteria, it has been hypothesised that the high case-fatality rate among young African children is due to extracellular bacterial growth unchecked by bactericidal antibodies (MacLennan et al., 2008). Since our findings indicate a lack of bactericidal activity in mouse serum against *Salmonella*, this could contribute to the sensitivity of BALB/c, C3H and C57BL/6 mice to infection with *S*. Typhimurium. Different mouse strains differ in their ability to handle infection with *Salmonella* and this may be due to a variety of reasons including differences in complement function, but also, importantly, the innate genetic susceptibility of each strain to *Salmonella* as discussed earlier. Variations in complement function between strains can be complex. For example, A/J mice are more effective at depositing C3b on *Salmonella* than C57BL/6 mice, but are deficient in the C5 component of complement, hence preventing complement-mediated bactericidal activity against *Salmonella* in such mice (Nakano et al., 1995).

The serum bactericidal assays used in this study where the same as used in our prior published human studies (Maclennan, 2010, MacLennan et al., 2008) in order to serve as the best comparison of relative bactericidal activity. They were designed to fulfil the recommendations of Taylor in his comprehensive review of serum bactericidal activity

(Taylor, 1983). The term 'relative bactericidal activity' is important to stress as the susceptibility of *Salmonellae* to serum, particularly in *in vitro* systems, is multifactorial. It is influenced by bacterial factors, such as the growth phase and growth medium; and also experimental factors, such as serum concentration, bacterial concentration, time of incubation, and washing of bacteria. *Salmonella* are exposed to a large range of environments and undergo multiple changes of environment during an infection, for example: from external environment to human gastro-intestinal tract; from intracellular in M cells or macrophages to extracellular in blood. Hence variance of serum resistance with growth conditions and environment means that replication of the serum resistance of the bacteria *in vivo* is complex. The ratio of components in a SBA is also important: excess bacteria can inhibit net killing, probably through exhaustion of complement, but also, too little bacteria with an excess of antibodies can inhibit killing. This effect, named the Neisser-Wechsberg (antibody prozone) phenomenon (Muschel et al., 1969) is most prominent in immunised sera which have high titres of antibody. It has been extensively studied through the early and middle 20th century but as yet no definitive explanation has been described.

In this study we observed the Neisser-Wechsberg effect in primed and boosted heat-killed *Salmonella* immunised mice and the day 35 live-attenuated immunised mice sera. Both had to be diluted between 10 and 100 times in PBS in order to exert a bactericidal effect. It has been reported that the artefact can be removed with addition of extra complement (Muschel et al., 1969) so it is thought unlikely to lead to inhibition of killing *in vivo*. Interestingly, we found difficulty in determining a dilution of day 35 live-attenuated immunised antibody that was bactericidal, despite it having similar antibody titres to the other immunised sera. This could indicate that the antibody response, in relation to antibody class, subclass, avidity or antigen target holds the explanation of the mechanism of the inhibitory prozone effect. Alternatively, it may be unlinked to any

prozone effect, but demonstrative of an antibody response that is basically just less bactericidal.

Considering the effect of conditions on bacterial serum resistance it may appear difficult to conclude definitively from *in vitro* work that mouse serum is unlikely to have bactericidal activity *in vivo*, particularly considering the concentration of bacteria we used was around 10 times higher than is usually reported in *Salmonella* bacteraemia in mice. However, crucially, there are considerations that help to give confidence in such a conclusion. Firstly, the bacteria we used for SBAs were grown rapidly in LB media and in log phase and washed, conditions which produce the most serum susceptible inoculums. Bacteria growing at sub-maximal rates *in vivo* are likely to be more resistant (Taylor 1978). Secondly, the format of the assay maximised potential bactericidal activity by using undiluted serum. Finally and perhaps most convincingly, the failure to kill exquisitely sensitive galE$^-$ shows a huge discrepancy in bactericidal activity with human sera, which can kill an inoculum 100 fold greater inside 15 minutes. These observations as well as sparse published data using different SBA systems (Rosenberg and Tachibana, 1962, Brown, 1943, Collins, 1967) allow us to conclude with a high degree of confidence that mouse serum (specifically complement) has much weaker bactericidal activity against *S.* Typhimurium than human serum which implies an absent or minimal role for cell-free complement-dependent killing in mice. Whilst this result is not unexpected considering the dogma concerning the inefficiency of the mouse complement system, there are a lack of published data about this and so we chose to investigate the phenomenon more fully. This is especially true as the striking bactericidal effects of human serum against *Salmonella in vitro* suggest this it is a modality of immunity that is likely to at least contribute to immunity against *Salmonella in vivo* in humans. According to Taylor in his review of the bactericidal effect: ‘The weight of published evidence indicates that resistance to the bactericidal action of antibody-

complement systems is likely to play a role in the pathogenesis of at least some infections due to gram-negative bacteria' (Taylor, 1983).

3.5 Conclusion

C57BL/6, BALB/c and C3H mice immunised with heat-killed *S.* Typhimurium strains D23580 (African invasive isolate), SL1344, and live-attenuated strain SL3261, produced a *Salmonella*-specific antibody response. Sera from these mice deposited reduced levels of C3 on *Salmonella* compared with human sera and were unable to kill both wild type and *galE*¯ rough mutant of D23580 indicating absent cell-free killing via classical and alternative complement pathways. Supplementing immune mouse sera with human complement enabled killing of *Salmonella*, while addition of human anti-*Salmonella* antibodies to immune mouse sera had no effect. These findings indicate that mouse serum cannot effect cell-free complement-dependent killing of *Salmonella*, because of reduced mouse complement ability to kill these bacteria compared with human complement. This difference in antibody-dependent immunity to *Salmonella* in mice and human clinical disease must be considered when applying findings from the mouse model of *Salmonella* disease and vaccination responses to man.

Chapter 4

The influence of antibody-dependent complement-mediated killing and growth-phase of bacteria on the *in vitro* kinetics of immunity against invasive African *Salmonella.*

4.1 Introduction

In healthy adults in developed countries salmonellosis is usually a self-limiting GI infection. However, deficiencies or immaturity in certain aspects of immunity can lead to severe *Salmonella* disease. A lack of specific-antibodies in African infants is associated with fatal invasive *Salmonella* disease (MacLennan et al., 2008). In contrast, individuals with defects IFNγ production pathways, which affect killing of *Salmonella* within phagocytes, typically develop non-fatal, though severe, recurrent focal infection in lymph nodes despite presence of specific antibodies (Lammas et al., 2002, MacLennan et al., 2004) and unpublished observations).

Proliferation, spread and distribution of *Salmonella* within a host is heterogeneous and not fully understood. Current models of NTS infection from experiments in mice suggest that upon infection, a proportion of *Salmonellae* gain access to the blood where complement-mediated clearance can proceed (Warren et al., 2002). In addition, spread from infected to uninfected cells during bacterial growth is likely to occur mainly by necrotic cell death and release of individual bacteria into the extracellular space (Mastroeni et al., 2009). Hence, a proportion of *Salmonellae* are likely to be, at least transiently, present in the extracellular space or blood. Here they are exposed to humoral components of immunity. Antibody-dependent complement-mediated killing of *Salmonella* is likely to be minimal in mice (Siggins et al., 2011). Though, in humans there is a powerful bactericidal complement activity in the presence of specific antibodies *in*

vitro (Taylor, 1983, MacLennan et al., 2008). As the majority of work on the spread of *Salmonella* within a host has been performed in mouse models, the potential contribution of cell-free complement killing to the dynamics of infection may have been under-estimated. Another complication in determining the impact of antibody-dependent complement-mediated killing of *Salmonella* is that the growth conditions and phase of bacteria have can alter potential virulence factors. In *Salmonellae* growth phase and pre-exposure to serum has been reported to modify LPS structure and alter resistance to serum-killing (Bravo et al., 2008, Murray et al., 2005).

Here we have performed serum bactericidal, phagocytosis, and antibody and complement bacterial-binding assays with human blood and sera to test the hypothesis that *in vitro* phagocytosis of *S*. Typhimurium by neutrophils and monocytes in blood occurs before all *Salmonellae* are killed by cell-free serum-killing in a comparable *in vitro* system. By comparing the relative timing of the deposition of humoral factors, serum-killing and phagocytosis uptake, we propose a model for the influence of antibody-dependent complement-mediated killing upon the spread of invasive African *S*. Typhimurium in humans. Because *in vivo* conditions and growth phase of *Salmonella* during infection probably vary and are difficult to predict we have tested the serum-susceptibility of an African invasive isolates of *S*. Typhimurium over a range of growth conditions to confirm the hypothesis that it can also vary in resistance to killing by human serum.

4.2 Statistical Analysis

One-way analysis of variance tests were used for comparisons of multiple groups. Pearson correlation coefficient was used to determine correlation. The D'Agostino

Pearson omnibus K2 normality test was used to help make an informed decision about the distribution of data.

4.3 Results

4.3.1 Kinetics of deposition of complement and binding of IgG to *S.* Typhimurium

All sera used were from healthy adult donors and had specific antibodies and bactericidal activity against *S*. Typhimurium. Maximal IgG deposition varied between donors (One way ANOVA $p > 0.05$). IgG deposition on *S*. Typhimurium was rapid for all sera and reached peak levels within 15 seconds (Fig. 4.1 *A*). Opsonisation of *Salmonellae* by antibodies alone is insufficient to effect optimal phagocytosis in humans and complement is also required for maximum uptake and killing efficiency (Gondwe et al., 2010). Deposition of the most abundant complement opsonin, C3, was slower to initiate than IgG binding (Fig. 4.1 *B*). The deposition rates varied between donors and the rank order for the four donors corresponded to the rank level of IgG deposition levels after 10 minutes. This is consistent with the observation that specific antibodies are needed for deposition of complement on smooth *Salmonellae* (MacLennan et al., 2010). Antibody-directed complement-mediated killing of *Salmonella* also requires the binding of additional complement factors to form a bactericidal C5b-9 membrane attack complex (MAC). C5b-9 deposition on *S*. Typhimurium was slower than both IgG binding and C3 deposition (Fig. 4.1 *C*). Again, the rank order of the rate of C5b-9 deposition by serum corresponded to the rank level of IgG binding. C3 and C5b-9 showed significant correlation (Pearson, $p < 0.0001$, $R^2 = 0.48$). Confocal microscopy images were recorded for IgG and C3 binding to *Salmonellae* and were consistent with flow cytometry measurement of the GMFI (Fig. 4.2).

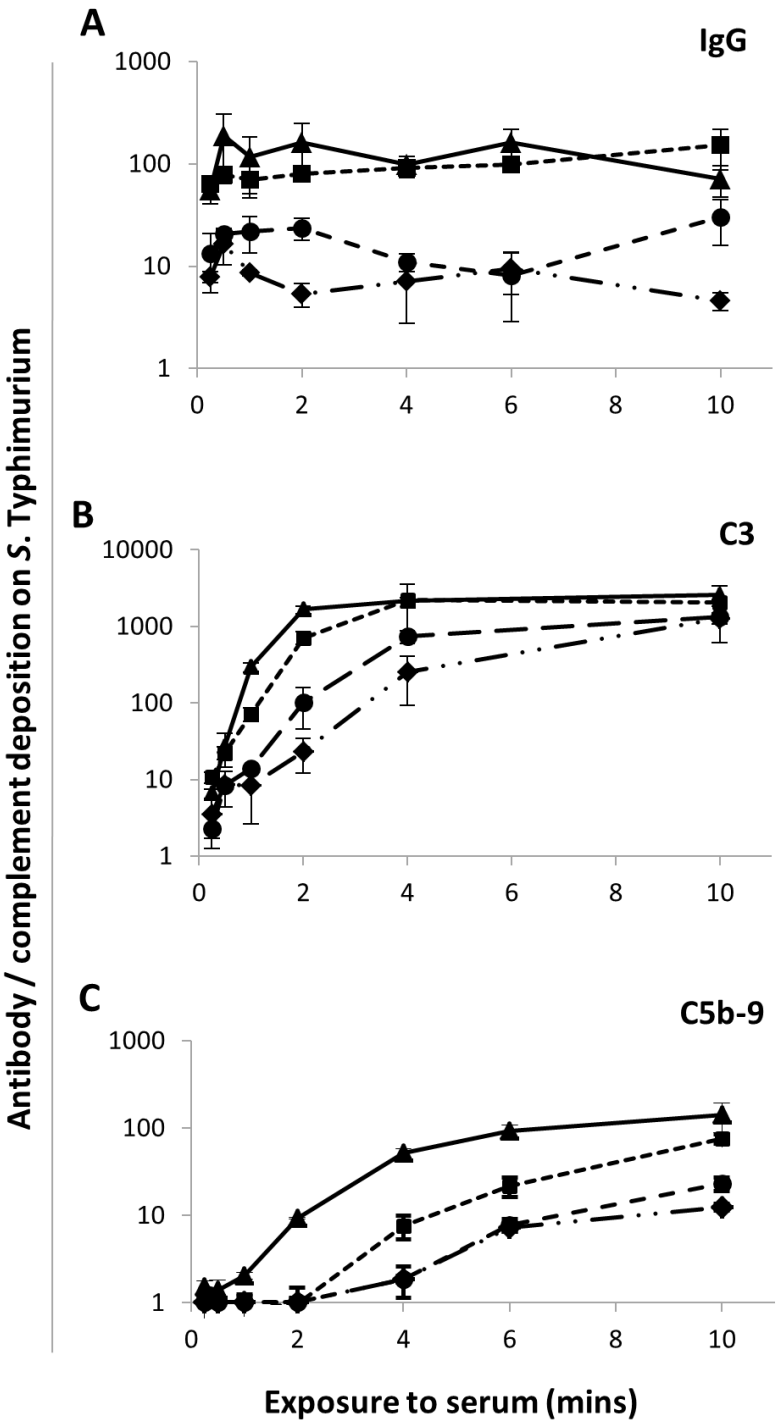


Figure 4.1: Time course of binding of IgG and deposition of complement components on *S.* Typhimurium. (A) IgG, (B) C3, and (C) C5b-9 deposited on African *S.* Typhimurium D23580 measured by flow cytometry (GMFI) following a time-course incubation in neat human serum. Each line represents one donor. Data are a mean of three experiments ± SD.

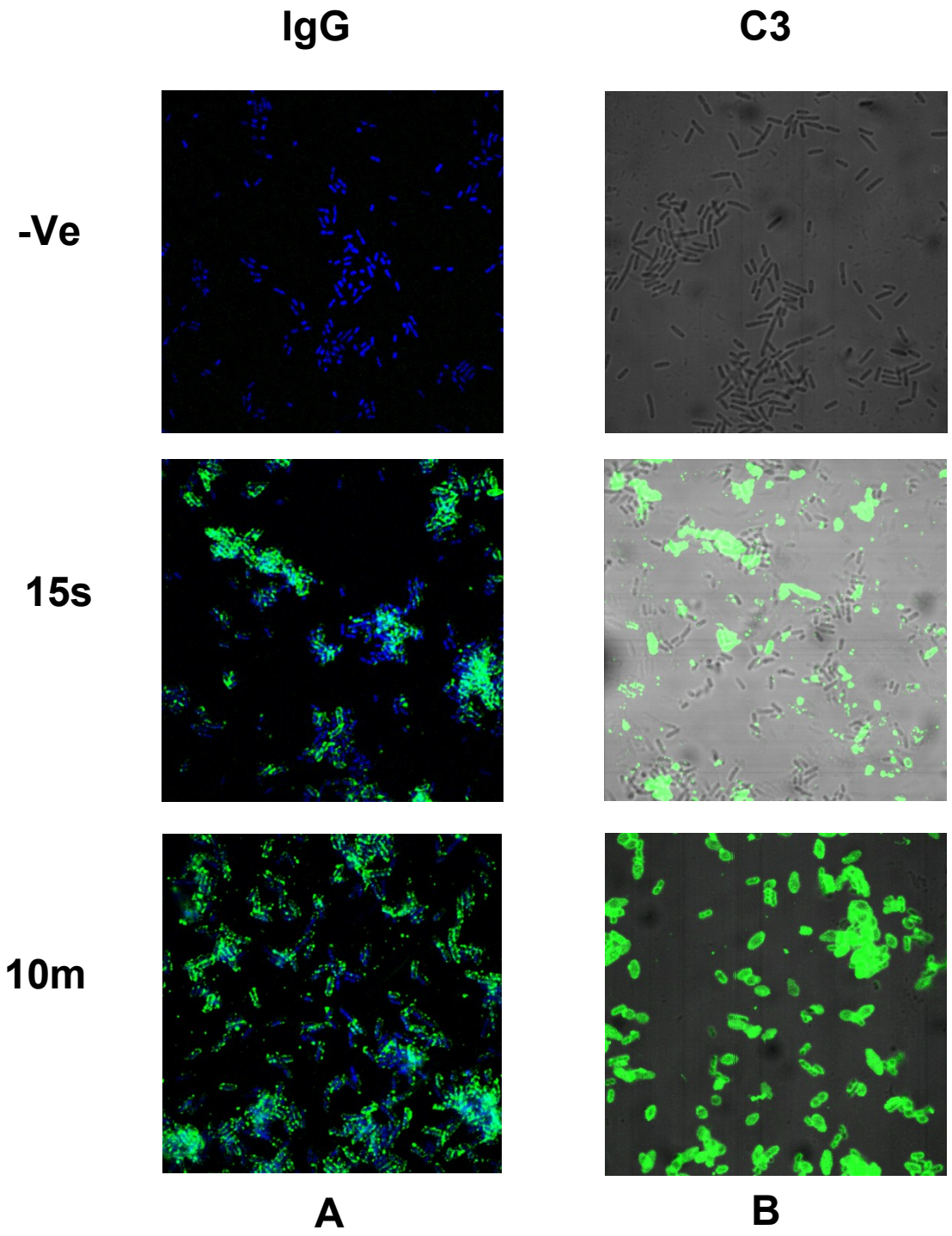


Figure 4.2: Confocal microscopy images of a time-course of binding of IgG and C3 to *Salmonellae*. 2x10^8 live *S*. Typhimurium D23580 were incubated in neat (C3) or dilute (IgG) serum from a healthy adult donor for 15 seconds or 10 minutes. Negatives were incubated in PBS. Samples fixed post incubation with 1% formaldehyde PBS solution. *Salmonellae* appear blue (DAPI) (A) or are imaged by transmitted light (B) and IgG and C3 stains appear green (FITC-conjugated anti-human antibody).

4.3.2 Kinetics of serum killing and phagocytosis of *S.* Typhimurium

Serum-killing was minimal for all donors over the first 5 minutes and even longer for some donors. Following this latency there is a rapid increase in killing rate. Such a latent period after exposure to serum has been observed in other bacterial species and it has been suggested that it corresponds to assembly of the MAC (Taylor, 1983). Our data supports this theory as maximal serum-killing only occurs once C5b-9 deposition has occurred. Serum-killing continued throughout the entire time course of 45 minutes, although the rate slowed near the end of the incubation period (Fig. 4.3 *A*).

Overall, there was a lack of clear correlation with ranked killing rate and binding and deposition of IgG, C3 and C5b-9. Though the serum with the highest deposition effected both the quickest kill and phagocytosis of *Salmonellae*. This suggests that not all antibody binding and complement deposition on *Salmonella,* detected by flow cytometry, may contribute to the bactericidal complex. Phagocytosis by neutrophils (Fig. 4.3 *B*) and monocytes (Fig. 4.3 *C*), determined by internalisation of FITC-labeled *S*. Typhimurium, began quickly and this early period coincided with the period of *Salmonella* survival as determined by SBA.

4.3.3 Production of a growth curve for *S.* Typhimurium D23580

To explore the effect of different growth phases on serum-susceptibility of our invasive African *S*. Typhimurium isolate, we first produced a 12-hour growth curve. The *Salmonellae* were grown as described in sections 2.1.2-3 according to 'Birmingham conditions'. Figure 4.4 A shows that logarithmic (log) phase growth starts at around 80 minutes. The log phase lasts around 5 hours. After approximately 390 minutes, the growth curve begins to flatten indicating late log and the approach of stationary phase.

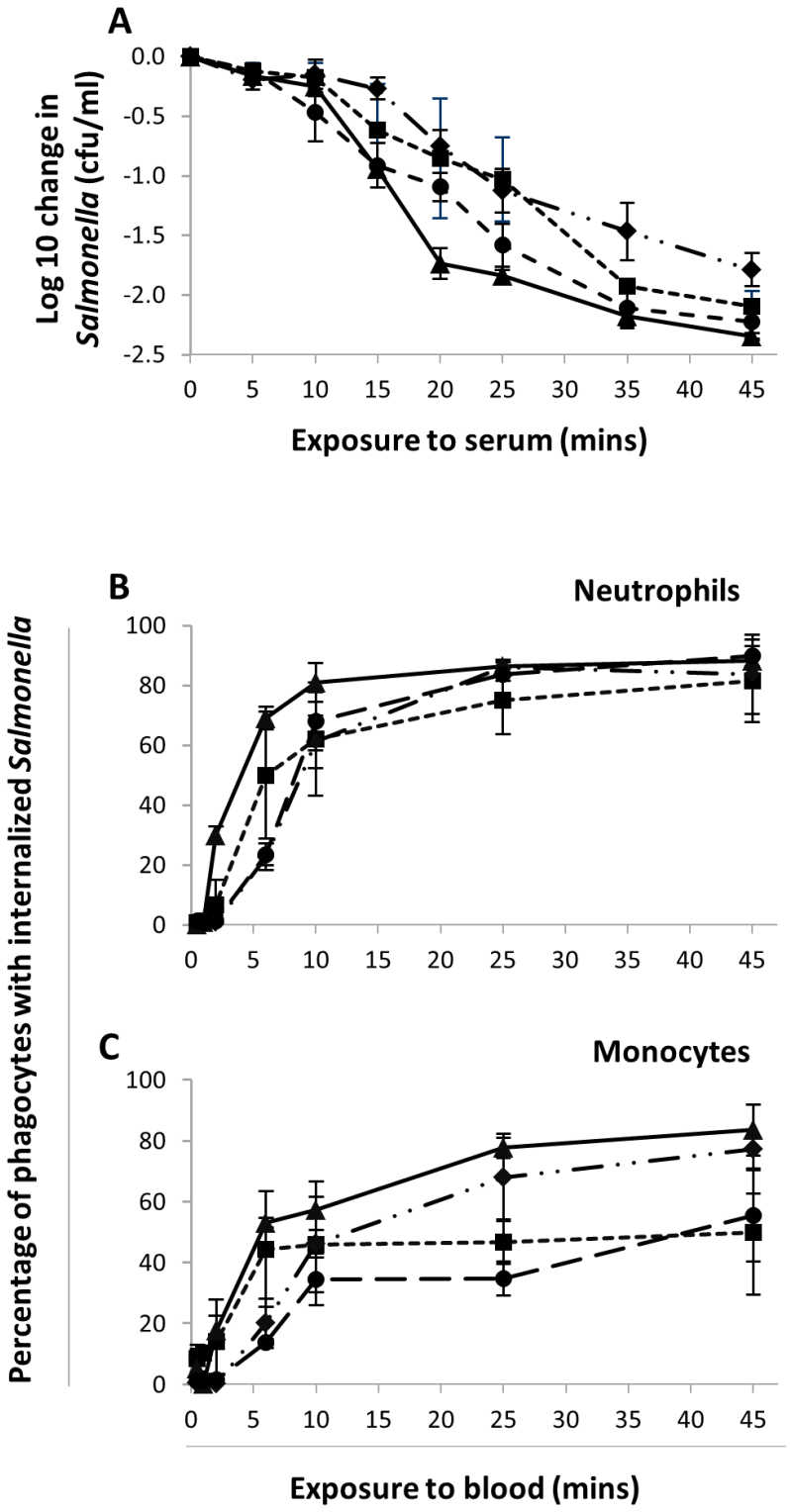


Figure 4.3*: In vitro* time-course of serum-killing and phagocytosis of *S.* Typhimurium. (A) Serum-bactericidal assay and (B) phagocytosis by neutrophils or (C) by monocytes of African *S.* Typhimurium D23580. Phagocytosis measured by flow cytometry. Each line represents a different donor. Data are mean of three experiments ± SD.

Our experiments mainly use *Salmonellae* grown for 120 or 180 minutes which corresponds to mid-log and late-log phases respectively. This difference could be important as LPS synthesis has been reported to vary between these growth-phases in *Salmonella* (Rojas et al., 2001) and growth-phase regulation of LPS OAg chain length can influence serum resistance in *Salmonellae* (Bravo et al., 2008). Optical density (OD) (Fig. 4.4 *B*) and viable colony counts were taken at half an hour intervals. OD values gave a strong positive correlation with cfu/ml counts (R^2=0.89), indicating that OD values can potentially be used to indicate both colony number and growth phase.

4.3.4 Growth medium affects *Salmonella* resistance to serum-killing

Serum-killing of *Salmonellae* grown in either heat-inactivated human serum (56°C for 30 minutes) or LB broth, to both log and stationary phase was examined. Log phase *S.* Typhimurium grown in heat inactivated serum and stationary phase *S*. Typhimurium, grown in both heat inactivated serum and LB broth had marked serum-resistance (log_{10} kill of ~ 0 – 0.5 after 180 minutes). (Fig. 4.5A) The results highlight the possibility that *Salmonella*-host interactions may be influenced by the conditions of growth *in vivo*. Bacteria can be capable of rapid growth in a nutrient-rich *in vitro* environment, such as LB media. In contrast, in vivo the same bacteria may achieve only very low rates of cell division when present in tissue or body fluids (Maw and Meynell, 1968, Meynell and Subbaiah, 1963). Our data is consistent with prior suggestions that bacteria dividing at submaximal rates may be less susceptible to serum than more rapidly dividing cells (Taylor, 1978)

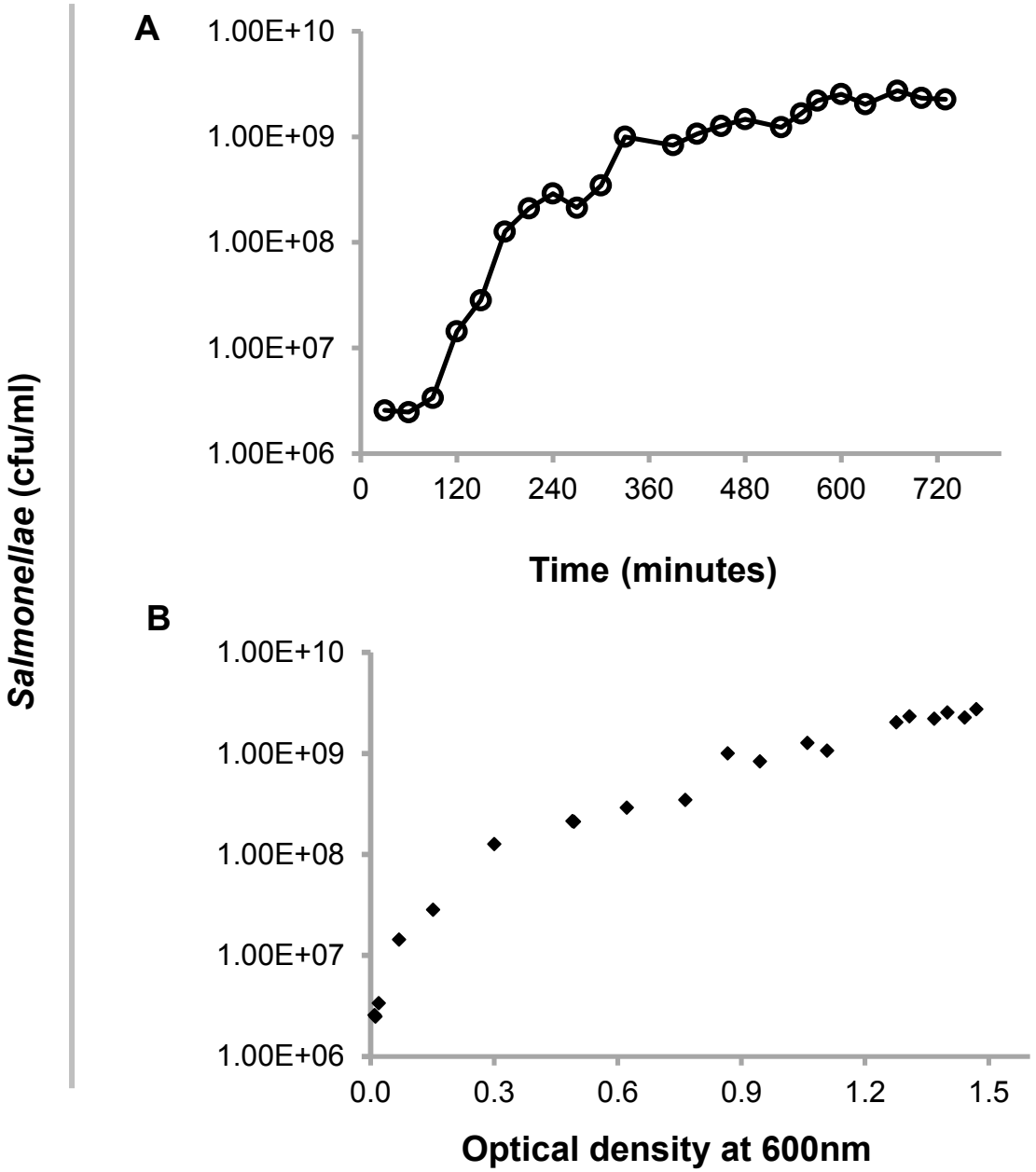


Figure 4.4: **Growth curve of *S.* Typhimurium D23580** at 37°C in LB broth with shaking, cfu/ml determined by viable colony counts. (**A**) Growth curve from early log through to stationary phase. (**B**) Optical density measured on a spectrophotometer at 600nm.

4.3.5 Growth-phase affects *Salmonella* resistance to serum-killing

An SBA was performed using *Salmonella* growths representative of lag, early log, mid log, late log phases and stationary phases (1, 2, 3, 4 and ~16 hour growths). The one and two hour growths exhibited comparable killing (log_{10} kill of ~2.00 after 90 minutes), but marked resistance to serum killing was seen for three hour (log^{10} kill of ~0.20 after 90 minutes), four hour (log_{10} growth of ~ 0.02) and overnight inoculums (log^{10} kill of ~0.50 after 90 minutes) (Fig. 4.5B). This represented a statistically significant difference between groups ($p = 0.0233$ 1-way ANOVA). This variable serum-susceptibility is a consideration in understanding the *in vivo* protective role of bactericidal antibodies and complement in salmonellosis. The resistance to serum-killing of the 4 hour late-log *Salmonella* growth could negate the contribution of bactericidal antibodies to immunity. However, it should be noted that the serum still has a potentially important bacteriostatic effect on even the most serum-resistant *Salmonella* growth.

4.4 Discussion

These results are consistent with *Salmonellae* entering the bloodstream being rapidly opsonised by specific antibodies and complement. Opsonisation facilitates faster uptake by phagocytes, particularly neutrophils (Gondwe et al., 2010), allowing the bacteria to escape from potential complement-damage mediated by C5b-9 formation. Around 90% of invasive NTS isolates in Malawi are sensitive to serum killing *in vitro*, so the ability of a proportion of *Salmonellae* to evade this cell-free bactericidal mechanism may be clinically important. Our data suggest that serum-susceptible *Salmonellae*, such as *S*. Typhimurium D23580, have a window of opportunity to escape the bactericidal environment of blood and move into phagocytes where they are adapted to survive by utili*s*ing virulence genes encoded by their pathogenicity islands (Vazquez-Torres et al., 2000).

Nevertheless, the effective C5b-9 deposition and antibody-dependent complement-mediated killing of *Salmonellae* suggests that sustained high grade bacteraemia in the presence of specific antibodies and functional complement is unlikely. Salmonellosis in IL-12/23-IFNγ (IFNγ) axis deficient patients presents extra-intestinally as focal disease, particularly in cervical lymph nodes.

Moreover, work in IFNγ$^{-/-}$ animals has demonstrated that intracellular densities of *Salmonellae* reach higher levels than in healthy controls (Mastroeni et al., 2009). Yet, despite the impact on cell-mediated immunity and associated increased *Salmonella* growth, IFNγ-axis-deficient patients do not develop fatal *Salmonella* bacteraemia. In contrast, salmonellosis in African infants that lack specific antibodies often presents as a fatal bacteraemia.

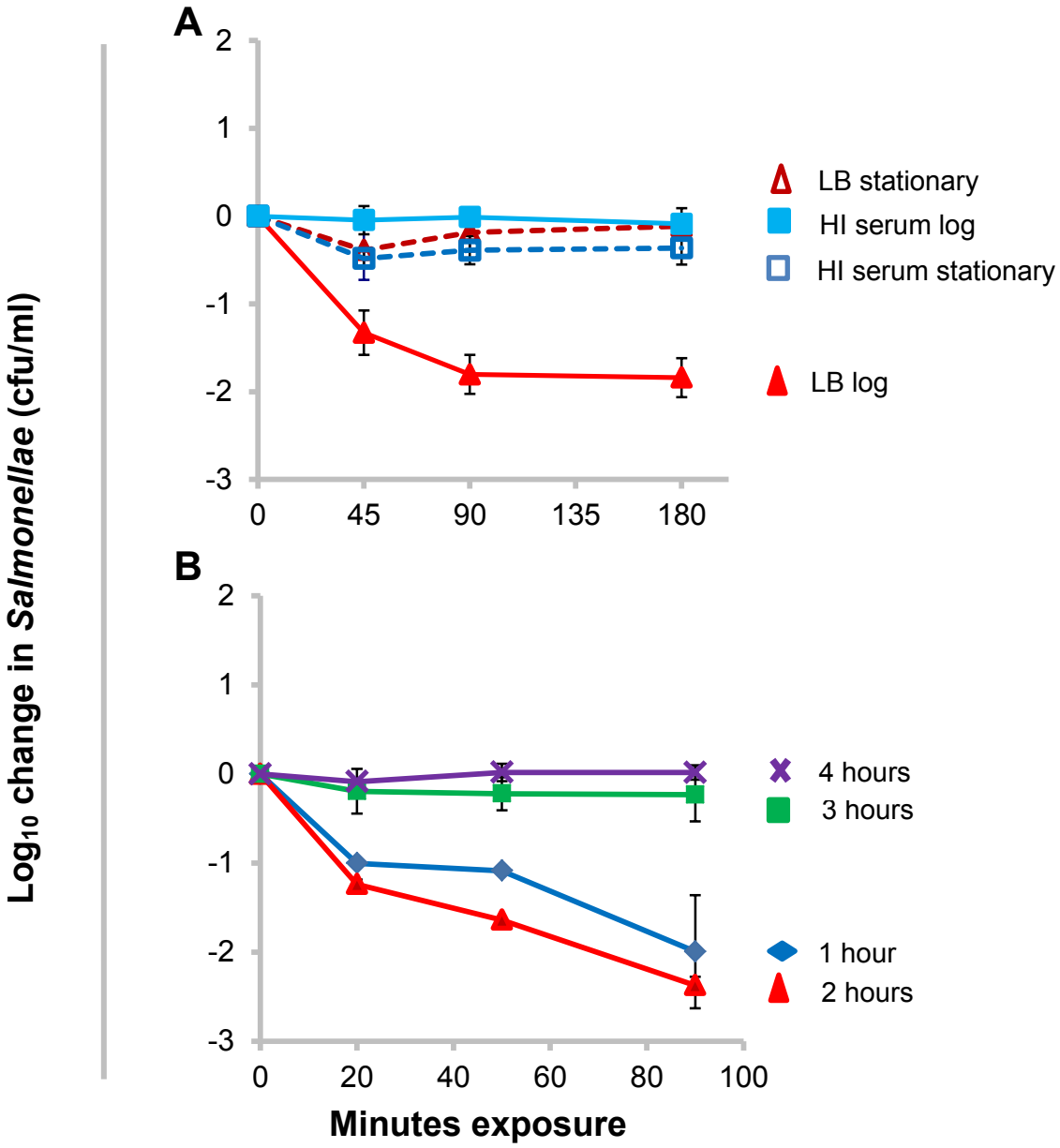


Figure 4.5: **Growth phase and media affects killing of *S.* Typhimurium by serum**. *In vitro* serum-killing assay of *S.* Typhimurium isolate D23580 10^6 /ml. (A) Logarithmic phase (red) and stationary phase (blue) *S.* Typhimurium grown in heat inactivated human serum (dashed lines) or LB broth (solid lines). (B) Serum-killing of *S.* Typhimurium grown for one hour (blue diamonds), two (red triangles), three (green squares) or four hours (purple crosses). Data are a mean of four (A) or two (B) experiments ± SD.

It is likely that the inability of these children's sera to deposit complement on *Salmonellae* prevents antibody-dependent complement-mediated-killing and effective phagocytosis. As such, these patients are predisposed to fail to clear *Salmonella* from their blood and are therefore susceptible to fatal bacteraemia.

The role of antibody-dependent complement-mediated killing of *Salmonellae* is further complicated by the variation in serum-susceptibility exhibited by *Salmonellae* grown in different conditions and to different growth-phases. Our data using an African invasive *S*. Typhimurium isolate are consistent with previous findings using laboratory *Salmonellae* strains demonstrating that that growth-phase can alter serum-susceptibility (Murray et al., 2005, Bravo et al., 2008).

We have reported that growth in heat-inactivated serum also lead to development of serum resistance. Crucially, our bactericidal experiments used 100% human serum and a representative African invasive strain of *S*. Typhimurium, which gives a closer representation of what occurs *in vivo* in NTS bacteraemia in Africans. Considering these points, and also how readily D23580 can lose its susceptibility to serum, resistance to complement-mediated serum killing could be a true phenotype of NTS during an infection. While the range of potential growth conditions of *Salmonella in vivo* makes testing of every possible condition unfeasible, the existence of variation should be considered upon drawing conclusions from models. Ideally, a more directed analysis of variance in expression, such as a gene expression-profiling microarray of *ex vivo* bacteria could be used to give an informed estimate of the metabolic and virulence states of *Salmonella* during infection.

Nevertheless, even serum-resistant *Salmonella* did not undergo a net growth in human serum that contains anti-*Salmonella* antibodies and functional complement during incubation at 37°C. This contrasts with the ~1 $\log_{10}$ growth seen when, even serum-susceptible, *Salmonellae* are incubated in serum that lacks anti-*Salmonella* antibodies (Fig 3.7). This implies a bacteriostatic role for antibody-dependent complement-mediated immunity, which could be important in limiting *Salmonella* concentration, and could assist preventing development of disease.

Our data display kinetics of complement and antibody binding, serum-killing and phagocytosis which are consistent with these clinical observations. The relative slowness of serum-killing compared to opsonisation and phagocytosis could allow the escape and survival of a sufficient percentage of *Salmonella* to continue infection. This perhaps explains how of complement-fixing specific antibodies are unable to prevent severe salmonellosis despite their mediation of bactericidal processes. However, near-instantaneous binding of antibodies to *Salmonella*, in combination an opsonic and bactericidal contribution from complement factors could prevent persistence of *Salmonellae* in the blood. In an in vivo setting this contribution of antibodies could be life-saving and thus a vaccine inducing an antibody response, even in the absence of a strong cellular response, may dramatically reduce mortality caused by invasive NTS disease in Africa.

4.5 Conclusion

Nontyphoidal strains of *Salmonella* are a major cause of morbidity and mortality worldwide. Infection in African children that lack specific antibodies frequently results in fatal bacteraemia, whereas IFNγ-deficient patients can suffer from serious extra-intestinal, yet non-fatal disease. Mouse models of salmonellosis may not consider the impact of antibody-dependent complement-mediated immunity on bacterial spread. We have used serum bactericidal, phagocytosis, and antibody and complement deposition assays with human blood and sera to assess the contribution of cell-fee antibody-dependent complement-mediated-killing of invasive African *Salmonella*. Phagocytosis overlapped *Salmonellae* survival in serum, potentially creating a window in the kinetics in which a proportion of *Salmonellae* can escape from extracellular complement-fixing bactericidal antibodies and re-establish intracellular infection. However, C5b-9 is deposited on *Salmonellae* remaining in serum and leads to fast and efficient killing. This antibody-dependent complement-mediated bactericidal activity likely stops sustained and fatal bacteraemia. Such a model helps to explain how specific antibodies alone might, in the absence of a strong cellular immune response, not prevent salmonellosis, but could halt fatal development of the disease. Growth condition-dependent variation in serum-susceptibility of *Salmonellae* complicates interpretation of antibody-dependent complement-mediated immunity *in vivo*. Nonetheless, specific antibodies and complement had a bacteriostatic effect on even the most serum-resistant *Salmonella* growths we tested. The current findings support the protective effect of an antibody response against *Salmonella* disease.

Chapter 5

Adsorption studies demonstrate that the O-antigen of invasive African nontyphoidal *Salmonella* is a major target of complement-fixing bactericidal antibodies

5.1 Introduction

Anti-lipopolysaccharide (LPS) and particularly anti-O-antigen (OAg) antibodies have been reported to be protective in mouse models of salmonellosis (Watson et al., 1992, Carlin et al., 1987, Colwell et al., 1984). Such antibodies have also been shown to be bactericidal against NTS in a complement-fixing capacity (Schulkind et al., 1972). However, there are mixed reports on the ability of exogenous LPS remove bactericidal activity against *Salmonella* (Skarnes, 1978, Michael et al., 1962, Cundiff, 1941). The most recent data of this kind demonstrated that exogenous Omp was capable of removing bactericidal activity against *S*. Typhimurium, while LPS was not (MacLennan et al., 2010). As well as this, a lack of bactericidal activity against invasive African NTS isolates in the sera of a sub-set of HIV-infected patients was due to the inhibitory effect of large titres of anti-OAg antibodies was reported. In contrast anti-Omp antibodies, generated by immunisations in mice, had bactericidal complement-fixing activity against *S*. Typhimurium. These anti-Omp antibodies have shown to be protective in challenge studies in C57BL/6 mice (Cunningham et al., 2007).

A major challenge in developing a vaccine against NTS for Africa is to identify the key molecular targets of the protective immune response against *Salmonella*. An OAg conjugate based approach to a NTS vaccine is seen as a relatively quick and cost-effective solution. However, the above findings have brought some doubt concerning the role of anti-OAg antibodies in at least one modality of immunity against invasive NTS disease in Africa.

Antibody adsorption

Specific-antibody free serum is a useful research tool to help determine specificity of bactericidal antibodies. However, anti-*Salmonella* antibody-free human sera are not common. The only natural sources are young children or those with antibody deficiency disorders. It is difficult and unethical to take large volumes of blood from children for study and patients that lack antibody are only a transient source, if a source at all, as they typically start treatment soon after diagnosis of the condition. In healthy humans development of acquired and/or natural antibodies, reactive against *Salmonella,* to a level required to effect serum-killing occurs by 2 years of life (MacLennan et al., 2008).

Adsorbing out specific antibodies from serum using bacteria is a simple process that has been frequently used since around the turn of the 20th century by researchers such as Lipstein in 1902 (Pandit, 1923, Mackie and Finkelstein, 1930, Collins, 1967). However, despite the age of the system, or perhaps because of it, the effects on the serum have never been fully characterised, with researchers mainly only relying on SBAs as demonstration of antibody removal.

Here, we have characterised the effect of adsorption of serum using whole *Salmonella* on specific *Salmonella* antibodies and complement activity of serum. To help identify the antigenic targets of bactericidal antibodies in human serum we have performed serum adsorptions, serum bactericidal assays (SBA) and flow cytometry antibody and complement-deposition assays, using a range of NTS isolates, including African clinical invasive isolates, laboratory strains, LPS mutants and OAg chimera mutants

5.2 Statistical Analysis

Comparisons of two groups were performed using two tailed student's *t* tests. All statistical tests were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA).

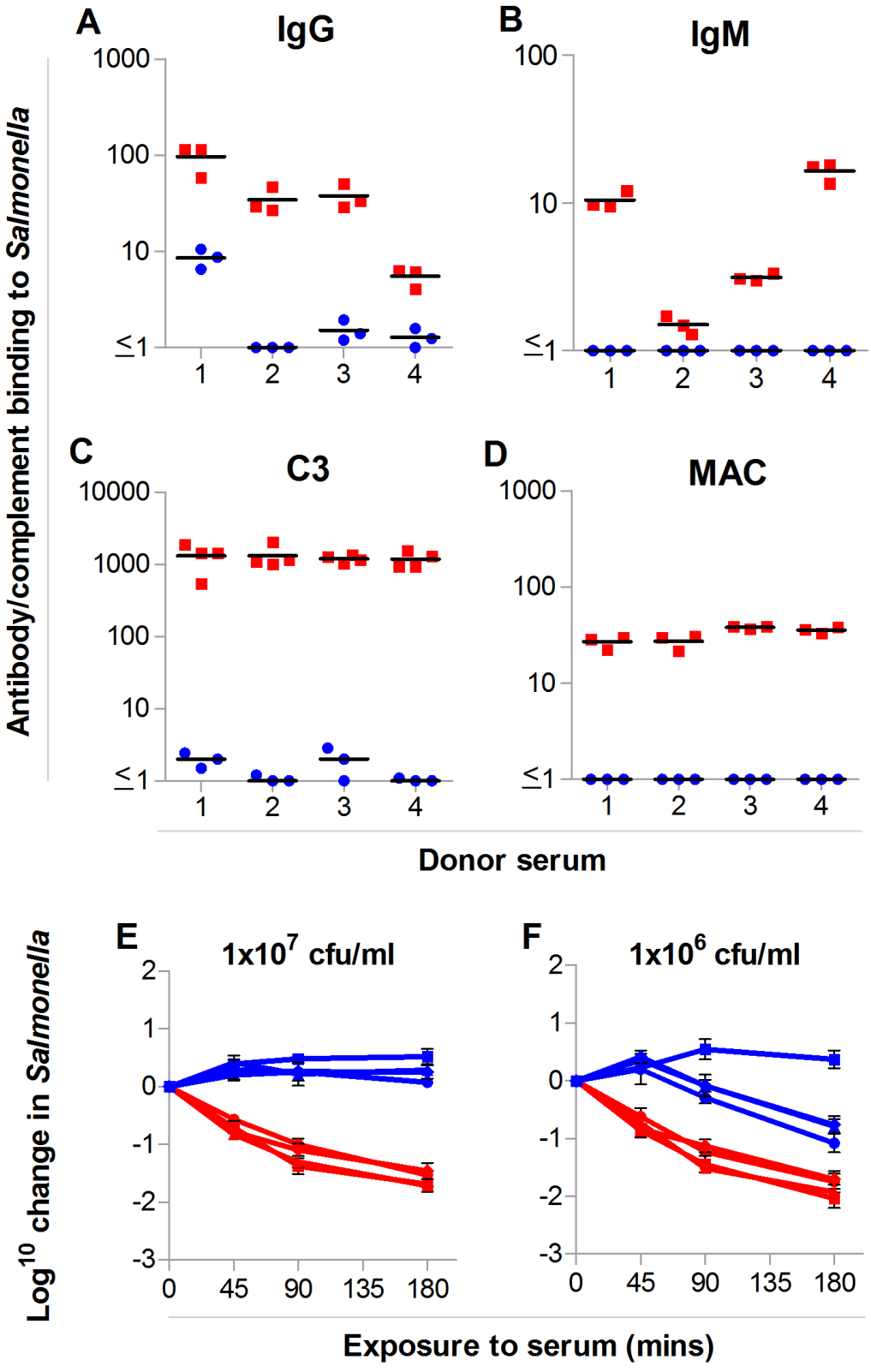
5.3 Results

5.3.1 Effect of adsorption on specific antibodies in serum

Sera from four healthy adults were assessed for their ability to deposit IgG, IgM, C3 and C5b-9 on the live African invasive *S*. Typhimurium D23580 isolate using flow cytometry. Cell-free bactericidal activity was determined by SBA. These sera were then adsorbed as described using a live suspension of $1x10^{11}$ cfu/ml of the homologous bacterial isolate. One adsorption cycle produced a marked and significant reduction of specific anti-*Salmonella* IgG (Fig. 5.1*A*) and IgM (Fig. 5.1*B*) in all four sera (Student's *t* test, all $p < 0.001$), though IgG was still detectable in the sera from three of the donors. Specific antibodies are required to deposit complement on smooth forms of NTS (MacLennan et al., 2008) and the removal of specific antibodies by adsorption produced an associated decrease in both C3 (Student's *t* test, all $p < 0.005$) and C5b-9 deposition (Student's *t* test, all $p < 0.001$) (Fig. 5.1*C* + *5.1D*). Following adsorption, C3 deposition was only detectable in two of the sera and C5b-9 deposition was absent in all sera. A serum killing assay using $1x10^7$ cfu/ml *S*. Typhimurium showed a net growth of up to ~0.5 $\log_{10}$ for adsorbed serum, contrasting with a ~1.5 $\log_{10}$ for un-adsorbed serum (Student's *t* test, all $p < 0.0005$) (Fig. 5.1*E*). However, with the usual 10-fold lower concentrations of *S.* Typhimurium used in previous SBAs (MacLennan et al., 2008) the reduction in bactericidal activity produced by pre-adsorption was reduced such that only three of the adsorbed sera were still bactericidal, producing ~1 $\log_{10}$ kill (Fig. 5.1*F*). The difference in kill between unadsorbed and absorbed sera was still significant for all donors (Student's *t* test, all $p < 0.01$).

Figure 5.1: Effect of adsorption of serum with *Salmonella* on antibody and complement binding and bactericidal activity. (A) IgG, (B) IgM, (C) C3 and (D) C5b-9 GMFI measured by flow cytometric analysis of binding to *S*. Typhimurium by four human sera. Serum was adsorbed with *S*. Typhimurium (blue circles) or unadsorbed (red squares). Each point represents one experiment. Horizontal bars indicate mean values. (E) *In vitro* serum bactericidal assay with 10^7 or (F) 10^6 *Salmonellae*/ml at 45, 90, and 180 minute time points using unadsorbed (red lines) or adsorbed (blue lines, adsorption as above) human serum from four healthy donors. Data are means of three experiments ± SD.

Figure 5.1

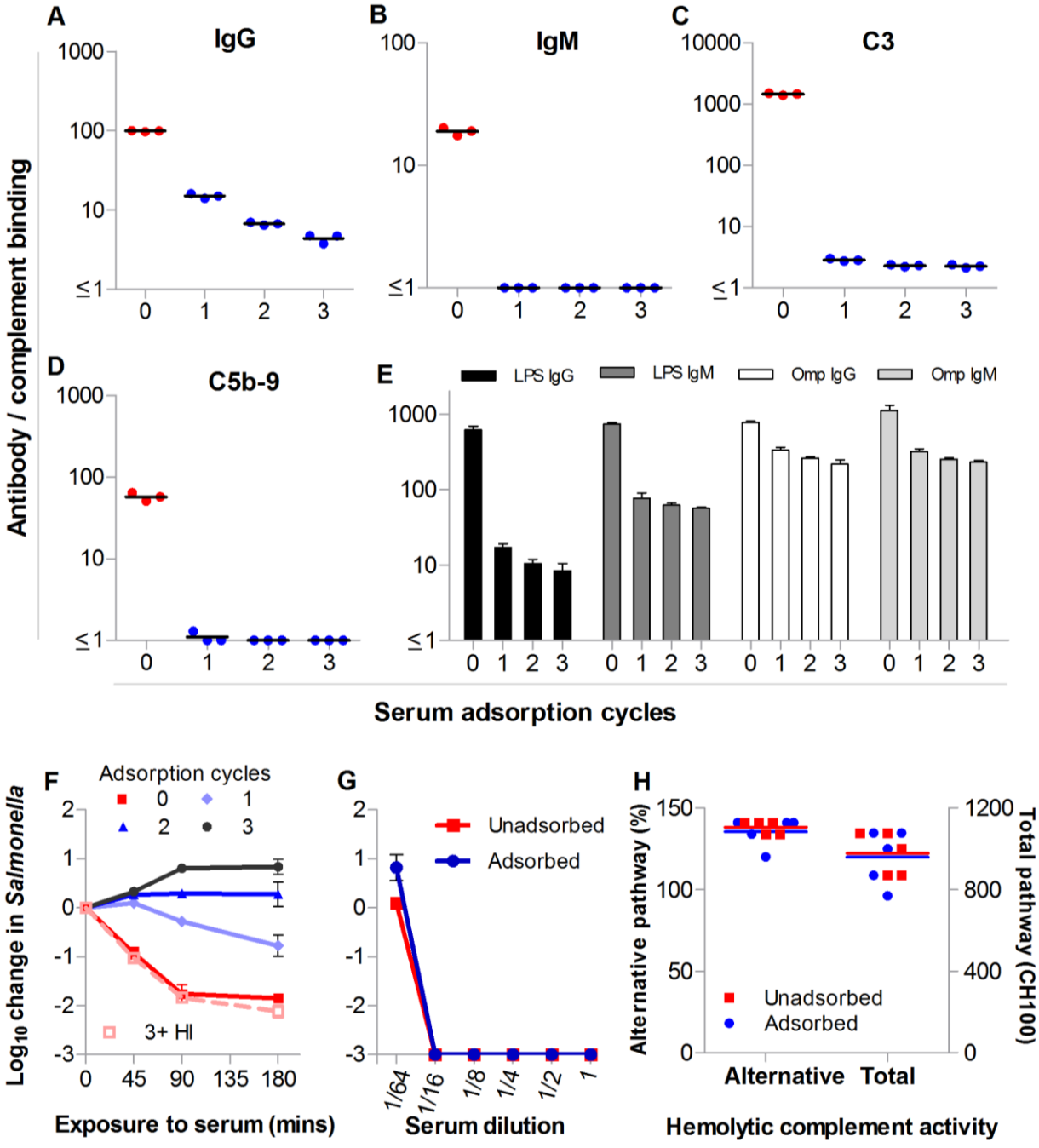

A
IgG
B
IgM
C
C3
D
MAC
1000
100
10
≤1
10000
1
2
3
4
Antibody/complement binding to Salmonella
Donor serum
E
1x10^7 cfu/ml
F
1x10^6 cfu/ml
2
1
0
-1
-2
-3
0
45
90
135
180
Log10 change in Salmonella
Exposure to serum (mins)

5.3.2 Effect of multiple adsorption on specific antibodies in serum

To increase the efficiency of the adsorption protocol, the serum with the highest antibody levels and bactericidal activity after one adsorption cycle was chosen and subjected to additional adsorption cycles. As Figure 5.2 *A-D* shows, the first adsorption cycle removed the majority of specific antibody and complement binding. Specific IgG titre was slightly lowered further by a second and third adsorption cycle (Student's *t* test, both $p < 0.005$). In addition to flow cytometry analysis of antibody and complement binding, an ELISA was performed using highly pure exogenous LPS and a preparation of *S.* Typhimurium outer membrane as capture antigens (Fig. 5.2*E*). The LPS capture format gave similar results to the flow cytometry assay, with adsorption producing a ~100 fold reduction in specific IgG and a ~10 fold reduction in specific IgM. Outer membrane capture showed a more modest decrease of ~3 fold. The decrease in specific antibodies after one cycle of adsorption was highly significant (Student's *t* test, all $p < 0.0001$) and subsequent cycles produced small but significant reductions in antibody titers (Student's *t* test, $p < 0.05$). These small reductions in specific antibodies achieved by additional adsorptions translated to a large difference in serum killing (Fig 5.2*F*). Two adsorption cycles removed net bactericidal activity and resulted in a net growth of ~0.5 $\log_{10}$ after 180 minutes (Student's *t* test, $p < 0.005$ compared to one adsorption), three cycles showed a net growth of ~1 $\log_{10}$ (Student's *t* test, $p < 0.005$ compared to two adsorptions).

Figure 5.2: Effect of multiple adsorption cycles on antibody and complement binding to *Salmonella* and bactericidal activity. (A) IgG, (B) IgM, (C) C3 and (D) C5b-9 GMFI measured by flow cytometric analysis of binding to *S*. Typhimurium by human serum. Serum was either unadsorbed or adsorbed with *S*. Typhimurium one, two or three times. Each point represents one experiment. Horizontal bars indicate mean values. (E) IgG and IgM titres against either exogenous LPS or outer membrane antigens measured by ELISA. (F) *In vitro* serum bactericidal assay with 10^6 wild type *Salmonellae*/ml at 45, 90, and 180 minute time points using unadsorbed or adsorbed human serum (adsorbed as above). Numbers in legend correspond to number of adsorption cycles performed on serum. Killing activity restored when heat inactivated (HI) serum was added as a complement source. Data are means of three experiments ± SD. (G) Serum bactericidal assay as above but using *galE*$^-$ *S*. Typhimurium, which is susceptible to killing by specific-antibody free serum, and a single 180 minute time point. Dilutions of adsorbed (blue lines, prepared as above) and unadsorbed (red lines) serum made in PBS. Data are means of three experiments ± SD. (H) Haemolytic alternative and total complement function determined by radial immunodiffusion assays. Serum was adsorbed with *Salmonella* (blue circles) or unadsorbed (red squares). Each point represents one experiment and horizontal bars indicate mean values.

Figure 5.2


A
IgG
B
IgM
C
C3
D
C5b-9
E
LPS IgG
LPS IgM
Omp IgG
Omp IgM
Antibody / complement binding
Serum adsorption cycles
F
Adsorption cycles
0
1
2
3
3+ HI
Log10 change in Salmonella
Exposure to serum (mins)
G
Unadsorbed
Adsorbed
Serum dilution
H
Alternative pathway (%)
Total pathway (CH100)
Unadsorbed
Adsorbed
Alternative
Total
Hemolytic complement activity

5.3.3 Adsorption has minimal detrimental effect on complement activity.

Adsorption was performed at 4°C to minimize the binding and consumption of complement components. Adsorptions were carried out for three cycles. Reconstituting the adsorbed non-bactericidal serum with heat-inactivated serum, as an antibody source, restored normal killing (Fig. 5.2*F*) (Student's *t* test, heat-inactivated or adsorbed alone compared with heat-inactivated plus adsorbed $p < 0.0001$). Additionally, the killing of *galE*⁻ *S*. Typhimurium (which, unlike wild type, is susceptible to killing in the absence of specific antibodies) was identical in adsorbed and unadsorbed serum even at dilutions in excess of 1/8 serum in PBS (Fig. 5.2*G*). Finally, hemolytic assays showed no significant difference in either alternative or total activity between unadsorbed and adsorbed serum (Fig. 5.2*H*). These results demonstrate the complement in adsorbed serum is still functional.

5.3.4 Adsorption of specific antibodies and removal of bactericidal activity is serovar-specific.

To test whether bactericidal antibodies were serovar or strain-specific, serum was adsorbed for three cycles with a range of laboratory and invasive clinical isolate strains of *S*. Typhimurium and *S*. Enteritidis (Table 5.1). All sera adsorbed with *S*. Typhimurium strains produced a significant decrease in bactericidal activity against *S*. Typhimurium D23580 (Fig. 5.3*A*) (Student's *t* test, all $p < 0.0001$), whereas all sera adsorbed with *S*. Enteritidis failed to remove killing against *S*. Typhimurium (Fig. 5.3*B*). This result was reflected in antibody binding assays, which demonstrated that all serum pre-adsorptions with *S*. Typhimurium strains produced a significant, complete or near-complete removal of both specific IgG and IgM (Student's *t* test, all $p < 0.0001$), while in contrast, *S*. Enteritidis strains produced a much smaller reduction (Fig. 5.3*C* + *D*). The effect was not limited to binding to *S*. Typhimurium, and when whole *S*. Enteritidis was used as the

capture bacteria, the opposite result was seen (Fig. S1 *A-C*). This suggests that bactericidal antibodies mediating cell-free complement-killing are serovar-specific.

Table 5.1 *Salmonella* strains and isolates used for adsorption study

Serovar	Strain name	Isolation
Enteritidis	ATCC4931	Human gastroenteritis, Copenhagen, Denmark
Enteritidis	D21685	Human systemic, Malawi
Enteritidis	D24359	Human systemic, Malawi
Enteritidis	D24953	Human systemic, Malawi
Enteritidis	D24954	Human systemic, Malawi
Enteritidis	P125109	Human gastroenteritis isolate
Typhimurium	D22477	Human systemic, Malawi
Typhimurium	D23005	Human systemic, Malawi
Typhimurium	D23580	Human systemic, Malawi
Typhimurium	LT2 (ATCC 700720)	Unknown natural source (1948)

Figure 5.3: Effect of adsorption with different *Salmonella* strains and isolates on antibody and complement and bactericidal activity. (A) *In vitro* serum bactericidal assay with 10^6 wild type *Salmonellae*/ml at 45, 90, and 180 minute time points using serum adsorbed with *S*. Typhimurium strains or (B) *S*. Enteritidis and *S*. Typhi strains. Strain names indicated in legend. Data are means of three experiments ± SD. (C) IgG or (D) IgM GMFI measured by flow cytometric analysis of binding to *S*. Typhimurium by human serum adsorbed with either a strain of *S*. Enteritidis, *S*. Typhi or *S*. Typhimurium (indicated on X axis) or unadsorbed (NHS). Each point represents one experiment. Horizontal bars indicate mean values.

Figure 5.3

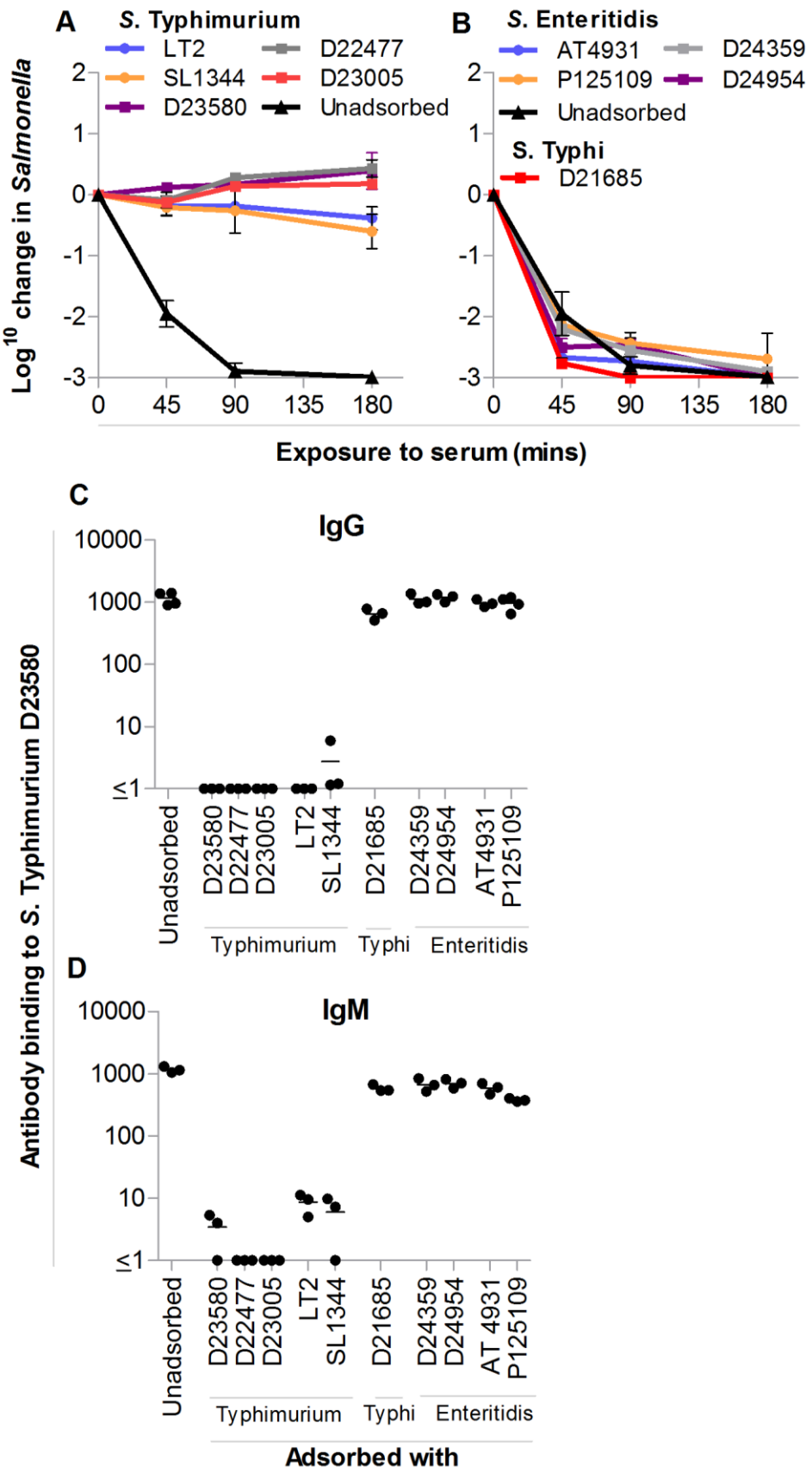

A
S. Typhimurium
LT2
SL1344
D23580
D22477
D23005
Unadsorbed
B
S. Enteritidis
AT4931
P125109
D24359
D24954
Unadsorbed
S. Typhi
D21685
Log10 change in Salmonella
Exposure to serum (mins)
C
IgG
Antibody binding to S. Typhimurium D23580
Unadsorbed
D23580
D22477
D23005
LT2
SL1344
D21685
D24359
D24954
AT4931
P125109
Typhimurium
Typhi
Enteritidis
D
IgM
Adsorbed with

Table 5.2: LPS knock-out *Salmonella* strains used in study.

Serovar	Strain Name	Knockout Gene
Enteritidis	P125109	*galE-*
Enteritidis	P125109	*wbaP*$^{-}$
Typhimurium	D23580	*galE-*
Typhimurium	D23580	*waaC*$^{-}$
Typhimurium	D23580	*waaF*$^{-}$
Typhimurium	D23580	*waaG*$^{-}$
Typhimurium	D23580	*wbaP*$^{-}$
Typhimurium	SL1344	*wbaP*$^{-}$

Figure 5.4

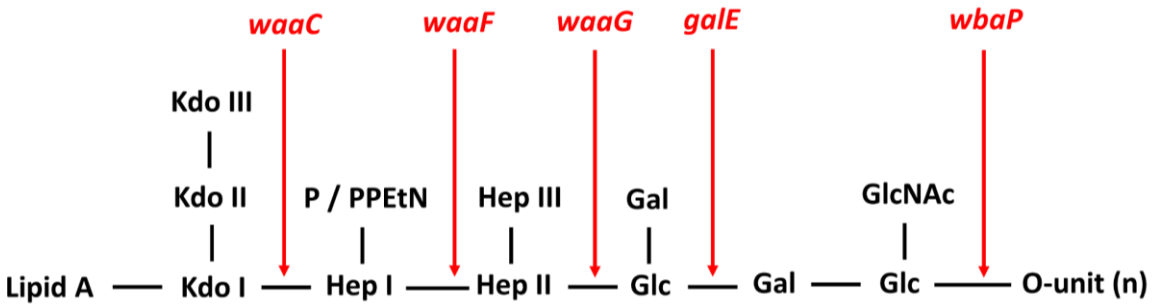


Figure 5.4: Schematic representation of *S.* Typhimurium lipopolysaccharide molecule and sites of truncation of mutants. (A) Lines indicate the level of LPS truncation resulting from each mutation. (Gal: Galactose; Glc: glucose; GlcNAc: *N*-acetylglucosamine; Hep: Heptose; Kdo: 3-deoxy-D-mannooctulosonic acid; P: phosphate; PPEtN: pyrophosphorylethanolamine;

5.3.5 Rough mutants lacking OAg are unable to remove bactericidal activity against the wild type parent strain.

One of the main immunogenic differences between serovars of *Salmonella* is the OAg expressed. We used a range of knockout mutants that had truncations of their LPS and all lacked OAg (Table 5.2 + Fig. 5.4) to investigate the contribution of OAg to the serovar specificity reported. Mutants cannot bind antibody against the LPS components they lack and so comparison of binding across a range of *Salmonella* with varying completeness of LPS gives insight into antigen targets (Fig. 5.5). Adsorptions were carried out for three cycles. All sera pre-adsorbed with the OAg knockouts of *S*. Typhimurium and *S*. Enteritidis failed to remove killing against a wild type strain of *S*. Typhimurium (Fig. 5.6*A*) (Student's *t* test, all $p < 0.0001$ compared to wild type *S*. Typhimurium adsorbed). Flow cytometric analysis of antibody binding to wild type *S*. Typhimurium demonstrated no significant difference between unadsorbed serum and serum adsorbed with OAg-knockout strains for either IgG (Fig. 5.6*B*) or IgM (Fig. 5.6*C*) (Student's *t* test). This is in clear contrast to the large and significant reduction achieved by adsorption with a wild type *S*. Typhimurium strain (Student's *t* test, $p < 0.0001$). In addition, non-bactericidal wild type-adsorbed serum had high levels of IgG and IgM specific against the OAg knockout strains remaining (Fig. 5.7 *D*-*I*). This suggests that either antibodies against the exposed surface of OAg knockout strains of NTS are not bactericidal, or that bactericidal antibodies specific to those targets are not present in the normal sera we tested. Addition of highly pure exogenous *S*. Typhimurium LPS was able to reduce IgG binding to *S*. Typhimurium (Student's *t* test, all $p < 0.0001$), and *S*. Enteritidis LPS did not produce a significant reduction (Fig. 5.6*D*). However, the decrease in specific IgG was much less than achieved with whole, live *S*. Typhimurium (Student's *t* test, all $p < 0.0001$). As well as this, adsorption with *Salmonella* LPS failed to produce a reduction in serum bactericidal activity against *S*. Typhimurium (Fig. 5.6*E*).

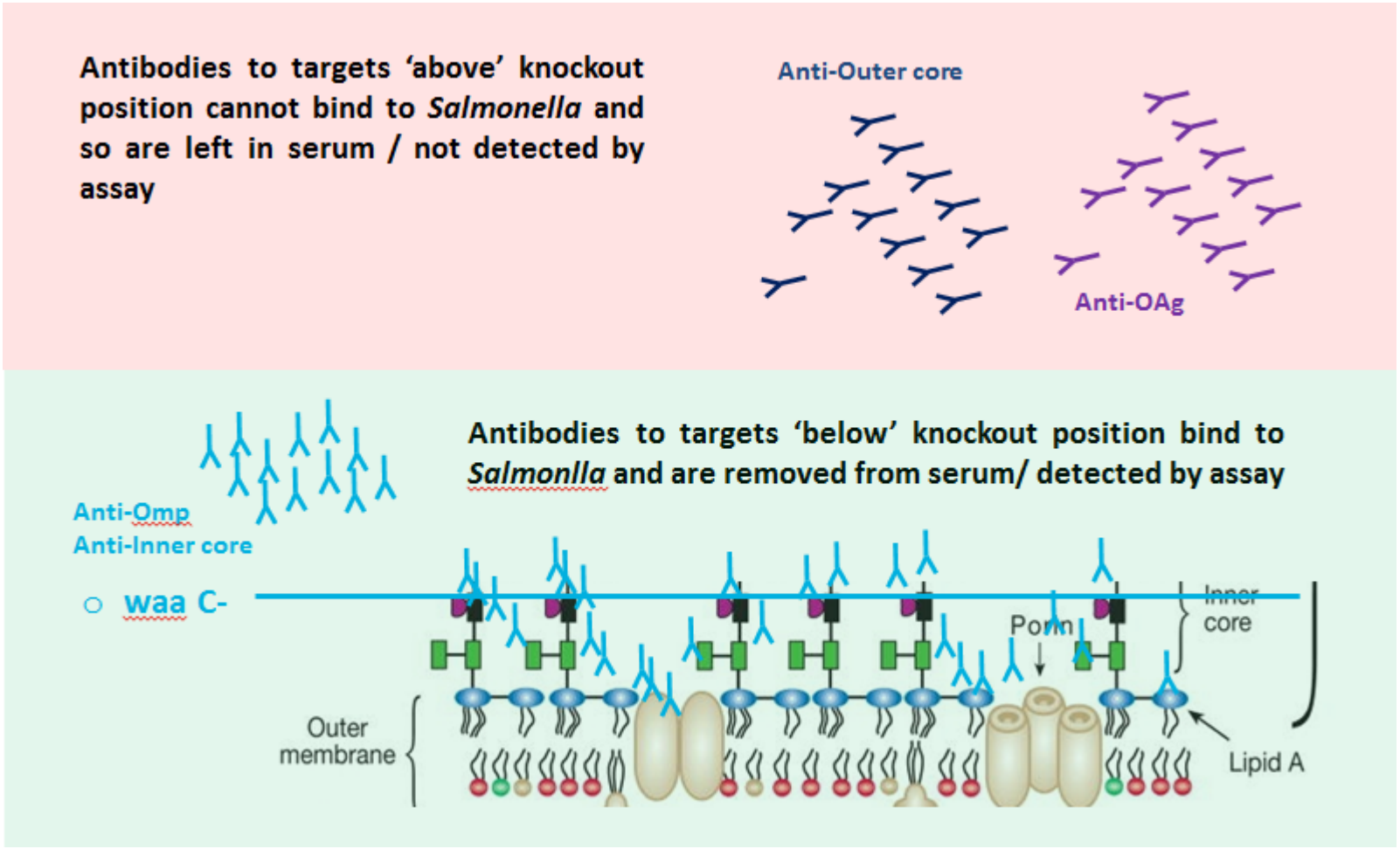
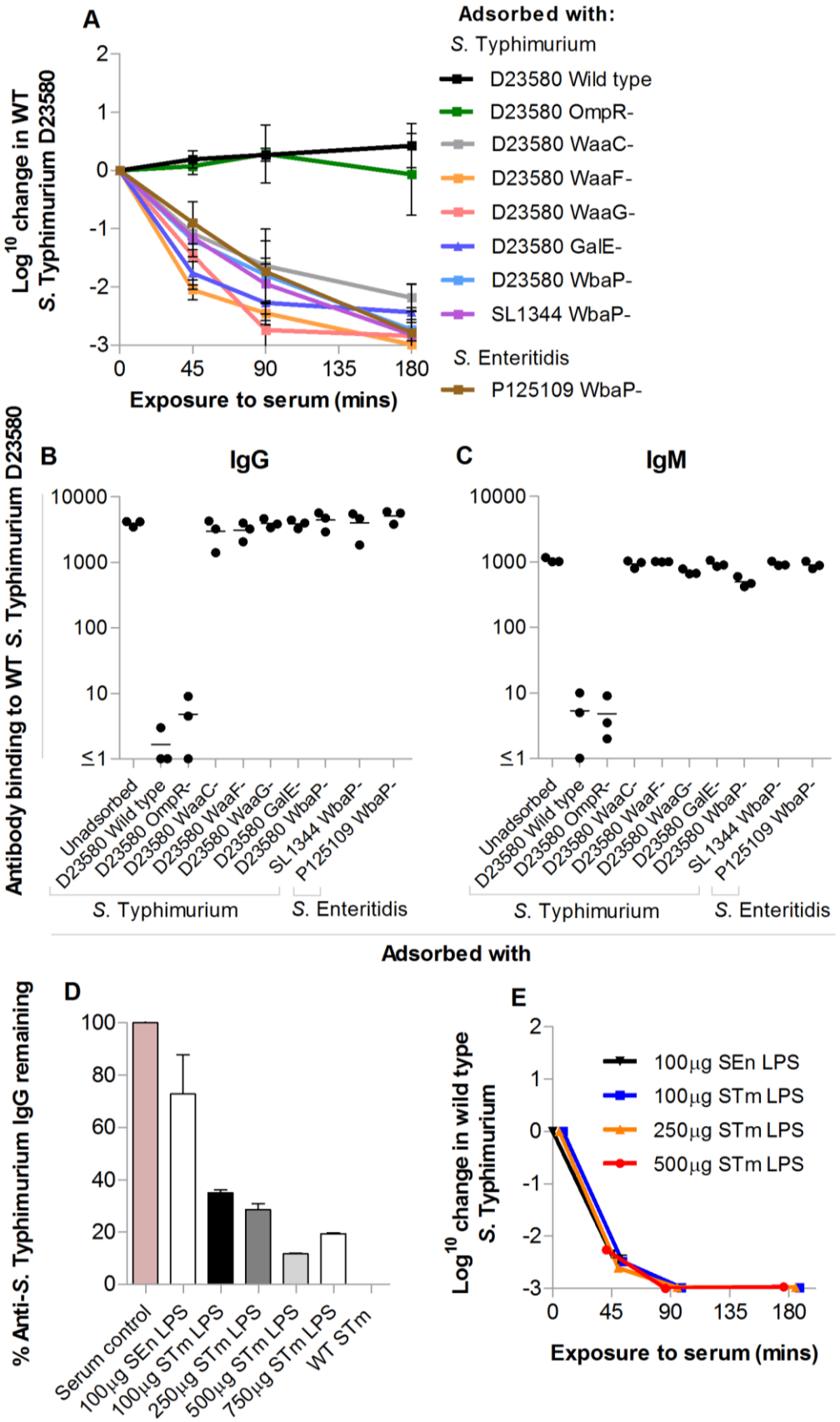


Figure 5.5: Rationale of LPS mutant serum adsorption / antibody capture systems

Figure 5.6: Effect of adsorption with LPS mutant *Salmonellae* or lipopolysaccharide on antibody and complement and bactericidal activity. (A) *In vitro* serum bactericidal assay with 10^6 wild type *Salmonellae*/ml at 45, 90, and 180 minute time points using serum adsorbed with mutant OAg knockout *Salmonellae*. Knockout names indicated in legend. Data are means of three experiments ± SD. (B) IgG or (C) IgM GMFI measured by flow cytometric analysis of binding to smooth wild type *S*. Typhimurium by human serum adsorbed with rough strains of *Salmonella*, all lacking OAg and possessing truncated LPS (knockout indicated on X axis). Each point represents one experiment. Horizontal bars indicate mean values. (D) Percentage of specific anti-*S*. Typhimurium IgG remaining in serum after adsorption with exogenous *S*. Typhimurium or *S*. Enteritidis LPS. IgG GMFI measured by flow cytometric analysis of binding to smooth wild type *S*. Typhimurium. Data are means of three experiments ± SD. (E) Serum bactericidal assay as A, but using serum adsorbed with exogenous *S*. Typhimurium or *S*. Enteritidis LPS. Data are means of three experiments ± SD.

Figure 5.6

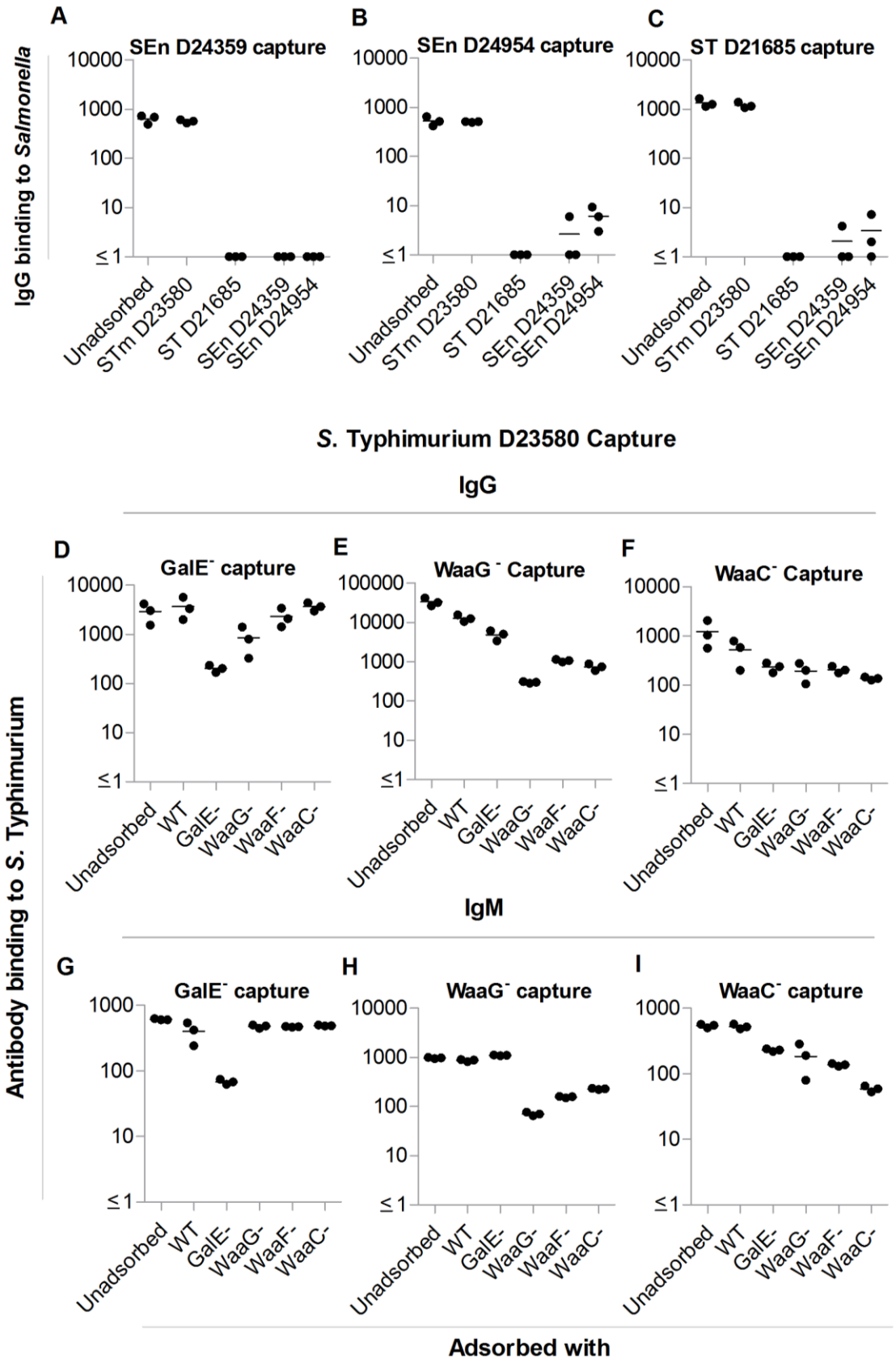

A
Adsorbed with:
S. Typhimurium
D23580 Wild type
D23580 OmpR-
D23580 WaaC-
D23580 WaaF-
D23580 WaaG-
D23580 GalE-
D23580 WbaP-
SL1344 WbaP-
S. Enteritidis
P125109 WbaP-
Log10 change in WT S. Typhimurium D23580
Exposure to serum (mins)
B
IgG
C
IgM
Antibody binding to WT S. Typhimurium D23580
Unadsorbed
S. Typhimurium
S. Enteritidis
Adsorbed with
D
% Anti-S. Typhimurium IgG remaining
Serum control
100µg SEn LPS
100µg STm LPS
250µg STm LPS
500µg STm LPS
750µg STm LPS
WT STm
E
100µg SEn LPS
100µg STm LPS
250µg STm LPS
500µg STm LPS
Log10 change in wild type S. Typhimurium
Exposure to serum (mins)

5.3.6 Adsorption with an OmpR$^-$ mutant removes bactericidal activity and specific antibody as wild type.

In addition to LPS mutants, Fig. 5.6 shows that adsorption (for 3 cycles) with an OmpR$^-$ *S*. Typhimurium strain, deficient in both OmpC and OmpF was able to remove specific IgG and IgM antibodies as well as serum bactericidal activity (Student's *t* test, all $p < 0.0001$). This suggests that antibodies against OmpC and OmpF are not present or not contributing to bactericidal activity in the sera tested.

Figure 5.7: Antibody binding to *Salmonellae* and LPS mutant *Salmonellae* after adsorption with rough and smooth *Salmonellae*. (A) IgG GMFI measured by flow cytometric analysis of binding to *S*. Typhi D21685, *S*. Enteritidis (B) D24954 and (C) D24359, (D) *S*. Typhimurium *galE*$^-$, (E) *waaG*$^-$, or (F) *waaC*$^-$ by either unadsorbed serum (NHS) or serum adsorbed with *Salmonellae* (listed on X axis). (G) IgM measured as above but binding to *S*. Typhimurium *galE*$^-$, (H) *waaG*$^-$, or (I) *waaC*$^-$ Each point represents one experiment. Horizontal bars indicate mean values.

Figure 5.7


A
SEn D24359 capture
B
SEn D24954 capture
C
ST D21685 capture
IgG binding to Salmonella
10000
1000
100
10
≤1
Unadsorbed
STm D23580
ST D21685
SEn D24359
SEn D24954
S. Typhimurium D23580 Capture
IgG
D
GalE⁻ capture
E
WaaG⁻ Capture
F
WaaC⁻ Capture
100000
WT
GalE-
WaaG-
WaaF-
WaaC-
IgM
G
GalE⁻ capture
H
WaaG⁻ capture
I
WaaC⁻ capture
Antibody binding to S. Typhimurium
Adsorbed with

5.3.7 Adsorption with mosaic OAg S. Enteritidis and S. Typhimurium demonstrate that OAg, rather than serovar, is the key determinant of bactericidal activity.

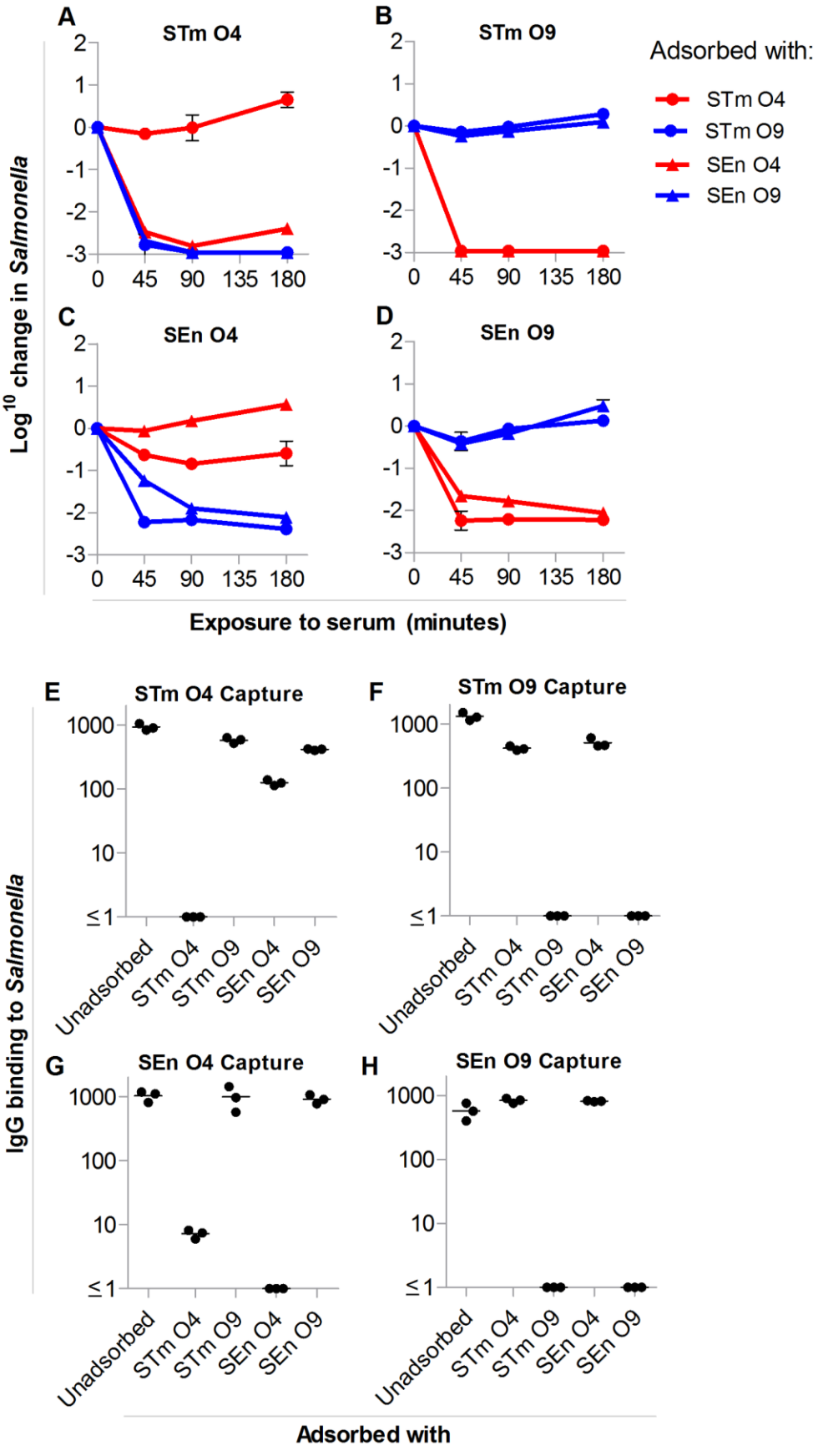
To help clarify the contribution of antibodies against OAg in serum killing, we obtained mutant strains of *S*. Enteritidis and *S*. Typhimurium that expressed either O:4 or O:9 (Table 5.3) and used them to adsorb serum (for 3 cycles). We used these adsorbed sera for serum bactericidal (Fig. 5.8 *A-D*) and specific antibody binding assays (Fig. 5.8 *E-H*) against each of the four strains. Adsorption of bactericidal activity was achieved by *Salmonella* with a homologous serovar and OAg for all strains (Student's *t* test, all $p < 0.0001$). However, when the alternative OAg was expressed, no reduction in killing was recorded, irrespective of serovar. Conversely, expression of the homologous OAg on the heterologous serovar produced a significant adsorption of bactericidal activity (Student's *t* test, both $p < 0.0001$). For O:9 expressing strains, adsorption with heterologous serovar-homologous OAg NTS removed killing as effectively as the completely homologous strain (Student's *t* test, both $p < 0.0001$). For O:4 expressing strains homologous NTS adsorbed killing most effectively, but the homologous OAg-heterologous serovar strains adsorbed killing more effectively than homologous serovar-heterologous OAg strains (Student's *t* test, both $p < 0.0001$). This pattern of removal of bactericidal activity corresponded to the results for adsorption of specific IgG binding.

Table 5.3: *Salmonella* LPS chimera strains

Serovar	Parent Strain	Strain number	O-Antigen	Name
Enteritidis	Thirsk	SL7488	4	SEn T O4
Enteritidis	Thirsk	SL7490	9	SEn T O9
Typhimurium	C5	SL5559	4	STm C5 O4
Typhimurium	C5	SL5560	9	STm C5 O9

Figure 5.8: Effect of adsorption with chimera OAg *Salmonellae* on antibody and complement and bactericidal activity. (A) *In vitro* serum bactericidal assay with 10^6 *S*. Typhimurium O4, (B) *S*. Typhimurium O9, (C) *S*. Enteritidis O4 or (D) *S*. Enteritidis O9 /ml at 45, 90, and 180 minute time points using serum adsorbed with each of the above *Salmonellae* (indicated in legend). Data are means of three experiments ± SD. (E) IgG GMFI measured by flow cytometric analysis of binding to *S*. Typhimurium O4, (B) *S*. Typhimurium O9, (C) *S*. Enteritidis O4 or (D) *S*. Enteritidis O9 by either unadsorbed serum (NHS) or serum adsorbed with *Salmonella* listed on X axis. Each point represents one experiment. Horizontal bars indicate mean values.

Figure 5.8


A
STm O4
B
STm O9
C
SEn O4
D
SEn O9
Adsorbed with:
STm O4
STm O9
SEn O4
SEn O9
Log10 change in Salmonella
Exposure to serum (minutes)
E
STm O4 Capture
F
STm O9 Capture
G
SEn O4 Capture
H
SEn O9 Capture
IgG binding to Salmonella
Unadsorbed
Adsorbed with

5.4 Discussion

We have described and characterised a simple method for adsorption of anti-*Salmonella* antibodies for the generation of specific-antibody free serum that retains complement activity. This is beneficial for research into complement and antibody-mediated mechanisms of immunity against NTS because anti-*Salmonella* antibody-free human serum is rare. While the adsorption method is essentially similar to those first described in the early 1900s (Mackie and Finkelstein, 1930, Pandit, 1923), the consequence of adsorption has been tested more rigorously than was possible for earlier researchers. We employed a range of assays, both bacterial and erythrocyte based, to test complement activity. The specificities and levels of antibodies were investigated using a combination of flow cytometry, ELISA and LPS mutant *Salmonella*. These were used to measure important factors in the context of cell-free complement-killing: specific IgG or IgM is required for killing; cleavage of C3 and deposition of C3b is the central event in all complement activating pathways; and C5b-9 formation indicates the formation of a membrane attack complex required to kill bacteria

The data demonstrates that removal of specific bactericidal antibodies from serum could be achieved by the incubation with live *Salmonella* at 4°C without detriment to the complement activity of the serum. For our SBA system, which conforms to Taylor's recommendations on SBAs (Taylor, 1983) and has been utilised in a number of recent publications on serum bactericidal activity against NTS (MacLennan et al., 2010, Siggins et al., 2011, MacLennan et al., 2008, Gondwe et al., 2010), removal of bactericidal activity required two adsorption cycles. However, as many as five adsorption cycles were performed without a reduction in complement activity.

Antibody adsorption

The specific-antibody depleted serum generated by the described adsorption process was used to investigate the antigen specificities of complement-fixing bactericidal antibodies in human serum. Although adsorption with bacteria is a low-tech approach, use of live bacteria allows the interaction of antibodies with bacterial antigens in their native conformation *in situ* on the bacterial surface. As such, the steric hindrance of antigenic sites (Pollack et al., 1989) in LPS and on the bacteria is potentially closer to that present in physiological conditions during bacteremia. Use of live bacteria avoids processing which can alter immunogenicity. For example, boiling of smooth enterobacteria exposes rough epitopes and permits binding of antibodies to core glycolipids that would otherwise not occur (Aydintug et al., 1989) (Reviewed in (Greisman, 1997)). These advantages mean that adsorption is a productive method of investigation in combination with animal immunisations and more specific column adsorptions using isolated antigens.

We have demonstrated that adsorption of serum bactericidal activity with smooth wild type NTS is serovar specific. Our work supports previous literature on the contribution of *Salmonella* serovar to protection in mice (summarised in Table 5.4) and in adsorption of bactericidal activity from serum (Collins, 1967, Michael et al., 1962). It also adds a novel aspect to prior adsorption reports through the incorporation of a range of clinically relevant African invasive isolate strains as well as laboratory stains. *S*. Typhimurium D23580 was chosen for this study because it is a typical invasive strain of NTS from Africa. It is representative of >90% of NTS strains isolated from bacteraemic individuals in Malawi and has a distinct genotype (Kingsley et al., 2009). Such genotypic differences might also correspond to immunogenic differences and so the use of clinical strains from endemic areas of disease is a valuable strategy for vaccine research.

Host species	Immunisation with serovar (immuno-dominant O-antigen)	Immunisation provides protection against serovar (immuno-dominant O-antigen)	Immunisation provides no protection against serovar (immuno-dominant O-antigen)	Reference
Mouse	*S.* Dublin (O9)	*S.* Dublin (O9)	*S.* Typhimurium (O4)	Lindberg *et al.* (1993)
Mouse	*S.* Enteritidis (O9)	*S.* Enteritidis (O9)	*S.* Typhimurium (O4)	Hormaeche *et al.* (1996)
Mouse	*S.* Gallinarum (O9)	*S.* Enteritidis (O9)	*S.* Typhimurium (O4)	Collins *et al.* (1966)
Mouse	*S.* Typhimurium (O4)	*S.* Typhimurium (O4)	*S.* Enteritidis (O9)	Hormaeche *et al.* (1996) Norris and Bäumler (1999) Hormaeche*et al.* (1991)
Mouse	*S.* Typhimurium (O4)	*S.* Typhimurium (O4)	*S.* Dublin (O9)	Lindberg *et al.* (1993) Coynault and Norel (1999)
Chicken	*S.* Enteritidis (O9)	*S.* Enteritidis (O9)	*S.* Typhimurium (O4)	Cooper *et al.* (1994)
Chicken	*S.* Gallinarum (O9)	*S.* Enteritidis (O9)		Nassar *et al.* (1994)
Chicken	*S.* Gallinarum (O9)	*S.* Gallinarum (O9)	*S.* Infantis (O7) *S.* Typhimurium (O4)	Silva *et al.* (1981)
Human	*S.* Typhi (O9)	*S.* Typhi (O9)	*S.* Paratyphi A (O2)	Simanjuntak *et al.* (1991)

Table 5.4: Lack of cross serovar protection after immunisation with *Salmonella.*
(Kingsley and Baumler, 2000)

Antibody adsorption

The serovars *S*. Enteritidis and *S*. Typhimurium, used in this investigation, are highly conserved in terms of surface features: both express important Omps; C, D and F (Gil-Cruz et al., 2009) and the primary structure of LpfA fimbrial proteins varies by only one amino acid (Nicholson and Baumler, 2001). The LPS and OAg backbone are also well conserved, though their dideoxyhexose branch sugars have an important distinction, with abequose in group O:4 (B) and tyvelose in O:9 (D) (Curd et al., 1998). Other key antigen differences are present in 'H' flagella antigens, however antibodies against flagella are as they are not thought to be relevant to cell-free complement killing (Taylor, 1983), leaving OAg as the key distinction for this aspect of immunity.

Immunisation of mice with rough *Salmonella* mutants, that lack OAg, does not provide protection against wild type parent strains (reviewed in (Greisman, 1997)). Our results agreed with this finding, showing that adsorption of serum with a range of LPS mutant strains that lack OAg failed to remove killing against smooth parent strains. Mosaic OAg *Salmonella* strains have previously been used to look at protection in challenge studies of mice (Hormaeche et al., 1996). The authors found a contribution of OAg to immunity, but ultimately concluded that 'LPS alone cannot fully account for the specificity of protection.' Our work isolates cell-free complement-mediated killing, and for this mechanism of immunity it seems that OAg makes a more significant contribution.

Genetic, immunological and serological evidence also supports an important role for OAg and anti-OAg antibodies in *Salmonella* immunity. OAgs are assembled by a set of sugar synthases and transferases encoded by genes clustered in the chromosomal *wba* locus. The *wba* locus is a hypervariable DNA region across serovars (Reeves, 1993). The high level of variability in the *wba* locus suggests that OAg polymorphism is driven by large selective pressure, from a source such as the immune system (Kingsley and

Baumler, 2000). In addition, analysis of the serologic response against serovars *S*. Typhimurium and *S*. Enteritidis shows that the immune-dominant determinants of their LPS are O:4 and O:9 antigen respectively (Smith et al., 1995, Barrow et al., 1992, Nicholson and Baumler, 2001).

However, adsorption with exogenous LPS failed to remove bactericidal killing. Use of *S*. Typhimurium LPS did lower antibody titres against *S*. Typhimurium, but not to the same extent as after adsorption with wild type *S.* Typhimurium. It is probable that an artefact of the LPS adsorption experiment is preventing full removal of specific antibodies. For adsorptions with bacteria, the bacteria itself and therefore all bound antibodies are removed from the serum after incubation. However, for adsorptions with LPS, the LPS and its bound antibodies remain in the serum for the subsequent assay. Therefore, dissociation of the antibodies from the LPS and binding to the bacteria can occur. Alternatively, it could be that the processing of the isolated LPS means it does not resemble the native form on the bacterial surface and as such does not adsorb the relevant antibodies. The amphiphilic nature of LPS may lead to self-aggregation when is not anchored, which could mask some OAg epitopes. There also could be differences in the quantity of OAg per weight of LPS between the exogenous preparation and on bacteria. NTS OAg consists of a repeating unit with typically bimodal distribution between ‘long’ and ‘very long’ lengths (Bravo et al., 2008), the lengths of these OAg repeats and also the microheterogeneity of LPS, due to factors such as acetylation, can vary and may impact antibody binding.

The majority of data reported here strongly suggests that antibodies against OAg are the key effectors of bactericidal activity in normal human serum. This may be positive news for vaccine design. It appears that the anti-OAg inhibitory antibodies discovered in a

sub-set of HIV-infected individuals, which brought doubt to the use of an OAg based vaccine, might be inhibitory because of another factor other than antigen specificity. The lack of bactericidal activity of these antibodies and sera could be a result of HIV-infection interfering with antibody development. Although, there are a number of reports in the literature from the pre-HIV era detailing patients with chronic infections with Gram-negative bacteria, including NTS, whose sera lacked bactericidal activity against the homologous bacteria (Waisbren and Brown, 1966, Adler, 1953). The inhibitory factor in these cases was a large titre of antibodies against the bacteria and so perhaps it is the excess of antibodies that results in inhibition of bactericidal activity. Such an effect has long been acknowledged, though not explained, in *in vitro* SBA systems (Muschel et al., 1969).

Experiments with LPS mutants did not provide evidence that antibodies to antigens other than OAg were key contributors to bactericidal component of the sera we tested. A mutant lacking OmpC and OmpF (but not OmpD) did not influence the effect on adsorption of bactericidal activity. However, antibodies against Omps have been shown to be both bactericidal (MacLennan et al., 2010) and partially protective against *Salmonella* in the mouse (Gil-Cruz et al., 2009). As such, it is possible that, like with bactericidal anti-factor H-binding protein antibodies against meningococci, the levels of antibodies present in normal serum (without vaccination) are insufficient to produce a bactericidal effect (Pizza et al., 2008). If this is the case, then it may in fact be a positive for hopes of an Omp-based vaccine generating pan-serovar-specific protection. The absence of a prominent bactericidal anti-Omp antibody population may indicate that these antigens they are under less selective pressure by the host immune system. If this was so it would increase the chance of a higher degree of conservation in Omp antigenic sites. The sera we used was from healthy adult donors and all contained

bactericidal activity and antibodies specific against *S*. Typhimurium D23580, though none had a confirmed history of *Salmonella* disease. It may be that the lack is due to how B cell populations respond to different antigens. Perhaps proliferation of long-lived plasma cells is greater in response to OAg than Omps and convalescent serum might show a different antibody profile with a natural role for bactericidal anti-Omp antibodies.

An overview of antibody-targeting against protein antigens in systemic salmonellosis using sera from Malawian children with *Salmonella* bacteraemia and immune mouse serum has recently been generated (Lee et al., 2012). To support this, adsorption analysis of such sera could help to determine specifically which epitopes complement-fixing bactericidal antibodies are generated against. This would build upon the work presented here and towards a vaccine that fully harnesses the potential contribution of cell-free complement-mediated immunity to immunity.

Large titers of anti-OAg antibodies that inhibited bactericidal activity against NTS in a sub-set of HIV-infected Africans have recently been described (MacLennan et al., 2010). Because a glycoconjugate OAg-based vaccine is an obvious candidate for a NTS vaccine in Africa, clarification of the effector functions of antibodies against OAg is needed. This study clearly demonstrates the bactericidal capacity of complement-fixing antibodies against the OAg of invasive African *Salmonella* LPS. Moreover, we report that such antibodies can be major effectors of antibody-dependent complement-mediated bactericidal activity against invasive African isolates of *Salmonella*. These findings support the development of an OAg based vaccine.

5.5 Conclusion

Nontyphoidal strains of *Salmonella* are a major cause of fatal bacteremia in Africa. Antibodies are important for protection against *Salmonella* and an important role has been found for antibodies in cell-free complement-mediated bactericidal activity against *Salmonella* in Africans. Determining the antigen targets of these bactericidal antibodies is a key consideration in developing a vaccine against nontyphoidal *Salmonellae* and the OAg of lipopolysaccharide is an obvious candidate for a glycoconjugate vaccine. However, the discovery of inhibitory anti-OAg antibodies in a subset of HIV-infected patients has cast doubt on the suitability of OAg as a vaccine agent. We used a range of invasive African *Salmonella* isolates, laboratory *Salmonella* strains and *Salmonella* LPS mutants to adsorb human serum in order to investigate the targets of bactericidal complement-fixing antibodies. The effect of adsorption on specific-antibodies and complement binding were assessed by flow cytometry and ELISA, and hemolytic and bactericidal activity of sera was determined. Adsorption of serum with whole *Salmonella* produced an effective serovar-specific removal of both antibodies against surface elements of *Salmonella* and bactericidal activity. Adsorption did not adversely affect the activity of complement. *galE*$^{-}$, *waaC*$^{-}$, *waaF*$^{-}$, *waaG*$^{-}$, and *wbaP*$^{-}$ mutants, which all lack OAg, failed to remove *Salmonella* serovar-specific antibodies and killing against their parent strains. *S*. Typhimurium and *S*. Enteritidis OAg chimera strains, expressing either native or heterologous OAg (O:4 or O:9) demonstrated that removal of antibodies and bactericidal activity against O:9-expressing strains was entirely dependent on the OAg of the adsorbing strain rather than other serovar-specific antigens. O:4-expressing strains showed a similar, but less pronounced effect. These data indicate that anti-OAg antibodies are major effectors of complement-mediated killing of invasive African nontyphoidal *Salmonella* by normal human serum. These findings support the development of an OAg based vaccine.

Chapter 6

Analysis of antibody and complement binding to *Salmonella* by confocal and electron microscopy

Electron microscopy work represents collaboration with Dr. David Goulding in Professor Gordon Dougan's laboratories at the Wellcome Trust Sanger Institute in Cambridge.

6.1 Introduction

Killing of Gram negative bacteria by complement requires formation of a stable C5b-9 membrane attack complex on the outer membrane (OM) (Joiner et al., 1982a, Joiner et al., 1982b, Inoue et al., 1968) and death is dependent on damage to the inner membrane (IM) (Wright and Levine, 1981, Feingold et al., 1968). Therefore, deposition of terminal complement components at locations too far from the bacterial membrane surface to exert killing has been theorised as a reason for the resistance of some bacterial strains to serum-killing (Taylor, 1983). As such, the 'long' and 'very long' chain O-antigens (OAg) of *Salmonellae* (Murray et al., 2005, Bravo et al., 2008) have been suggested to contribute to inhibition of serum-killing by binding anti-OAg antibodies at their distal end and blocking access to internal sites. Furthermore, the recent description of a sub-set of HIV-infected patients who had large titres of anti-OAg antibodies which inhibited serum-killing (MacLennan et al., 2010) was consistent with this hypothesis. Yet, we reported in chapter 5 that anti-OAg antibodies were a major source of bactericidal antibodies in the healthy adult sera we examined. Although anti-OAg antibodies are not inhibitory *per se*, the binding of antibodies and associated complement deposition may sterically hinder access to bactericidal sites on the *Salmonellae* in serum with a high titre of anti-OAg antibodies. Alternatively, (or additionally) complement may be exhausted by this deposition in non-bactericidal sites (MacLennan et al., 2010).

Here, we have developed a confocal microscopy assay to image antibody binding and complement deposition on *Salmonellae*. We have then used transmission electron microscopy (TEM) to determine complement and antibody binding requirements for effective serum-killing of an African *S*. Typhimurium clinical isolate. In addition, we have imaged this serum-mediated damage against NTS in high resolution. Finally, we have compared the activity of both bactericidal and inhibitory HIV-infected sera to identify mechanisms responsible for inhibition of serum-killing. We hypothesise that HIV-infected inhibitory serum shows differences in the location and quantity of deposition of antibodies and complement on *S*. Typhimurium compared to bactericidal sera.

6.2 Statistical Analysis

Comparisons of two groups were performed using two tailed student's *t* tests. All statistical tests were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA).

6.3 Results

6.3.1 Antibody and complement deposition confocal microscopy assay

To develop a confocal assay for imaging of antibody and complement binding to *Salmonellae*, we adapted our flow cytometry assays (section 2.3.1-2). Only minor changes were made, most notably the bacterial solution was slightly concentrated post serum incubation. The sample was applied to charged glass slides and stained with DAPI. Dry and wet samples were prepared as described in the methods. Figure 6.1 shows that bacteria were detectable either by transmitted light (A) or fluorescence (DAPI) (B, C, and D). The confocal system was somewhat less sensitive than flow cytometry. While the abundant C3 binding was easily detectable, the less plentiful IgG, IgM and C5b-9 binding and deposition were only clearly distinguishable from

background levels in donor serum that contained the highest levels of these components (IgG shown in Fig. 6.2, other data not shown). There was no discernible increase in the magnitude of binding associated with using wet samples and so dry samples were chosen for further usage due to their relative ease for capturing images with a suitable quantity and distribution of bacteria in the same focal plane.

6.3.1.2 Influence of fixative solution

EM generally uses harsher fixatives that flow cytometry. In order to identify any potential problems with the interaction of the fixatives and bacteria, antibody and antibody/complement binding, we tested a typical EM fixative solution, common for pre-embedding transmission EM (TEM) 2% paraformaldehyde + 0.1 – 0.5% glutaraldehyde, with FITC conjugated anti-C3 and anti-IgG antibody using our confocal and flow cytometry data protocols. The flow cytometry showed little difference in binding between fixatives. The confocal images revealed more intense fluorescence with the formaldehyde solution than the glutaraldehyde solutions, which was due to high background values. It is well known that glutaraldehyde generates free aldehyde groups that can result in non-specific binding with antibodies (Kiernan, 2000). However, samples that were fixed only after labelling showed similar effects suggesting there was influence with the fluorochrome and that the effect was a confocal-specific problem

The timing of fixation had a large effect of fluorescence intensity. Prefixing the *Salmonellae* before incubation with serum reduced the signal recorded on confocal images and by flow cytometry for both C3 (Fig. 6.3) and IgG (data not shown). The effect was particularly prominent for C3 binding. It is likely that the fixation limits binding of antibody and complement by altering epitope conformations and samples should be fixed after incubation with serum.

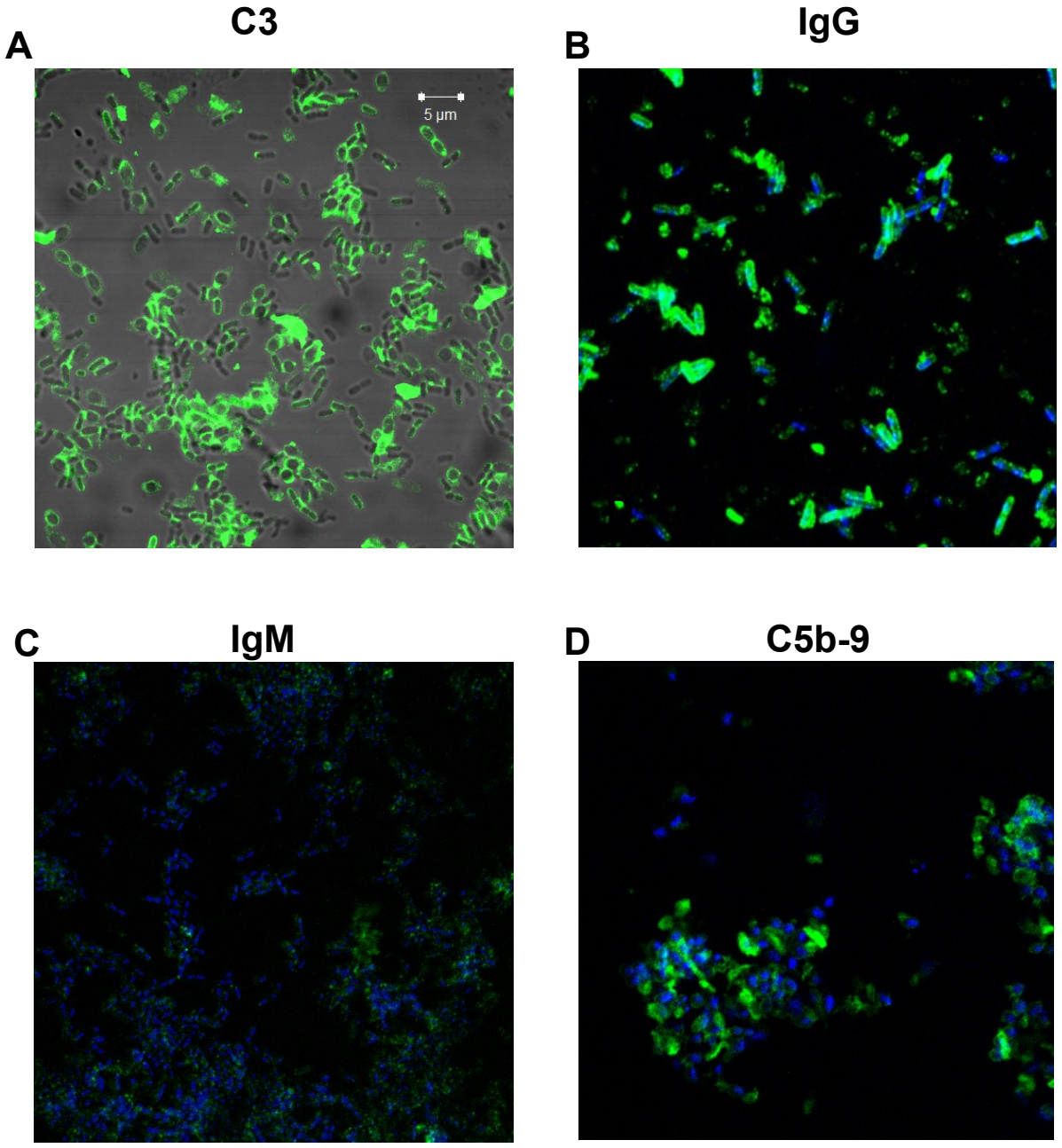


Figure 6.1: Antibody and complement binding on *Salmonellae* imaged by confocal microscopy. Binding of IgG, IgM and deposition of C3 and C5b-9 on *S.* Typhimurium D23580 by confocal microscopy. $2x10^8$ *Salmonellae* were incubated in healthy adult donor serum for 10 minutes. Negatives were incubated in PBS and used to determine settings for positive images. *Salmonellae* appear blue (DAPI) (B,C,D) or are imaged by transmitted light (A). C3 (A), IgG (B), IgM (C), and C5b-9 (D) are green (FITC-conjugated anti-human antibodies).

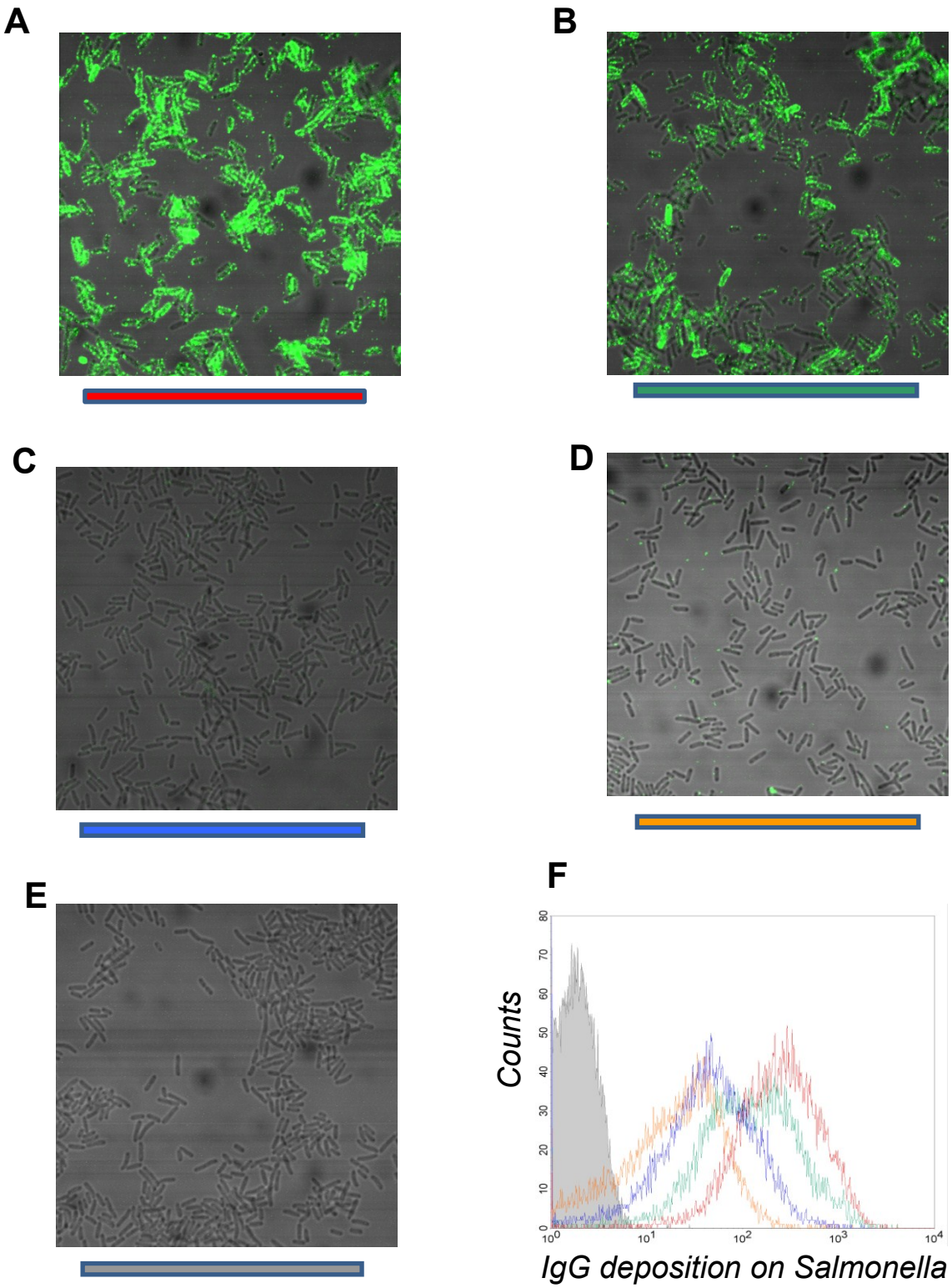


Figure 6.2: Variation in binding of IgG on STm D23580 between donor sera. 2x10^8 *S.* Typhimurium D23580 were incubated in serum from four healthy adult donors (A-D) for 10 minutes. Negative (E) was incubated in PBS. Analysis by confocal microscopy. *Salmonellae* imaged by transmitted light. IgG is green (FITC-conjugated anti-human IgG antibody). (F) Overlayed histograms of IgG binding of same four donor sera compared to baseline (grey line and shading) determined by flow cytometry.

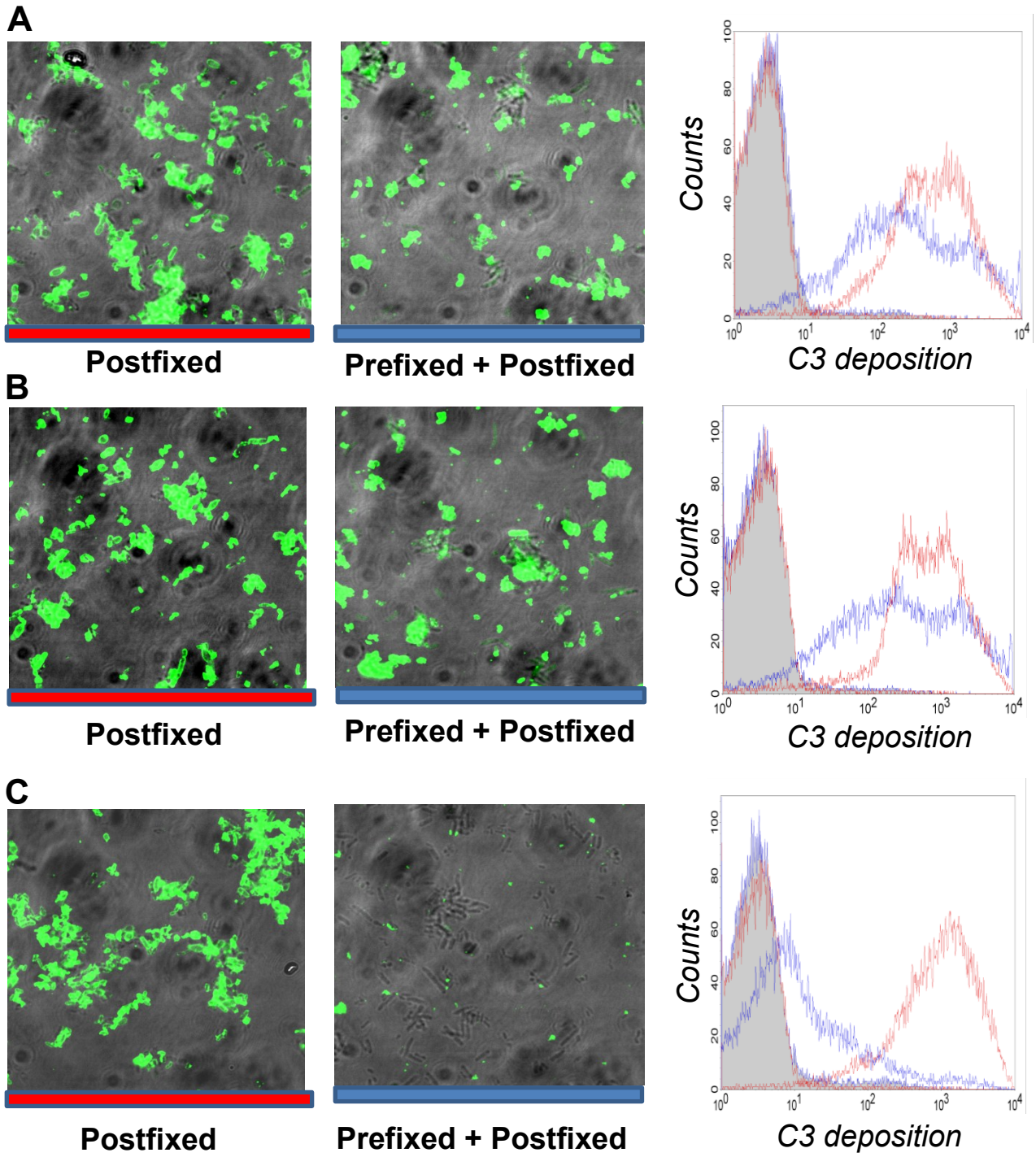


Figure 6.3: Effect of 'prefixing' bacteria on imaging C3 deposition on salmonellae by confocal microscopy. $2x10^8$ live (left panel) or prefixed (middle panel) *S.* Typhimurium D23580 incubated in serum. Samples were fixed in 2% paraformaldehyde PBS with (**A**) 0.1% (**B**) 0.2% or (**C**) 0.5% glutaraldehyde. Negative incubated in PBS and used to determine settings for positives. *Salmonellae* imaged by transmitted light. C3 is green (FITC-conjugated anti-human antibody). Right panel: histograms of C3 deposition of serum compared to negative baseline (grey line and shading) determined by flow cytometric analysis. Flow cytometry samples identical to confocal sample. Coloured lines correspond to coloured bar indicating fixative treatment.

6.3.2 Electron microscopy imaging of serum-mediated damage of *Salmonellae*

A resin pre-embedding TEM technique (section 2.5.2) was used to visualise the effects of incubating healthy Malawian pooled sera with clinical African isolate *S*. Typhimurium D23580. Figure 6.4 shows that observed damage was not uniform and presented in a number of stages. Healthy *Salmonellae* were also observed even in bactericidal serum (Fig. 6.4 A), though they were in the minority. Of the damaged cells, severe plasmolysis (Fig. 6.4 C), contraction and disruption of cytoplasm integrity (Fig. 6.4 D), breaks in both outer and inner membranes (Fig. 6.4 E-H), formation of ghost cells and spheroplasts (Fig. 6.4 I-K), and finally, complete destruction of the bacterial cell, leaving only curled membrane debris (Fig. 6.4 L) were all prominent. Plasmolysis did not always occur in a regular distribution around the cell, contrary to as is commonly observed with hypertonic solutions. Many *Salmonellae* had spheres of material in the widened periplasmic space (Fig. 6.4 C) and in some *Salmonellae*, the cytoplasm within the IM contracted away from the membrane edge. In other *Salmonellae* the entire cytoplasm contents, including the associated IM, escaped through a hole in the OM (Fig. 6.4 G, H). The exit holes in the OM in these cases are large, up to around 500nm in size, and are likely forced open by the passage of the cytoplasm. Bacteria lacking an OM can survive in balanced solutions, but in serum damage to the OM of *Salmonellae* appears to lead to lysis. In other *Salmonellae,* diminishment or disappearance of the cytoplasm precedes extensive OM damage and leads to the formation of membrane-only structures termed ghosts. It is likely that both ghosts and *Salmonellae* with extensively damaged OMs are not stable in serum and that they disintegrate into the tangles of membrane also visualised (Fig. 6.4 L).

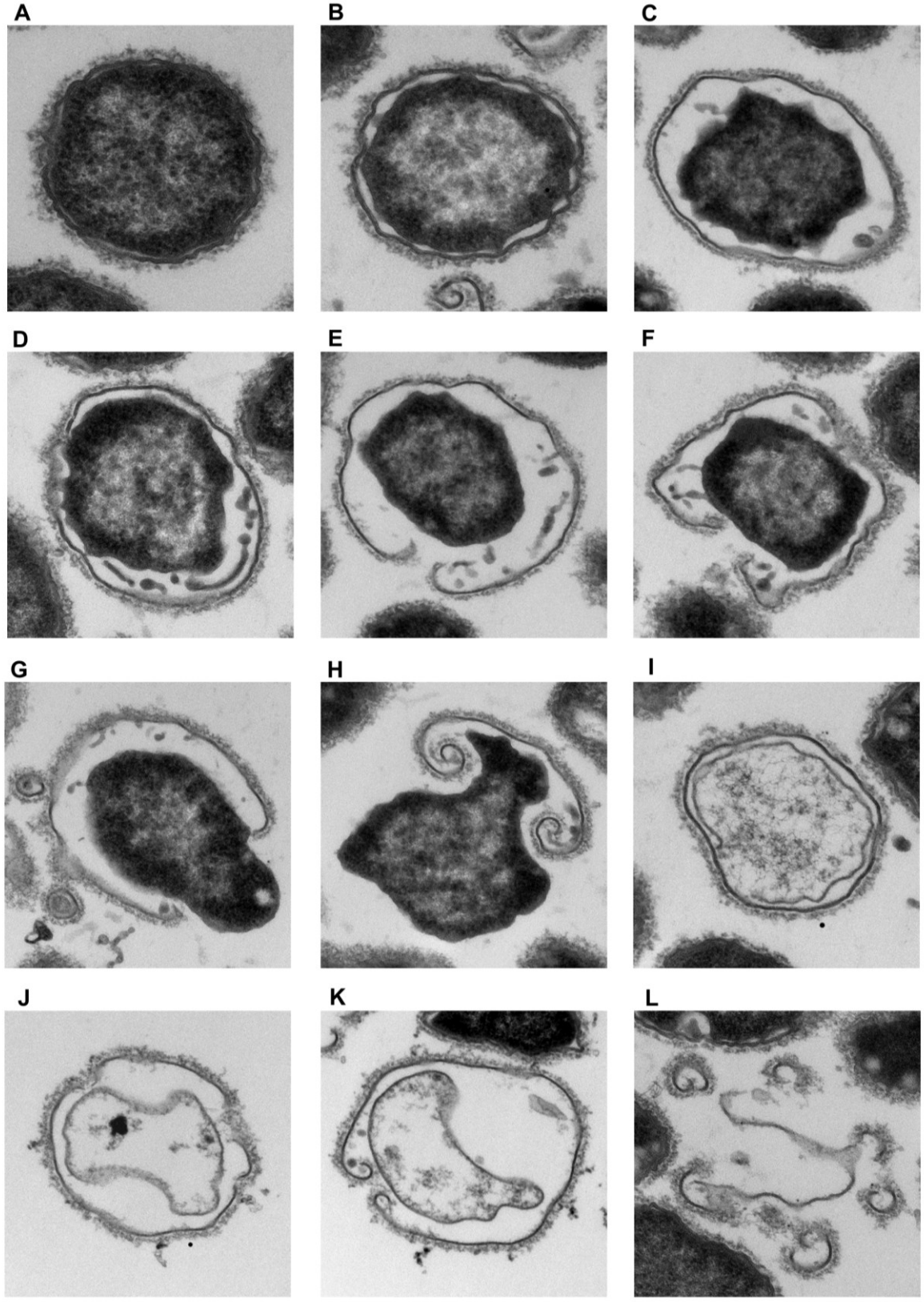


Figure 6.4: Serum-mediated damage to *Salmonellae*

TEM of pre-embedded *S*. Typhimurium D23580 after incubation in neat pooled Malawian sera. Images are representative of commonly observed damage in samples.

6.3.3 Comparison of damage to *Salmonellae* inflicted by bactericidal and HIV-infected inhibitory sera

Incubation of *S*. Typhimurium D23580 with HIV-infected inhibitory serum did not to lead to the same degree of damage described with bactericidal serum. The majority of *Salmonellae* lacked any physical signs of damage, which is consistent with the lack of killing reported from SBAs with the inhibitory serum (MacLennan et al., 2010). A minority of *Salmonellae* displayed damage, but there was a clear difference between bactericidal and inhibitory sera (Fig 6.5). No damage was observed in PBS controls.

6.3.4 Presence of 'blocking layer' on *Salmonellae* after serum-incubation

When a pre-embedding method (section 2.5.2) was used for TEM, *Salmonellae* incubated with neat serum showed a distinctive layer around the OM. This layer seemed to impede labelling of antibody and complement binding with secondary antibodies. C3 (Fig 6.6 D) and C5b-9 (data not shown) labels were seen on the outer edge of the layer, while IgG labelling was absent (Fig. 6.6 B). Dilution of the serum to 1 in 10 with PBS (10% serum is non-bactericidal in the SBA used, described in section 2.2) led to the disappearance of the layer on the majority of *Salmonellae* observed. In addition to this, C3 and C5b-9 deposition was no longer present (data not shown). In contrast, IgG labelling was present on 10% serum-incubated *Salmonellae*, in, or close to the OM (Fig 6.6 C). It is likely that the layer is made up from a build-up of deposition of complement components on the surface of the *Salmonellae*. To avoid this problem, we used a post-labelling method using cryosections (section 2.5.3). In which *Salmonellae* are sectioned after incubation with serum, but crucially, before the addition of secondary gold-conjugated antibodies. Although the layer should still remain, the secondary antibodies have access to internal areas of the bacterium and layer above and below the section and so detection of antibodies and complement bound to the *Salmonellae* is not impeded

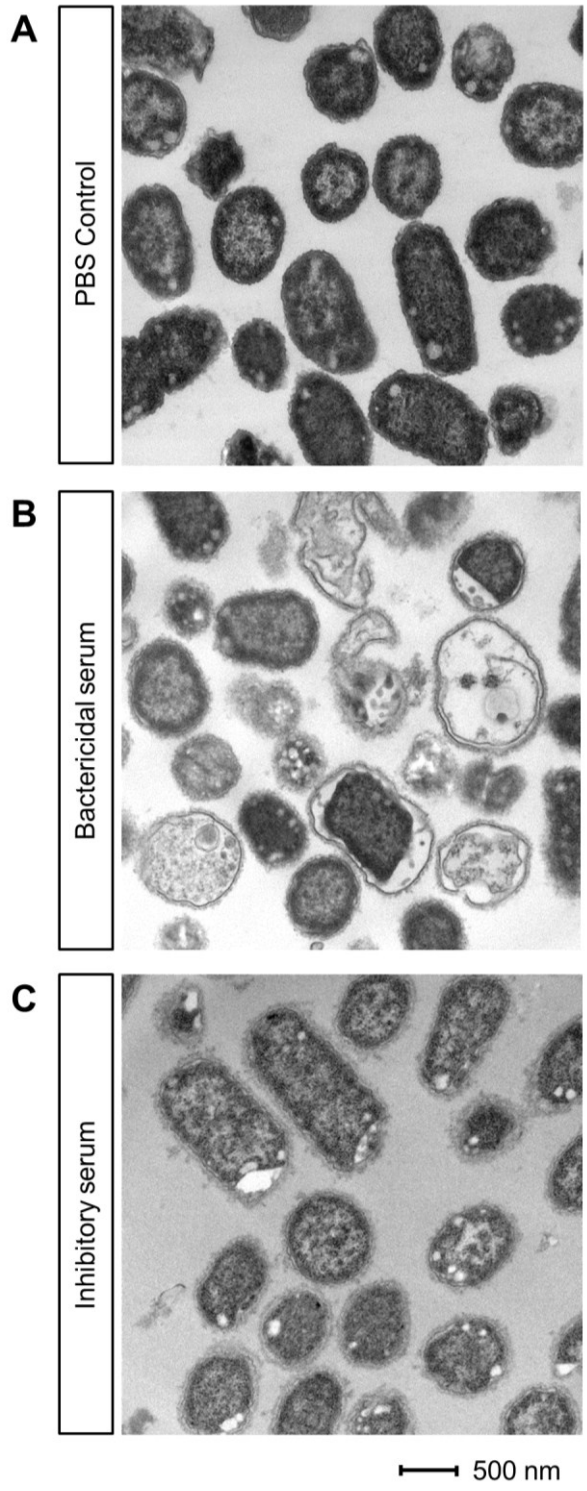


Figure 6.5: Serum-mediated damage to *Salmonellae*
TEM of *S*. Typhimurium D23580 after incubation in either PBS, neat pooled Malawian sera (Bactericidal serum) or HIV-infected inhibitory serum (Inhibitory serum). Images are representative of numerous observations.

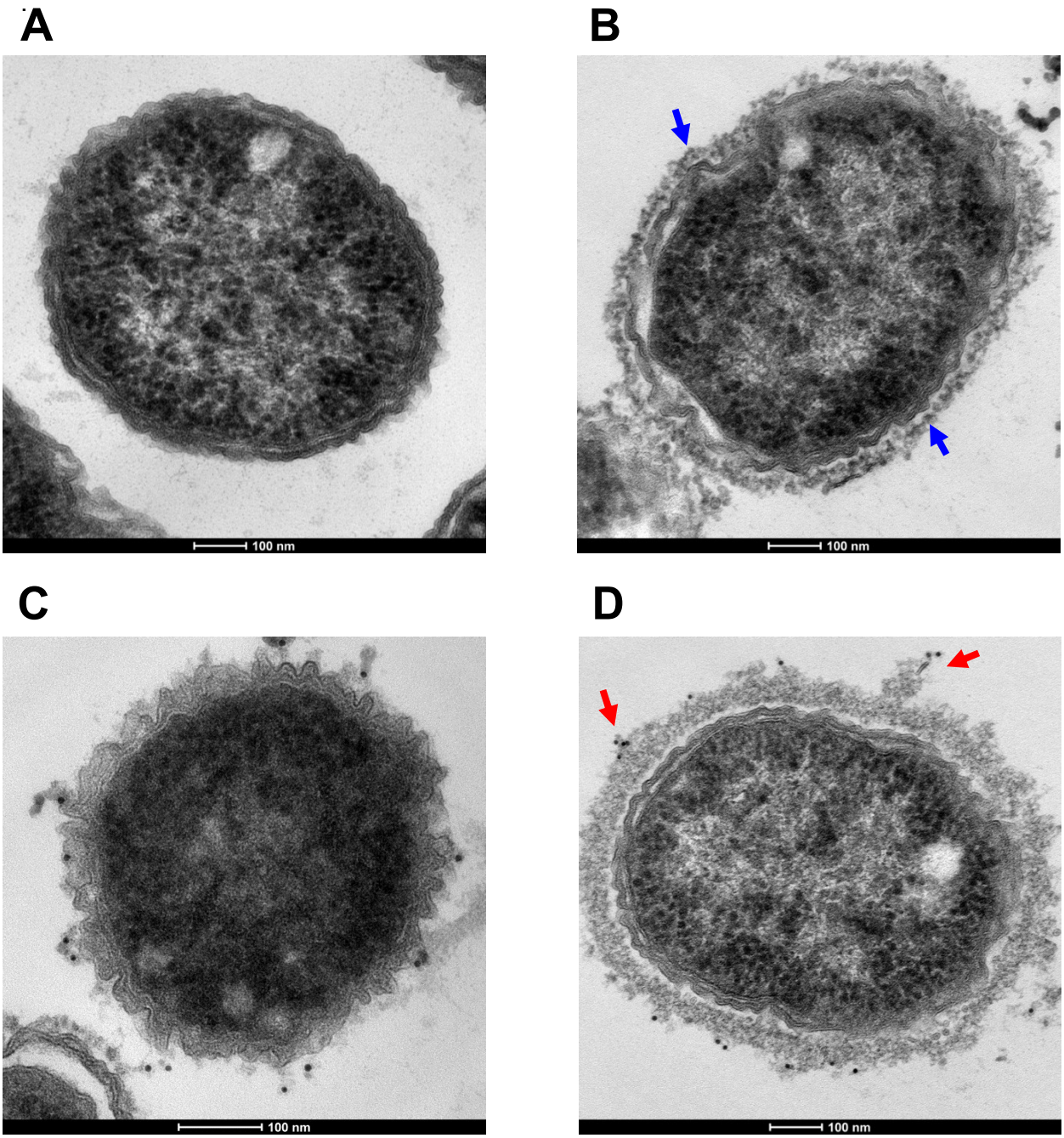


Figure 6.6: Effect of serum-deposit layer on labelling of *Salmonellae* with C3 and IgG. *S*. Typhimurium D23580 labelled with anti-IgG, incubated with PBS (A) or neat (B) or 10% serum (C). (D) Incubated with neat serum, labelled with C3. Immunogold labels have 10nm diameter. Red arrows highlight layer deposited by serum and associated anti-C3 antibody binding. Blue arrows show deposited layer and general absence of anti-IgG label binding.

6.3.5 Comparison of location of antibody binding and complement deposition on *Salmonellae* after incubation with bactericidal or inhibitory sera

The ultrastructure of cryosections was well preserved and the antibody labelling worked well, being very clean with high affinity. The controls were clear both of label and the complement layer.

Complement component C3 (Fig 6.7 A), membrane attack complex (C5b-9) (Fig 6.7 B) and IgG (Fig 6.8) were all observed on both the OM and the IM and even in the cytoplasm, as well as distal sites from the bacterial membrane of *Salmonellae* in both inhibitory and bactericidal sera. Overall, there were no striking differences in the location of complement deposition and antibody binding on *Salmonellae* between bactericidal and inhibitory sera.

However, whereas damaged *Salmonellae* all had C3 and C5b-9 deposition on the OM and IM, many healthy *Salmonellae* did not. Some healthy cells did show IM C3 deposition, though this was generally of a low level and probably represented the start of damage progression. The heaviest labelling of both complement components and IgG seemed to occur on damaged, particularly lysed, *Salmonellae* (scoring counts are being performed to statistically confirm these observations at the time of writing, as well labelling of both IgA and IgM antibodies). Furthermore, contraction of the IM seemed to be associated with C3 deposition on the IM. Where plasmolysis proceeded predominantly from one side, or area, the C3 deposition was focussed in that area on the leading edge of the withdrawing IM. This suggests the origin of damage allows an influx of complement components from serum from outside into the interior of the *Salmonella* cell.

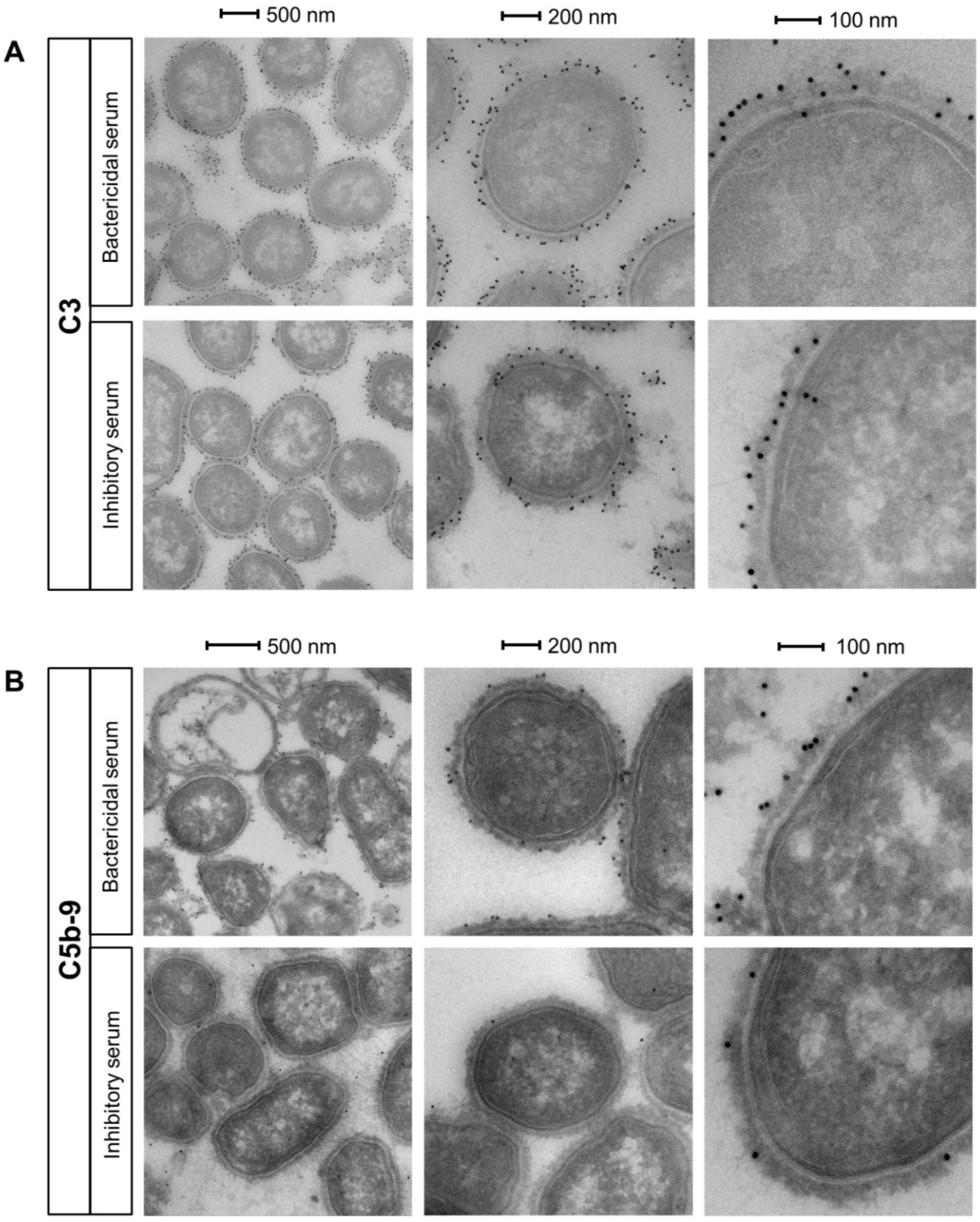


Figure 6.7: Deposition of complement on *Salmonellae*
TEM cryosections of *S*. Typhimurium D23580 after incubation in neat pooled Malawian sera (bactericidal serum) or HIV-infected inhibitory serum (inhibitory serum) labelled with anti-C3 or C5b-9 antibody. Immunogold labels have a 10nm diameter. Images are representative of numerous observations.

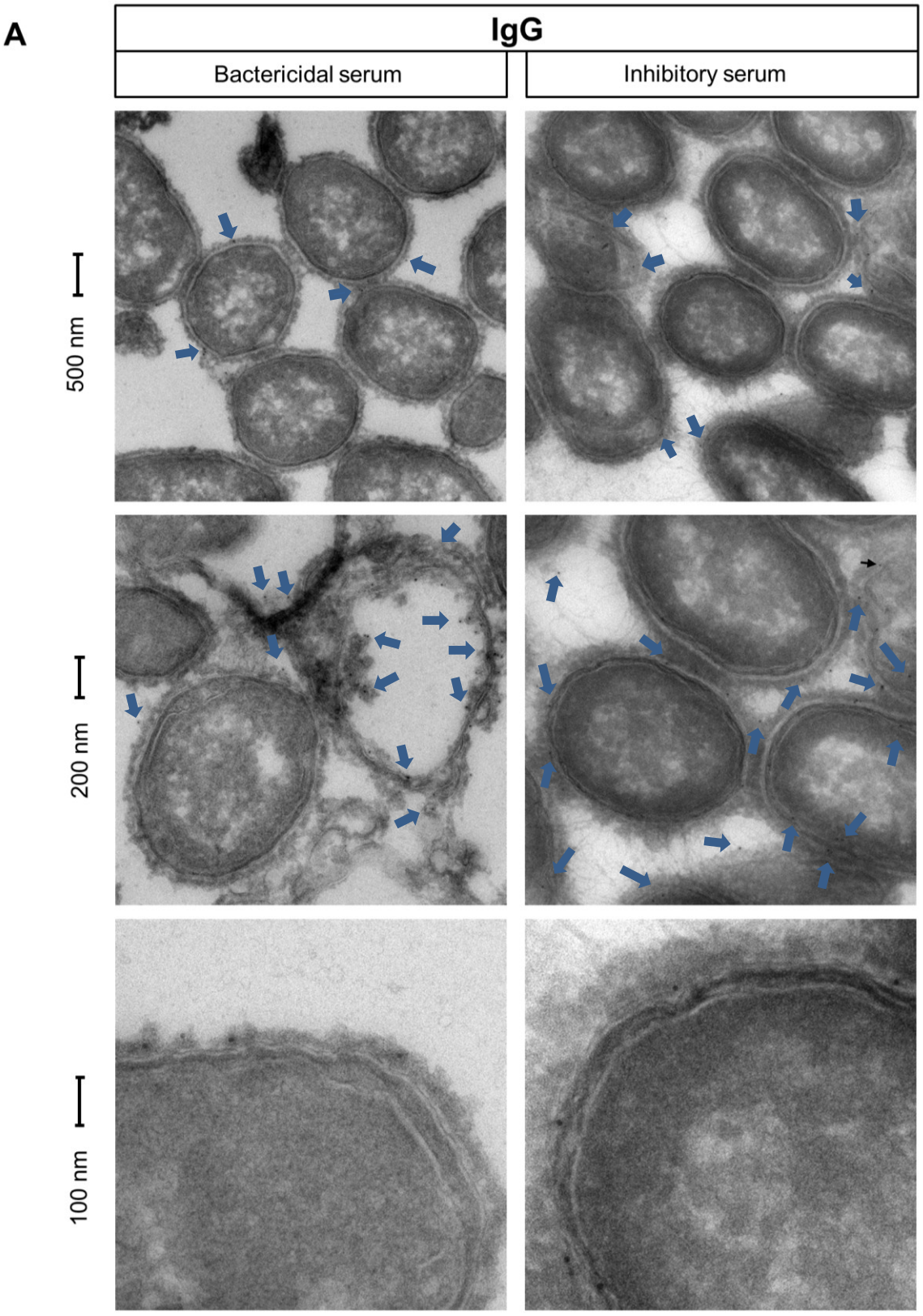


Figure 6.8: Binding of IgG to *Salmonellae*
TEM cryosections of *S*. Typhimurium D23580 after incubation in neat pooled Malawian sera (bactericidal serum) or HIV-infected inhibitory serum (inhibitory serum) labelled with anti-IgG antibody. Immunogold labels have a ~5nm diameter and some are indicated by blue arrows. Images are representative of numerous observations.

6.3.6 Comparison of quantity of antibody binding and complement deposition on *Salmonellae* after incubation with bactericidal or inhibitory sera

We scored the levels of deposition of C3, C5b-9 and IgG binding on randomly selected *Salmonella* in order to compare levels between inhibitory and bactericidal sera (Fig 5.9 A). As expected, binding of IgG was higher in HIV-infected inhibitory serum (Student's *t* test $p < 0.001$). However, contrary to results gained with flow cytometry (Fig 5.9 B), deposition of both C3 and C5b-9 on *Salmonellae* was reduced in HIV-inhibitory serum in comparison to bactericidal serum (Student's *t* test both $p < 0.001$). These results suggest that the lack of ability for inhibitory serum to kill Salmonellae is due to a reduced ability to deposit complement on *Salmonellae*. Scoring for IgA and IgM binding to *Salmonellae* and a detailed analysis of the complement deposition and antibody binding differences between damaged and undamaged *Salmonellae* is ongoing at the time of writing.

6.3.7 Evidence for dissociation of complement membrane attack complexes from *Salmonellae* incubated in inhibitory serum

Clumps of material, heavily labelled with C5b-9, distant from the surface of the bacterial OM were observed on some *Salmonellae*. These appeared more often in samples incubated in HIV-infected serum and may be evidence of dissociating C5b-9. However, firm evidence has not yet been collected.

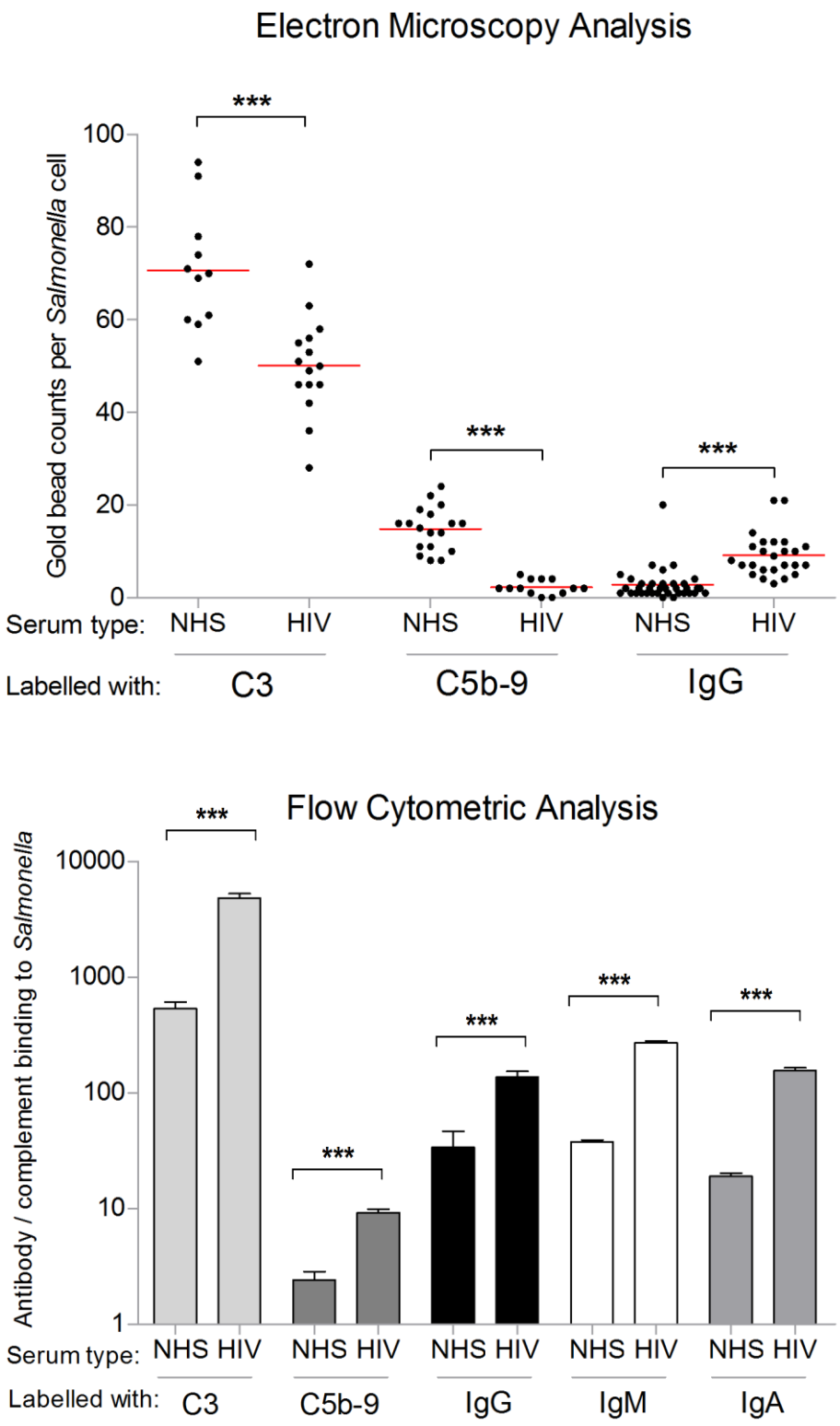


Figure 6.9: Quantification of complement and antibody binding to *S.* Typhimurium (A) C3, C5b-9 and IgG deposition on *S.* Typhimurium D23580 by bactericidal (NHS) and inhibitory (HIV) sera, measured by counting of gold-conjugated antibodies on randomly selected *Salmonellae* using TEM. (B) C3, C5b-9, IgG, IgM and IgA deposition (GMFI) measured by flow cytometry analysis of binding to *S.* Typhimurium D23580 by bactericidal (NHS) and inhibitory (HIV) sera. Data are means of three experiments ± SD. *** represents statistical significance of $p < 0.001$ determined by Student's *t* test.

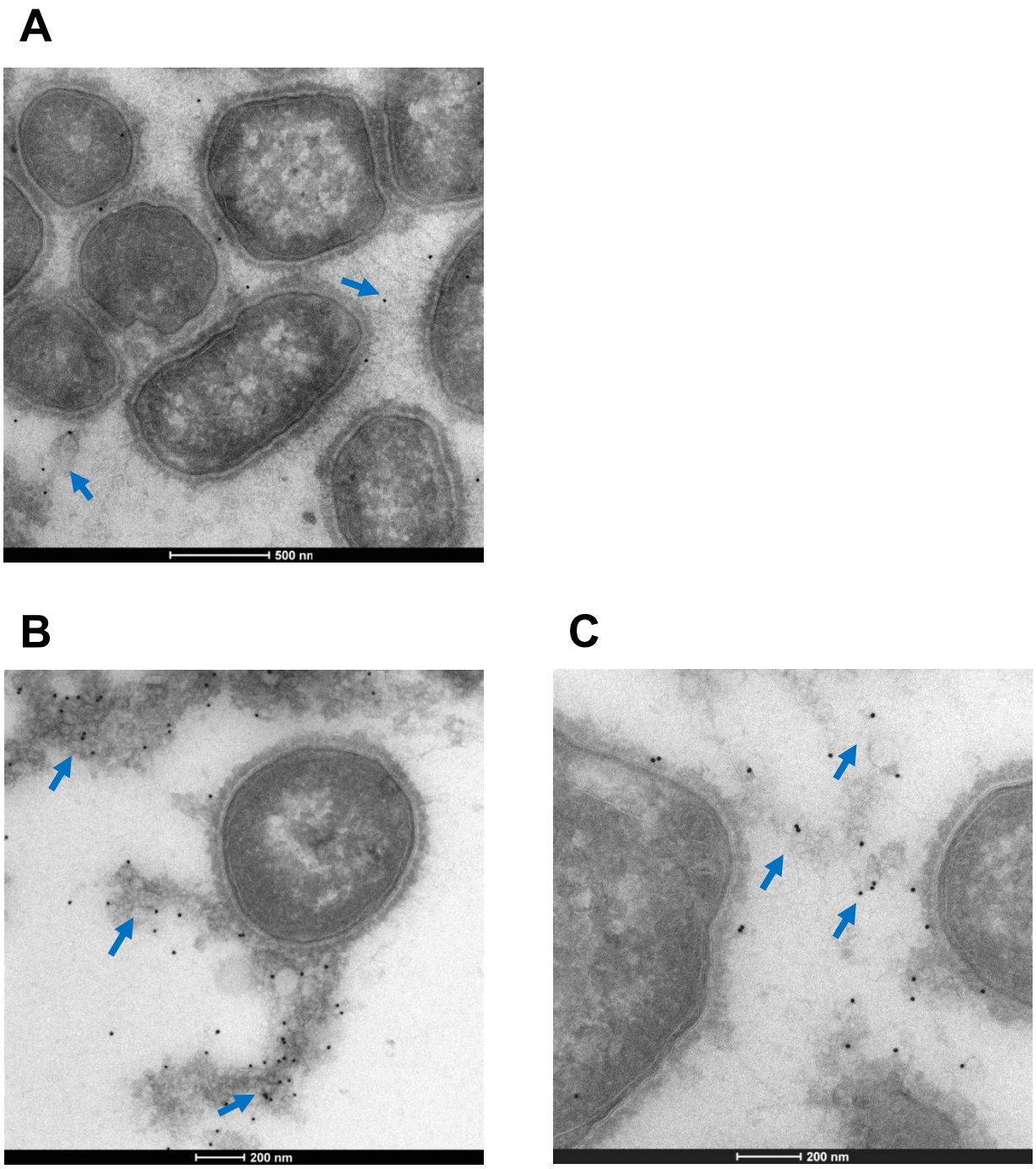


Figure 6.10: Potential dissociation of C5b-9 deposition from *Salmonellae* in inhibitory serum. *S*. Typhimurium D23580 incubated in HIV-infected 'inhibitory' serum. Cryosections labelled with anti-C5b-9, Imaged by TEM. Immunogold labels have a 10nm diameter. Blue arrows highlight some sites of C5b-9 deposition distant from the bacterial membrane.

6.4 Discussion

The primary purpose of the EM study was to look at the differences in antibody and complement binding on *Salmonellae* between normal bactericidal and HIV-infected inhibitory sera, in order to verify a role for anti-*Salmonella* 'blocking' antibodies, which was suggested by prior research (MacLennan et al., 2010). Despite the large difference in the number of damaged and lysing cells between the sera, in terms of the location of binding of antibodies and complement on *Salmonellae,* overall there was no obvious difference. Both bactericidal and inhibitory sera exhibited bound C3, C5b-9 and IgG in OM, IM, cytoplasmic sites, as well as distal from the *Salmonella* membrane. Separation of bacteria in terms of 'damaged' and 'undamaged' cells suggested some associations with complement-deposition location and damage, as discussed in section 6.3.2. Preliminary observations suggested that *Salmonellae* incubated in bactericidal serum had higher levels of C5b-9 deposition in OM, IM and inner cell locations than inhibitory serum. While scoring of complement and antibody deposition associated with *Salmonellae* was performed, more images would be needed to give suitable power to confidently discern site specific comparisons. If possible, scoring of complement and antibody deposition at sites potentially key in complement-mediated killing, such as the OM and IM, would represent a worthwhile addition to this study.

The main difference we saw between the sera was the quantity of IgG and complement-components deposited on *Salmonellae* (Fig. 6.10A). HIV-infected serum had higher levels of IgG binding, as expected from prior flow cytometry analysis (MacLennan et al., 2010) and the associations of HIV-infection with hypergammaglobulinemia. But, HIV-infected inhibitory serum also had reduced deposition of both C3 and C5b-9. This suggests that lack of killing of *Salmonellae* by this serum is due to its reduced ability of to deposit stable C5b-9. This is consistent with previous reports that binding of C9 to

serum-resistant *Salmonella* spp. and *E. coli* isolates is minimal (Joiner et al., 1982a, Joiner, 1988).

Previous reports on similar types of inhibitory 'blocking' antibodies against *Neisseria* reported the presence of inhibitory antibodies lead to increased complement consumption (Frank et al., 1987). Some EM images of *Salmonellae* incubated in inhibitory serum showed large clumps of material, heavily labelled with C5b-9, distant from the bacterial surface (Fig 6.10). These may be evidence of C5b-9 dissociation from a site closer to the *Salmonella* membrane. If so, this would provide an explanation for how increased consumption of complement without increased deposition on *Salmonellae* is possible. Shedding of bound C5b-9 has been reported to be associated with serum-resistance in *Salmonella* spp. and *E. coli* isolates (Joiner et al., 1982a, Joiner et al., 1982b, Joiner, 1988). Furthermore, complement-mediated LPS release has been reported in strains of *E.coli* (O'Hara et al., 2001). The relative and overall levels of bactericidal and blocking antibodies in serum determine the ultimate location and configuration of bound C5b-9 on the *Neisseria* bacterial surface (Joiner, 1988). This could apply in *Salmonella* too, demonstrating a mechanism by which dissociation of C5b-9 complexes was elevated in Salmonellae incubated in inhibitory serum.

Interestingly, flow cytometry analysis gave the opposite result to EM analysis, showing that HIV-infected serum deposited higher levels of complement on *Salmonellae.* However, our discovery, using EM, of a layer around neat serum-incubated *Salmonellae* (Fig 6.6) that binds high levels of anti-complement antibodies suggests that the flow cytometry results may be influenced by an artefact. The layer prevented the passage of secondary labelling antibodies. As the flow cytometry method uses whole *Salmonellae*, it would only record complement deposition on the outside of this layer rather than

complement associated with the *Salmonellae* themselves. Although the flow cytometry assay correlates well to serum bactericidal (SBA) data and predicts killing with bactericidal serum, this is probably a coincidence. Both anti-*Salmonella* antibodies and complement are required both for serum killing and deposition of the complement layer and so with bactericidal serum, the assay effectively measures an indirect surrogate of serum-killing. However, with serum-resistant *Salmonellae* or HIV-infected inhibitory serum antibodies and complement can bind to the bacterium but cannot effect killing and as such the indirect measurement of complement components at the edge of the complement layer is irrelevant in the prediction of bactericidal activity.

Our observation of complement-components deposited on the IM and in the cytoplasm is important. As recently as 1994 (recent in terms of the investigation in the mode of action of complement-mediated killing) it was stated that no direct evidence of any complement elements becoming associated with the inner membrane after cell killing has been found (Blanchard and Dankert, 1994). One study that did report the presence of complement components on the IM (Goldman and Miller, 1989) using sucrose density centrifugation was criticised as being potentially contaminated (Tomlinson et al., 1990). Our results, using TEM, which not at risk of such contamination, agree with the findings of Goldman. The key difference between studies supporting IM and inner cell deposition and those refuting it, is the use of lysozyme-free serum in the latter.

Lysozyme and complement components are a prerequisite for bacteriolysis (Inoue et al., 1968) and lysis of bacteria cannot occur in the absence of lysozyme (Taylor and Kroll, 1983, Wright and Levine, 1981), but there are mixed reports on the role of lysozyme in complement-killing. The key question in determining the mechanism of complement-mediated killing is whether the removal of lysozyme helps to determine the mechanism

or merely demonstrates a different process of serum-killing that does not take place *in vivo.* Some studies report killing without lysozyme can occur with no or minimal reduction in rate (Schreiber et al., 1979, Pramoonjago et al., 1992), while others report a small reduction (Taylor and Kroll, 1983) and others still reported a large reduction in bactericidal activity (Glynn and Milne, 1967). Much like the situation with SBAs (discussed in section 3.4) these differences are probably a result of varying assay conditions. Differences in bacteria used, rough or smooth and serum incubation conditions can account for large differences in bactericidal activity. Even factors such as insufficient sampling over a time course of killing can mask differences in killing rate (Taylor, 1983).

A criticism of removing lysozyme based on studies showing no difference in killing with and without would be that removing a component from a system does not necessarily validate a functional redundancy or irrelevancy, especially as it is difficult to determine what the true rate of serum-killing of an microorganism is and so the effect could be hidden. C9-deficient serum, which can effect killing of some bacteria (Pramoonjago et al., 1992, Harriman et al., 1981) and can haemolyse erythrocytes demonstrates how complement can effect non-optimal killing in the absence of certain components. Both processes occur to a lesser extent when compared to normal serum and it has been reported, at least for haemolysis, that the mechanisms may differ to that of C5b-9 haemolysis (Takeda et al., 1986). Complement may be able to exert a deadly effect on bacteria using distinct mechanisms either in the presence or absence of IM and cytoplasmic deposition. It could be that the primary killing mechanism doesn't require inner cell deposition of complement components but its presence can contribute to killing effectiveness or rate, as may be the case with haemolysis by C9-deficient serum (Takeda et al., 1986) Complement has multiple roles in homeostasis and disease

(Ricklin et al., 2010) and considering this diversity it has been suggested that complement may not possess a singular clear mode of action by which it exerts it bactericidal effect (Bhakdi and Tranum-Jensen, 1991).

Further to these considerations, the lysozyme removal process itself may create problems as processing of the serum can have unwanted side-effects, removal using bentonite (montmorillonite, MMT) can also remove any serum component that is basic (positively charged) such as C1q and 'beta-lysins' (Myrvik and Weiser, 1955, Bugla-Ploskonska et al., 2009). Removal of C1q can affect the classical complement pathway and beta-lysins have been reported to be required for optimal killing of some Gram-negative bacteria (Donaldson et al., 1974). Lysozyme-removal using the other widely-used method of anti-lysozyme antibody has been suggested as a cause of complement diversion (Taylor, 1983).

6.5 Conclusion

Evidence for important complement-fixing bactericidal and opsonising antibody roles has been identified in Africans. However, high titers of anti-O-antigen antibodies, capable of inhibiting the bactericidal action of serum, have been discovered in a subset of HIV-infected patients. This has cast doubt on the suitability of inducing large antibody responses against some antigens. We incubated invasive African *Salmonella* in both healthy bactericidal and HIV-infected inhibitory sera. Next, using transmission electron microscopy (TEM) we imaged the resulting bacterial damage. In addition, we recorded the location and quantity of antibody binding and complement deposition on the *Salmonellae.* Consistent with viable counts from serum bactericidal assays, HIV-infected inhibitory serum showed substantially less damaged and lysed cells than bactericidal serum. Damage was not uniform and presented as severe plasmolysis, contraction and disruption of cytoplasm integrity, breaks in both outer and inner membranes and formation of ghost cells and spheroplasts. Finally, complete destruction of the bacterial cell, leaving only curled membrane debris occurred. IgG, complement component C3 and membrane attack complex C5b-9 were all observed on both the outer and inner membranes, as well as distal sites from the bacterial membrane of *Salmonellae* in both inhibitory and bactericidal sera. However, whereas damaged *Salmonellae* all had C3 and C5b-9 deposition on the OM, many healthy cells did not. Furthermore, bactericidal serum deposited greater quantities of C3 and also C5b-9 on the *Salmonellae.* This suggests that the failure of inhibitory serum to kill *Salmonellae* is due to a reduced ability to deposit bactericidal complement complexes mediated by the high titre of antibodies.

7. General discussion

A vaccine is urgently needed for invasive nontyphoidal *Salmonella* (NTS) disease in Africa. Antibodies have a protective effect against NTS and prior *In vitro* work has demonstrated important roles for both complement-fixing bactericidal antibodies (MacLennan et al., 2008) and opsonising antibodies (Gondwe et al., 2010) in African children. In this thesis we have investigated the mechanisms by which these antibodies direct complement-mediated bactericidal activity and how this modality of immunity contributes to protection against *Salmonella.* Understanding antibody-dependent immunity against *Salmonella* has significant implications in developing an efficacious vaccine for NTS in Africa.

In Chapter 3 we demonstrated undetectable bactericidal activity of mouse sera against NTS. As well as the lack of antibody-dependent complement-mediated bactericidal action in the mouse, we showed reduced complement component C3 deposition on the surface of *Salmonellae*. Complement deposition is vital for effective opsonisation and respiratory burst of *Salmonellae* in human blood (Gondwe et al., 2010, Shaio and Rowland, 1985, Vreede et al., 1986). Therefore, mouse models may underestimate the potential protective effect of antibodies against *Salmonella* in humans, although antibodies alone are enough to enhance phagocytosis, oxidative burst, and killing of *Salmonellae* in mouse macrophages (Uppington et al., 2006). Future work using an animal model with complement activity more similar to levels in humans could give a better prediction of the protection mediated by antibodies in humans. But, selecting an alternative model is difficult. Few commonly used research animals have complement activity equivalent to humans. Ish and co-workers surveyed complement activity of sera from 25 species using haemolysis against all the erythrocytes from each species and also antibody-coated human tumour cells (Ish et al., 1993). They found that popular

laboratory animals such as mice, guinea pigs and rabbits had relatively weak complement systems. Although complement-mediated killing of some Enterobacteriaceae by calf, chicken, guinea pig, pig and rabbit sera has been recorded (Kenny and Herzberg, 1967, Collins, 1967, Ogata and Levine, 1980, Taylor, 1983), comparisons show human serum to possess much more effective bactericidal activity (Ogata and Levine, 1980, Collins, 1967, Schwab and Reeves, 1966).

Another problem with selecting an alternative animal model is *Salmonella* serovar species specificity. The two most common serovars causing invasive disease in Malawi and much of tropical Africa are the generalist serovars *S*. Typhimurium and *S*. Enteritidis (Brent et al., 2006, Graham et al., 2000). Generalist *Salmonellae* can infect a wide range of mammals, but the manifestation of the disease they cause can greatly vary between host species. This in turn can alter the immune response, for example, vaccination of cattle, chickens, pigs, and humans with live attenuated strains of the corresponding host-specific serovars leads to strong immune responses that guard against reinfection (Zhang-Barber et al., 1999, Van Immerseel et al., 2005). In contrast, vaccination against non-host-specific *Salmonella* serotypes, such as *S*. Typhimurium in chickens, has shown mixed rates of success (Zhang-Barber et al., 1999, Van Immerseel et al., 2005).

The dominance of the mouse in modelling systemic NTS disease somewhat limits the value in expanding our study to other species. The use of the bovine enterocolitis models may warrant a repeat in the investigation conducted in Chapter 3 using immune serum from cows, though this would present logistical problems as facilities capable of researching NTS infection in cows are rarer. Our investigation included three of the most commonly used mouse strains (PubMed, 2006). These strains cover those that have be used in some of the most important antibody related discoveries in the context of NTS

infection (Cunningham et al., 2007, Xu et al., 1993, Hormaeche, 1979, McSorley and Jenkins, 2000, Mastroeni et al., 1993, Gil-Cruz et al., 2009) and so give a good coverage for investigation. All the strains we tested showed similar performance despite variance in a number of factors relevant to *Salmonella* infection (Section 3.4).

Confirming the contribution of opsonising activity in the mouse is important. Antibodies have a well-established protective effect against NTS in the mouse (reviewed in Dougan et al., 2011) and our work suggests that this protection is not mediated through bactericidal antibodies. Using immune mouse blood and serum for phagocytosis (Section 2.7), respiratory burst and whole blood cell killing assays (Gondwe et al., 2010) would demonstrate if opsonising antibodies are responsible for protection. Such a study would also allow comparison of the relative efficiency of mouse and human phagocytes in killing NTS. This could both help to explain host-specific differences in presentation of the disease, as well as highlight key mechanisms of immunity in humans. The latter is significant in helping determine the key immunological responses a successful vaccine would need to evoke.

Unlike mice, humans have a complement system that has a powerful bactericidal action against range of Gram negative bacteria *in vitro* (Reviewed in (Taylor, 1983)). However, the extent of the contribution of bactericidal antibodies against *Salmonellae* in humans *in vivo* is unknown (discussed in depth in Section 3.4). It is difficult to determine the degree of cell-free complement-mediated bactericidal activity in *vivo*. Separating the contributions of other aspects of immunity is troublesome, particularly because (as discussed above) antibodies can activate both cell-free and cell-mediated bactericidal activity. Furthermore, as previously reported in the literature, and confirmed for our representative invasive African *S*. Typhimurium isolate D23580 (Chapter 4), serum-

susceptibility of bacteria can vary dependent on the conditions and the growth phase of the bacteria. Yet, even for the most serum-resistant growths of *S*. Typhimurium D23580, the presence of specific antibodies and complement had a bacteriostatic effect. *Salmonella* infections are occasionally described in complement-deficient patients, but the association is much weaker than with other major causes of bacteraemia, such as meningococcal and pneumococcal diseases (Morgan and Walport, 1991, Ross and Densen, 1984, Botto et al., 2009, Taylor, 1983). The lack of correlation may be due to the complex immunity to *Salmonella* and the redundant nature of the immune system, meaning that other immune responses can prevent the development of disease.

In Chapter 4 we also modelled the *in vitro* kinetics of the deposition of antibodies and complement on *Salmonellae,* as well as phagocytosis by neutrophils and monocytes and complement-mediated cell-free killing. This model suggested that the speed that a proportion of the *Salmonellae* exited the extracellular bactericidal environment of the blood meant that an infection could continue even in the presence of bactericidal anti-*Salmonella* antibodies. Individuals with defects in IFNγ production pathways typically develop severe recurrent *Salmonella* infections, but strikingly, the disease is focal and non-fatal (MacLennan et al., 2004, Lammas et al., 2002). We suggest that specific antibodies protect against the development and spread of a fatal bacteraemia in those with certain cell-mediated deficiencies. This is consistent with the suggestion that antibodies contain the spread of *Salmonella* infection in mice (Mastroeni et al., 2009).

In Chapter 5 we demonstrated that the O-antigen (OAg) of *Salmonella* is a major target of bactericidal antibodies in human sera examined. As discussed, this is positive news for OAg-based vaccine design. Anti-OAg inhibitory antibodies described in a sub-set of HIV-infected individuals had cast doubt on the suitability of an OAg-based vaccine

(MacLennan et al., 2010). However, our data suggests that anti-OAg antibodies are not inhibitory *per se*. As expanded upon in Section 3.4, it is possibly an example of an *in vitro* artefact due to a gross excess of antibodies, termed the Neisser-Wechsberg phenomenon (Neisser, 1901). The effect appears to have a correlation with complement levels (Muschel et al., 1969) and it is not thought to be a problem for humans *in vivo.* The poor complement-fixing effector ability of some antibody subsets potentially contributes to the inhibition of serum-killing observed. For instance, IgA, IgG2 and IgG4 are poor binders of C1q (Flanagan and Rabbitts, 1982). Typically, HIV-infection (stages III and IV) is associated with decreased IgG2 and IgG4 levels. Titres of the more effective complement-fixing IgG subclasses IgG1 and IgG3 are increased (Parkin et al., 1989, Bartmann et al., 1991). Though, it could be that in HIV-infected patients with recrudescent *Salmonella* infections, the repeated exposure to the polysaccharide antigens of the *Salmonellae* OAg increases production of IgG2 (Siber et al., 1980). Chapter 6 further strengthens the link of inhibitory antibodies to complement. We show using electron microscopy that the major difference between bactericidal and inhibitory serum is that bactericidal serum deposits greater quantities of both C3 and the C5b-9 membrane attack complex on the *Salmonellae* than inhibitory serum.

Exploration of the classes and sub-classes of antibodies raised to *Salmonellae* and the specific antigen targets of these antibodies is a natural progression of this thesis. The use and comparison of acute, convalescent and healthy bactericidal sera could provide details on the antibodies required for both immunity and protection. Polysaccharide antigens, such as OAg, are typically T-independent and immunisation of humans with several encapsulated bacteria results in a predominantly IgG2 anti-polysaccharide response (Siber et al., 1980). An exception is observed in young children under the age ~2-3 years who can produce anti-polysaccharide antibodies in the IgG1 subclass (Morell

et al., 1990). IgG2 deficiency is associated with decreased responses to infections with encapsulated bacteria and after immunisation with polysaccharide antigens (Siber et al., 1980, Umetsu et al., 1985). As OAg is a major target of bactericidal antibodies, T-independent responses could be central to development of immunity.

Antibody responses and immunity against NTS disease naturally develop as African children age (MacLennan et al., 2008). Lack of specific antibodies early in life could be due to insufficient exposure to *Salmonella* antigens. It is also likely that immaturity of the infant immune system, particularly with regard to T-independent responses (Siegrist and Aspinall, 2009), is an important factor in the lack of anti-*Salmonella* antibodies in young children under ~2 years of age. To form effective protection in these children, a vaccine might have to produce a different response to the one naturally produced upon exposure to the pathogen. A proportion of children under 2 years old do have measurable titres of anti-*Salmonella* antibodies and bactericidal activity against *Salmonella* (MacLennan et al., 2008). Analysis of anti-*Salmonella* antibody sub-classes and antigen targets in this young antibody-producing population would help determine if a vaccine produces a different response in young children compared to older children and clarify what antibody repertoire constitutes an effective antibody response. For example, children immunised with an unconjugated meningococcal polysaccharide vaccine, develop an antibody response, but it is non-bactericidal (Plotkin, 2010). However, not all elements of the immune systems of African children function differently than adult immune systems. Phagocytes from young African children that lacked anti-*Salmonella* antibodies were just as effective at phagocytising and killing *Salmonellae* when given a source of anti-*Salmonella* antibodies (Gondwe et al., 2010). Hence, generation of specific antibodies in these children, through vaccination, should enable mechanisms of both cell-free and cell-mediated immunity against NTS. This increases the likelihood that a vaccine that

induces an antibody response will protect against invasive NTS disease in African children.

It is feasible that both opsonising and bactericidal activities are effected by the same antibodies. Opsonic antibodies need to be bound by FCγ receptors on host immune cells and so need to bind to a location on the pathogen that is accessible. OAg is distal from the bacterial membrane on the outer 'visible' surface and so would be a clear antigen target for opsonising antibodies. Opsonisation of *Salmonellae* with antibodies alone failed to enhance phagocytosis and complement-fixation is required for both bactericidal and opsonic functions of antibodies against *Salmonellae* (Gondwe et al., 2010). This means it is unlikely that significant opsonisation activity in the blood is mediated by antibody classes that do not fix complement well or at all, such as IgG4. It is possible that many antibodies mediating both opsonisation and bactericidal activity are of the same sub-class, or even the very same antibodies. As antibodies are required for binding of C3 to smooth-form *Salmonella* (MacLennan et al., 2008) and complement is required for opsonisation of this type of bacteria, it is even possible that the principal opsonic effect is due to complement deposition, rather than direct opsonisation by the antibodies themselves.

Almost all current vaccines work through antibodies in serum or on mucosa that block infection or bacteraemia (Plotkin, 2010). For this reason, the antibody response to *Salmonella* is essential to study. As well as quantity of response, the functional properties of antibodies in order to determine protection. It will also be helpful to determine useful correlates of protection for an NTS vaccine so that efficacy can be accurately determined. Many vaccine correlates and surrogates of protection are determined by measurement of antibodies. For three major bacteraemia causing

pathogens *Haemophilus influenzae* type B, meningococci and pneumococci, serum bactericidal assays and opsonphagocytic assays are used for correlates of protection (Siber et al., 2007, Maslanka et al., 1998). Binding antibody titres, measured by ELISA, can also sometimes be useful as surrogates of protection (Kayhty et al., 1983). The manifestation of NTS disease in Africa and the susceptibility of *Salmonellae* to antibody-dependent mechanisms of immunity imply these assays also function as useful correlates or surrogates of protection for a *Salmonella* vaccine. We have observed that serum containing binding antibodies, measured by flow cytometry, always have bactericidal activity against NTS. However, clear correlates of protection for vaccines against some intracellular bacterial diseases such as the Tuberculosis Bacille Calmette Guerin vaccine are not currently known (Fletcher, 2007).

The work contained in this thesis and the continuation and extension of projects described, in classifying antibody responses and functionality, will play a key supportive role to the development of a vaccine against NTS for Africa. This thesis has particularly focussed on antibody-dependent complement-mediated immunity and as such, immunity against *Salmonella* in the blood. Study of possible antibody-mediated protection at the intestinal epithelium, the site of the initial pathogen: host contact, could further assist vaccine development and would be a valuable area of further study.

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Absent Bactericidal Activity of Mouse Serum against Invasive African Nontyphoidal *Salmonella* Results from Impaired Complement Function but Not a Lack of Antibody

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Absent Bactericidal Activity of Mouse Serum against Invasive African Nontyphoidal *Salmonella* Results from Impaired Complement Function but Not a Lack of Antibody

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Nontyphoidal strains of *Salmonella* are a major cause of fatal bacteremia in Africa. Developing a vaccine requires an improved understanding of the relevant mechanisms of protective immunity, and the mouse model of *Salmonella* infection is useful for studying immunity to *Salmonella* in vivo. It is important to appreciate the similarities and differences between immunity to *Salmonella* in mice and men. Ab is important for protection against nontyphoidal *Salmonella* in both species, and we have previously found an important role for Ab in cell-free complement-mediated bactericidal activity against *Salmonella* in Africans. It is unclear whether this modality of immunity is relevant in the mouse model. C57BL/6, BALB/c, and C3H mice immunized with heat-killed *Salmonella* Typhimurium strains D23580 (African invasive strain) and SL1344 and live-attenuated strain SL3261 produced a *Salmonella*-specific Ab response. Sera from these mice deposited reduced levels of C3 on *Salmonella* compared with human sera and were unable to kill both wild-type and *galE*$^-$ rough mutant of D23580, indicating absent cell-free killing via classical and alternative complement pathways. Supplementing immune mouse sera with human complement enabled killing of *Salmonella*, whereas addition of human anti-*Salmonella* Ab to immune mouse sera had no effect. These findings indicate that mouse serum cannot effect cell-free complement-dependent killing of *Salmonella*, because of the reduced mouse complement ability to kill these bacteria compared with human complement. This difference in Ab-dependent immunity to *Salmonella* in mice and men must be considered when applying findings from the mouse model of *Salmonella* disease and vaccination response to man. *The Journal of Immunology*, 2011, 186: 2365–2371.

Nontyphoidal *Salmonella* (NTS) strains, in particular *Salmonella enterica* serovar Typhimurium, are emerging as a major cause of fatal bacteremia among African children (1–3) and HIV-infected adults (1, 4, 5). The minimum incidence of NTS bacteremia is 175 out of 100,000 in Kenyan children under 5 y of age (3), and the case-fatality rate exceeds 20% (2, 6). Partly because of the requirement for blood-culturing facilities in the diagnosis of NTS bacteremia (2), the scale of the problem caused by this disease has been unappreciated until recently, and currently no vaccine is available. Increasing levels of antibiotic resistance among African strains of NTS (1) and the rapid demise of many children with NTS once they present to hospital indicate that a vaccine is urgently needed. An improved knowledge of the relevant protective mechanisms of immunity against *Salmonella* is required for the development of an effective vaccine against NTS, and this involves complementary studies with human tissues and animal models of salmonellosis. Because *S.* Typhimurium causes invasive disease in the mouse, this animal provides a useful model for the study of NTS infections in vivo.

Salmonellae are adapted to survive within macrophages (7). It has long been appreciated from mouse studies (8, 9) and more recently for studies of patients with rare primary immunodeficiencies (10, 11) that cell-mediated immunity is important for protection against invasive *Salmonella* disease. From studying NTS bacteremia in Malawi, we have recently shown that NTS bacteremia particularly affects African children between 4 mo and 2 y of age, the period in which Ab levels to NTS are low or absent (12). Ab can potentially protect against *Salmonella* disease in several ways. First, Ab can protect in a cell-independent manner through complement-dependent bactericidal activity. Second, it can protect through a cell-dependent mechanism involving the oxidative burst by opsonizing bacteria for uptake and killing by phagocytic cells. In our studies on African children, we have found evidence for an important role for both bactericidal (12) and opsonic Ab (13) in protecting against NTS. Other ways in which Ab can protect against *Salmonella* disease include Ab-dependent cellular cytotoxicity and blockade of uptake by cells in the gastrointestinal tract.

A second major challenge in developing a vaccine against NTS for Africa is to identify the key molecular targets of the protective immune response against *Salmonella*. Because much of the assessment of vaccine candidates needs to be performed in vivo, for which animal models of infection are required, it is important to understand the similarities and differences between the immune

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Abbreviations used in this article: CFD, complement fixation diluent; NTS, nontyphoidal *Salmonella*.

responses to *Salmonella* in mice and men. Studies of *Salmonella* infections in mice have shown that blood Ab titers correlate with protection against *Salmonella* (14). Ab has been shown to be important for protecting innately susceptible *Nramp*^{s} mouse strains (15, 16), such as C57BL/6 and BALB/c, both in adoptive transfer studies (17, 18) and in studies in T cell-impaired or -deficient mice (19, 20). Recently, studies in C57BL/6 mice have indicated that the outer membrane proteins (19), in particular OmpD (20), are promising targets for vaccine development. Ab has previously been shown to have an important role in opsonizing *Salmonella* for phagocytosis and cellular killing in C57BL/6 mice (21). However, its ability to kill *Salmonella* using the cell-independent complement-mediated mechanism described in Africans is uncertain, particularly in view of the recognized limited activity of mouse complement (22, 23).

To address this question, we studied the bactericidal capacity of serum, Ab, and complement from C57BL/6, BALBc, and C3H mice immunized with heat-killed *S.* Typhimurium strains D23580 and SL1344 and live-attenuated strain SL3261.

Materials and Methods

Salmonella

S. Typhimurium D23580 is an invasive African strain of NTS isolated from a bacteremic girl aged 26 mo who was admitted to Queen Elizabeth Central Hospital in Blantyre, Malawi, in 2004 (12, 24). This strain is sensitive to killing by human adult serum, undergoing a 1–3 $\log_{10}$ kill over a 3-h time course (see *Salmonella* killing assays below). It is representative of >90% of NTS strains isolated from bacteremic individuals in Malawi since 2002. A rough *galE*$^-$ mutant of D23580 that has truncated LPS lacking *O*-Ag polysaccharide has previously been described (12). SL1344 is a laboratory strain of *S.* Typhimurium that has been used in many experimental studies. Both strains have been sequenced by the Sanger Core Sequencing Facility at the Wellcome Trust Sanger Institute (24, 25). SL3261 is an attenuated mutant of *S.* Typhimurium SL1344 with a deleted *aroA* gene and was kindly provided by B.A.D. Stocker. For immunizations, *Salmonellae* were grown in Luria-Bertani media to log phase, washed twice with PBS, and heat-killed as required at 72°C for 1 h. No exogenous adjuvants were used for immunizations.

Mice

Animal studies had ethical and home office approval. C57BL/6, BALBc, and C3H mice were age and sex matched, housed in pathogen-free conditions, and used between 6 and 12 wk of age. Mice immunized with heat-killed *Salmonellae* were given 10^7 bacteria/animal via the i.p. route at day 0, boosted with the same dose of heat-killed bacteria at day 14, and exsanguinated at day 28 by cardiac puncture. Those immunized with live *Salmonellae* were given 5×10^5 bacteria i.p. at day 0 and exsanguinated at day 35. Blood was allowed to clot for 60 min, immediately separated, and frozen to preserve complement function. Aliquots of serum were stored at −80°C and not subjected to freeze-thaw cycles.

Materials

Unless otherwise stated, materials were from Sigma-Aldrich. Human sera were from African adults with anti-*Salmonella* Ab or were sera deficient in anti-*Salmonella* Ab. When required, serum was heat-inactivated at 56°C for 30 min.

Hemolytic classical pathway complement function assay

Washed rabbit erythrocytes were resuspended in complement fixation diluent (CFD) (Oxoid) at 2% (v/v) and sensitized by incubation for 30 min at room temperature with a 1:500 final dilution in CFD of mouse anti-rabbit erythrocyte antiserum. Sensitized erythrocytes were washed, resuspended at 1% in CFD, aliquoted into the wells of a 96-well plate (50 μl/well), and incubated at 37°C for 30 min with 50 μl dilutions of mouse serum in CFD. Zero and 100% lysis controls were included in all assays. Plates were centrifuged, and complement activity was assessed by hemoglobin release (absorbance at 415 nm). Percentage lysis for individual wells was calculated using the following formula: percent lysis = $(A_{415}[\text{test}] - A_{415}[\text{min}])/(A_{415}[\text{max}] - A_{415}[\text{min}]) \times 100$.

*Anti-*Salmonella *Ab assays and complement deposition assays*

These were performed by flow cytometry as described previously (12). Briefly, 5 μl *S.* Typhimurium D23580 in log-growth phase was mixed with 45 μl 10% serum for Ab determination or undiluted serum for complement deposition (final *Salmonella* concentration 2×10^8/ml). FITC-conjugated anti-mouse IgG and IgM Ab (Sigma-Aldrich) and FITC-conjugated anti-C3c Ab that binds both human and mouse C3 (DakoCytomation) were used for detection prior to FACS analysis on an FACSCalibur instrument (BD Biosciences).

Salmonella *serum bactericidal assays*

These were performed as described previously (12). Briefly, 5 μl viable *S.* Typhimurium D23580 in log-growth phase was added to 45 μl undiluted serum (final *Salmonella* concentration 1×10^6 CFU/ml) and incubated at 37°C with the number of viable *Salmonellae* determined after 45, 90, and 180 min. When mouse serum was supplemented with human Ab or complement, the mouse serum was mixed at a 1:1 ratio with either heat-inactivated human immune serum (as a source of anti-*Salmonella* Ab) or human serum lacking anti-*Salmonella* Ab (as a source of exogenous complement). When mixed with Ab-deficient human serum, mouse serum was diluted in PBS in a 10-fold dilution series to determine the minimum concentration that could still effect *Salmonella* killing.

Confocal microscopy

Salmonellae incubated in serum and labeled with FITC-conjugated anti-C3 were fixed with acetone on Superfrost Plus charged microscope sides (Leica Microsystems), mounted in Prolong-Gold DAPI (Invitrogen), and viewed under an oil immersion 100× objective lens with an Axiovert 100M confocal microscope (Zeiss).

Statistical analysis

Comparison of groups was performed using the Student *t* test.

Results

Immunization of mice with heat-killed African S. *Typhimurium strain D23580 induces a switched Ab response*

Groups of six C57BL/6, BALBc, and C3H mice were primed and boosted with heat-killed African *S.* Typhimurium D23580 or PBS (negative control groups). Another two groups of six C57BL/6 mice were either primed and boosted with heat-killed laboratory strain *S.* Typhimurium SL1344 or immunized with the live-attenuated vaccine strains SL3261. At 28 and 35 d, mice were exsanguinated and serum assessed for Ab response to *Salmonella*. Whereas no anti-*Salmonella* Ab was detected in serum from the control groups, all mice immunized with *Salmonella* except two produced IgG and IgM Ab to *Salmonella* (Fig. 1*A*, 1*B*). Serum from the two mice in which the immunizations had failed was discarded. Anti-*Salmonella* Ab titers were similar regardless of whether the Ab assay was performed using *S.* Typhimurium D23580, SL1344, or SL3261 (Supplemental Fig. 1). Sera prepared from the mice were shown to have intact complement function as demonstrated by lytic activity against sensitized rabbit erythrocytes using a hemoglobin release assay to assess classical pathway complement activity (Fig. 1*C*). This demonstrated that the processing and storage of the mouse sera had not adversely affected the quality of the complement in these sera.

Sera from S. *Typhimurium-immunized mice lack bactericidal activity against wild-type* S. *Typhimurium*

We next tested serum from the immunized mice for ability to kill viable *S.* Typhimurium D23580 in the log-growth phase. Our previous work with serum from African children has shown that, in the presence of specific IgG or IgM Ab, human serum is capable of Ab-dependent complement-mediated killing of D23580 (12). Despite the presence of anti-*Salmonella* Ab, sera from both immunized and unimmunized C57BL/6 (Fig. 2*A*), BALB/c (Fig. 2*B*), and C3H (Fig. 2*C*) mice were unable to kill wild-type D23580 in vitro

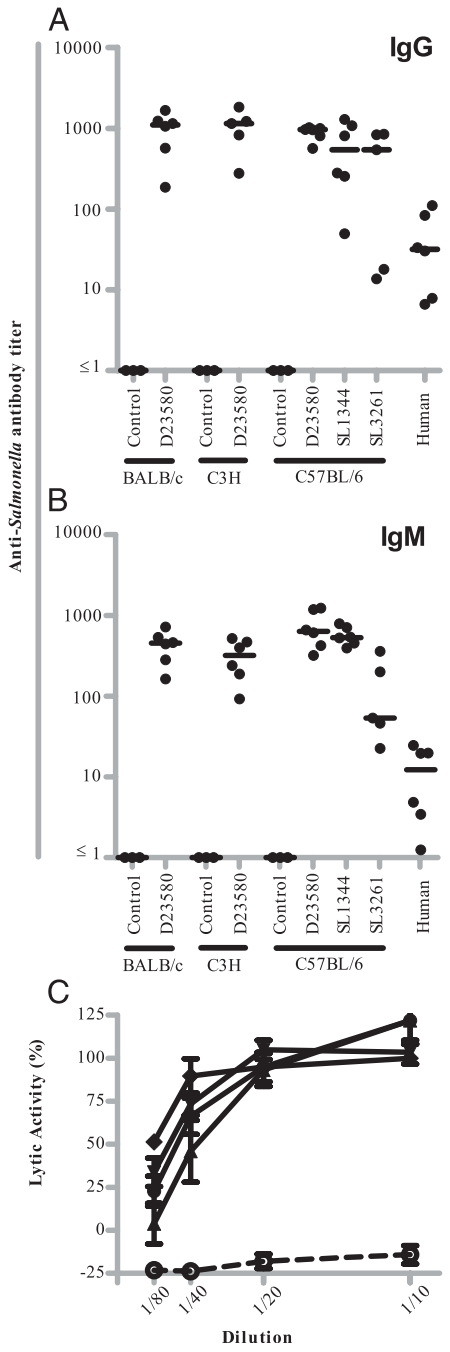

FIGURE 1. Ab response and classical pathway complement activity in mouse sera following immunization with African and laboratory strains of *S.* Typhimurium. Titers of anti-*Salmonella* Ab at 28 d after i.p. immunization with two doses of heat-killed *Salmonellae* (D23580 and SL1344) and at 35 d after i.p. immunization with live-attenuated *Salmonellae* (SL3261) and unimmunized (Control). Immunizations for heat-killed *Salmonellae* were with 10^7 heat-killed *S.* Typhimurium D23580 (African strain) and SL1344 (laboratory strain). Immunizations for live-attenuated SL3261 were with 5×10^5 *Salmonellae*. Groups of BALB/c, C3H, and C57BL/6 mice received D23580 immunizations, with groups of C57BL/6 mice also receiving SL1344 and SL3261. *A*, IgG. *B*, IgM. Ab measured by flow cytometric analysis of Ab-binding to fixed *S.* Typhimurium D23580. Each point represents serum from one mouse. Horizontal bars indicate median values. *C*, Lytic activity of mouse sera against rabbit erythrocytes sensitized with mouse anti-rabbit erythrocyte antiserum, measured using a hemoglobin release assay. Fresh C57BL/6 mice sera (diamonds); frozen freshly thawed mice sera: BALB/c (inverted triangles), C3H (triangles), and C57BL/6 (filled circles); and heat-inactivated C57BL/6 sera (empty circles). Data are means of experiments with sera from six mice ± SD.

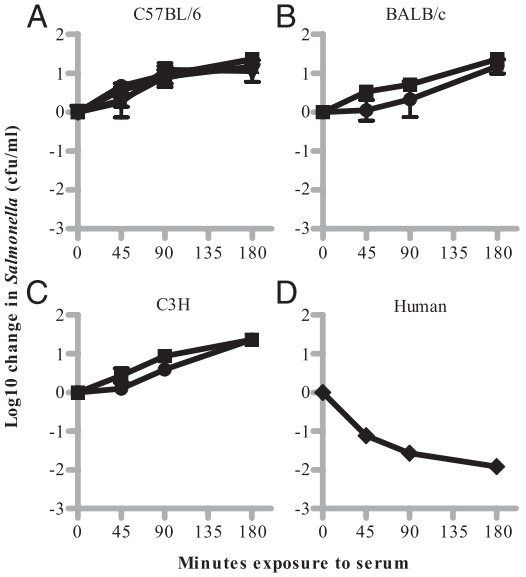

FIGURE 2. Absence of killing of African wild-type *S.* Typhimurium D23580 by mouse sera compared with human sera. In vitro serum bactericidal assay with 10^6 *Salmonellae*/ml at 45, 90, and 180 min time points using sera from C57BL/6 (*A*), BALB/c (*B*), or C3H mice (*C*) immunized with D23580 (squares) and unimmunized mice (circles). *A* also shows data using sera from C57BL/6 mice immunized with SL1344 (triangles) and SL3261 (inverted triangles). Data are means of experiments with sera from three mice ± SD. *D*, Killing of wild-type D23580 by control serum from an African adult. Lack of killing of *Salmonellae* by sera from all groups of mice compared with the killing of *Salmonellae* by human serum was highly significant (Student *t* test, $p < 0.0001$).

over a 3-h time course, regardless of the immunization strategy used. The concentration of viable *Salmonellae* increased by >1 $\log_{10}$ in this period. In contrast, African adult serum effected a 2 $\log_{10}$ kill over 3 h (Fig. 2*D*). The lack of killing of *Salmonellae* by sera from all groups of mice compared with the killing of *Salmonellae* by human serum was highly significant (Student *t* test, $p <$ 0.0001). These findings could result from the Ab induced following immunization of mice with D23580 or SL1344 lacking inherent bactericidal activity. This could occur if the Ab is unable to facilitate the deposition of complement membrane attack complex at the *Salmonella* outer membrane. Alternatively, mouse complement per se may lack the necessary bactericidal activity to kill *Salmonella*, as suggested by early experiments on mouse complement (22, 23).

Sera from S. *Typhimurium-immunized mice also lack bactericidal activity mediated through the alternative pathway of complement against a galE$^-$ rough mutant of* S. *Typhimurium*

To explore these possibilities, we repeated the serum killing assays using a *galE*$^-$ knockout mutant of D23580 that is unable to synthesize the *O*-Ag polysaccharide of LPS (12). Previously, we showed that this rough mutant is susceptible to killing by serum from African children lacking *Salmonella*-specific Ab via alternative pathway complement activity (12). There was no difference between the results from these experiments and those using wild-type D23580. No *Salmonella* killing occurred with sera from C57BL/6, BALB/c, and C3H mice immunized with D23580, SL1344, and SL3261 or from unimmunized mice (Fig. 3*A–C*). By contrast, African child serum lacking anti-*Salmonella* Ab was able to effect a 3 $\log_{10}$ kill of *S.* Typhimurium D23580 (Fig. 3*D*). Again, the lack of killing of *Salmonellae* by sera from all three

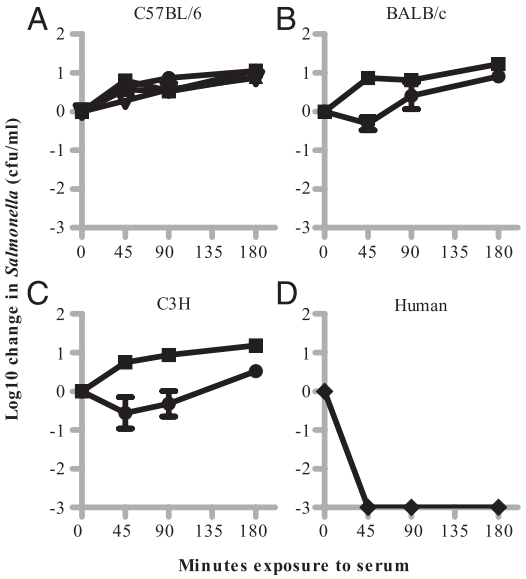

FIGURE 3. Absence of killing of African rough *galE*$^{-}$ mutant of *S.* Typhimurium D23580 by mouse sera compared with human sera. In vitro serum bactericidal assay with 10^6 *Salmonellae*/ml at 45, 90, and 180 min time points using sera from C57BL/6 (*A*), BALB/c (*B*), or C3H mice (*C*) immunized with D23580 (squares) and unimmunized mice (circles). *A* also shows data from C57BL/6 mice immunized with SL1344 (triangles) and SL3261 (inverted triangles). Data are means of experiments with sera from three mice ± SD. *D*, Killing of wild-type D23580 by control serum from an African adult. Lack of killing of *Salmonellae* by sera from all groups of mice compared with the killing of *Salmonellae* by human serum was highly significant (Student *t* test, $p < 0.0001$).

groups of mice compared with the killing by human serum was highly significant (Student *t* test, $p < 0.0001$). These findings strongly suggest that the lack of bactericidal activity is the result of comparatively limited mouse complement function against *Salmonella*, regardless of mouse species tested, the deficiency affecting both classical (Ab-dependent) and alternative pathways.

Sera from S. *Typhimurium-immunized mice deposit reduced levels of C3 complement on* Salmonella *compared with human sera*

Because cleavage of C3 and deposition of C3b is the central event in all complement activating pathways, we next looked at deposition of C3 on the surface of wild-type *S.* Typhimurium D23580 by sera from mice immunized with *Salmonellae* using flow cytometry. Whereas sera from unimmunized mice were unable to deposit C3 on *Salmonella*, sera from C57BL/6, BALB/c, and C3H mice immunized with D23580, SL1344, and SL3261 were able to deposit C3 on the surface of D23580, though ~1 $\log_{10}$ less than the amount of C3 deposited by immune African serum (Student *t* test, $p < 0.0001$) (Fig. 4*A*). Using confocal microscopy, qualitatively less C3 deposition was visualized on *Salmonellae* incubated with mouse serum compared with human serum (Fig. 4*B*). These data therefore indicate deficiency in C3 deposition on *Salmonella* by mouse serum via the classical pathway relative to human immune serum, but do not exclude the possibility of poor activity in the terminal pathway of complement.

*Mouse sera supplemented with human bactericidal anti-*Salmonella *Ab do not kill* S. *Typhimurium D23580*

To check whether the absence of killing of *Salmonellae* by mouse serum was due to a lack of bactericidal Ab, we supplemented sera from unimmunized C57BL/6, BALB/c, and C3H mice with heat-inactivated serum from African adults containing Ab to *Salmonella*. We had previously shown that sera from African children that lack Ab to *Salmonella* become bactericidal when exogenous anti-*Salmonella* Ab is added (12). Addition of human Ab did not correct the inability of mouse sera to kill D23580, the absence of killing of *Salmonella* being highly significant compared with the

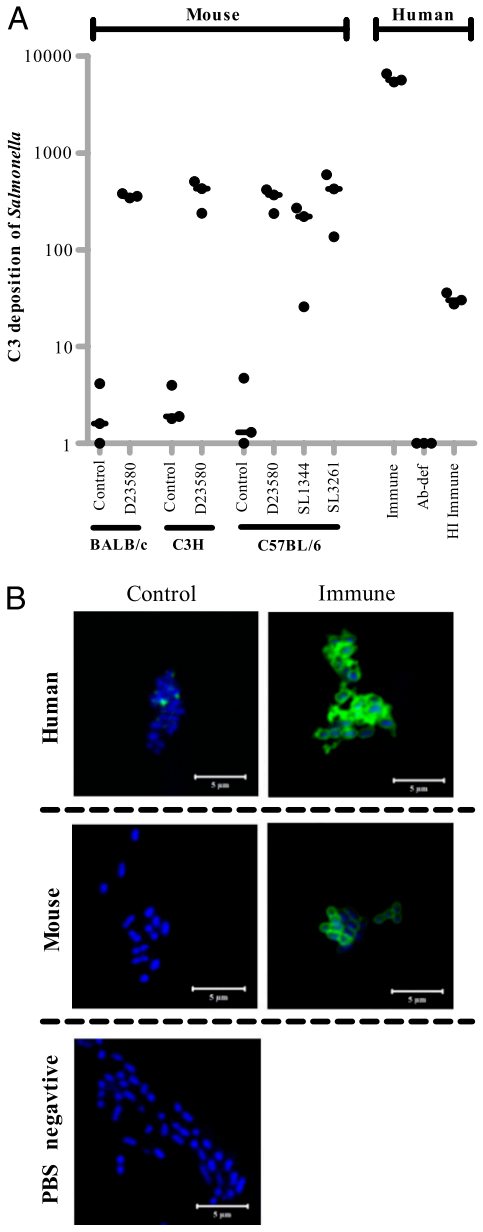

FIGURE 4. Deposition of complement C3 on *S.* Typhimurium D23580 by mouse sera compared with human sera. *A*, Titers of C3 deposited on *S.* Typhimurium D23580 following incubation in sera from BALB/c, C3H, and C57BL/6 mice immunized with *S.* Typhimurium D23580, SL1344, and SL3261 or unimmunized mice (Control) and immune human serum (Immune) ± heat inactivation (HI Immune) from a healthy African adult and Ab-deficient human serum (Ab-def). Each point corresponds to one experiment. Lines show median values for each group. Difference between C3 deposition with immunized mice sera compared with immune human sera was significant (Student *t* test, $p < 0.0001$). *B*, Confocal microscopy imaging of C3 deposited on *Salmonellae* using FITC-conjugated anti-C3 Ab (green) following incubation in mouse and human sera. *Salmonellae* are visualized using DAPI (blue). *Human panels*, Immune serum (Immune) and heat-inactivated serum (Control). *Mouse panels*, Serum from C57BL/6 mice immunized with heat-killed D23580 (Immune) and unimmunized (Control). *PBS negative panels*, *Salmonellae* incubated in PBS. All images acquired with identical confocal microscope settings. Scale bars, 5 μm.

killing by immune human serum (t test, $p < 0.0001$) (Fig. 5). This finding suggests that lack of killing of *Salmonellae* by immune mouse serum is not due to a lack of Ab function, further implicating limited mouse complement function against *Salmonella* as the reason for absent bactericidal activity.

Immune mouse sera supplemented with human complement can kill S. *Typhimurium D23580*

To confirm that impaired mouse complement function rather than impaired Ab function was the reason for absent killing of *Salmonellae*, we supplemented C57BL/6, BALB/c, and C3H mouse serum with human complement by mixing mouse serum with human serum lacking Ab to *Salmonella*. Mouse sera could now kill and/or prevent the growth of *Salmonella* at 10- or 100-fold dilutions with PBS (Fig. 6). This was statistically significant compared with both the lack of killing by the anti-*Salmonella* Ab-deficient human serum alone and immunized mouse serum alone (Student t test, $p < 0.0001$ for serum from mice immunized with heat-killed bacteria, and $p < 0.05$ for serum from mice immunized with live-attenuated bacteria). This experiment confirms that absent killing of *Salmonellae* by mouse sera is the result of limited mouse complement function against *Salmonella* and that the mouse Ab against *Salmonella* has potential bactericidal activity leading to complement-mediated killing of *Salmonella*, provided a suitable source of exogenous complement is available.

Discussion

The main finding from this study is that although C57BL/6, BALB/c, and C3H mice mount both an IgG and IgM Ab response to heat-inactivated African invasive *S.* Typhimurium strain D23580 and laboratory strain SL1344 and to live-attenuated *S.* Typhimurium

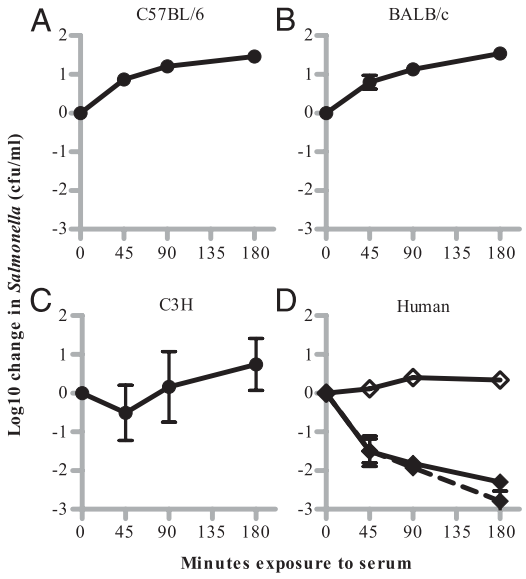

FIGURE 5. Absence of killing of *S.* Typhimurium D23580 following supplementation of mouse sera with human anti-*Salmonella* Ab. In vitro serum bactericidal assay with 10^6 *Salmonellae*/ml at 45, 90, and 180 min time points using sera from C57BL/6 (*A*), BALB/c (*B*), or C3H-unimmunized (*C*) mice all supplemented with heat-inactivated immune human serum containing both anti-*Salmonella* IgG and IgM. Data are means of experiments with sera from three mice ± SD. *D*, Killing of wild-type D23580 by immune human serum (diamonds) and Ab-deficient human serum supplemented with heat-inactivated immune human serum (diamonds with dashed line). Lack of killing by heat-inactivated human control serum alone (hollow diamonds). The lack of killing of *Salmonellae* by sera from all groups of mice compared with the killing of *Salmonella* by human serum was highly significant (Student t test, $p < 0.0001$).

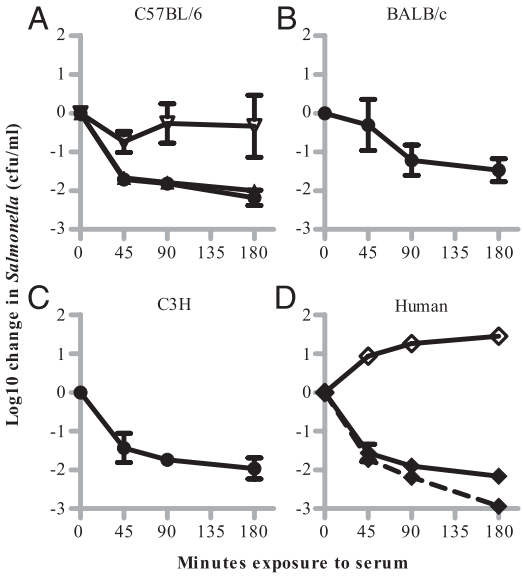

FIGURE 6. Killing of *S.* Typhimurium D23580 following supplementation of immune mouse sera with human complement. In vitro serum bactericidal assay with 10^6 *Salmonellae*/ml at 45, 90, and 180 min time points using sera from C57BL/6 (*A*), BALB/c (*B*), or C3H (*C*) mice immunized with D23580 (squares), SL1344 (triangles), and SL3261 (inverted triangles), all supplemented with human complement. Sera from C57BL/6 and BALB/c mice immunized with heat-killed bacteria killed at a dilution of 1:100. Sera from C3H bacteria immunized with heat-killed bacteria and C57BL/6 mice immunized with live bacteria killed/prevented growth of *Salmonella* at a dilution of 1:10. Data are means of experiments with sera from three mice ± SD. *D*, Lack of killing of wild-type D23580 by Ab-deficient human serum (hollow diamonds), killing by control immune human serum (diamonds), and Ab-deficient human serum supplemented with heat-inactivated immune human serum as a source of Ab (diamonds with dashed line). The killing of *Salmonella* by each mouse sera supplemented with human complement compared with the lack of killing of by corresponding mouse serum alone (shown in Fig. 2) was statistically significant (Student t test, $p < 0.0001$ for immunizations with heat-killed bacteria, $p < 0.05$ for immunizations with live bacteria).

strain SL3261, serum from immunized mice lacks bactericidal activity against *Salmonella*. The lack of killing appears to be due to a lack of specific complement activity against *Salmonella*, because the serum is able to lyse erythrocytes in a standard hemolytic assay. This contrasts with the 1–3 $\log_{10}$ killing of D23580, which is effected by African adult and child serum containing anti-*Salmonella* Ab. Serum from African children does not kill *Salmonella* when it lacks specific Ab, and this was the basis for previously describing bactericidal Ab in Africans as an effector of humoral immunity against NTS (12).

S. Typhimurium D23580 was chosen for this study because it is a typical invasive strain of NTS from Africa, is sensitive to killing in human serum by bactericidal Ab in the presence of complement, and has been sequenced at the Wellcome Trust Sanger Institute (24, 25). Experiments using *S.* Typhimurium laboratory diarrhogenic strains SL1344 and SL3261 were included to demonstrate that the lack of bactericidal activity was not restricted to African invasive NTS and was independent of whether the immunization was with heat-killed wild-type or live-attenuated *Salmonellae*. C57BL/6 mice were chosen because mechanisms of immunity to *Salmonella* have been studied extensively in these mice (19–21), and the Ab response to vaccination with *S.* Typhimurium in C57BL/6 mice has been shown to protect against subsequent infection with *S.* Typhimurium (19, 20). The experiments were also performed with BALB/c and C3H mice to show that

deficiency in serum bactericidal activity in *Salmonella* is not restricted to one inbred strain of mice. Our findings suggest that the protective effect of mouse anti-*Salmonella* Ab is not mediated by cell-independent bactericidal activity.

The *galE*$^-$ rough mutant of *S.* Typhimurium D23580 is sensitive to killing via the alternative pathway of complement by human serum lacking anti-*Salmonella* Ab (12). Absent killing of the *galE*$^-$ mutant by all mouse sera therefore implicates limited complement function against *Salmonella* rather than absence of bactericidal Ab itself as the reason for lack of killing with wild-type D23580. Limited complement function in mouse serum was first described >60 y ago (22). Indeed, our finding that serum from immunized mice supplemented with human complement can effect cell-free killing indicates that the Ab generated following immunization with D23580, SL1344, and SL3261 is capable of directing complement-deposition to the *Salmonella* surface at locations that enable membrane attack complex to kill the bacteria (12).

Our data suggest that complement from C57BL/6, BALB/c, and C3H mice is defective in killing D23580 at multiple points in the complement pathway. Because we have previously shown that deposition of complement and killing of wild-type D23580 is Ab dependent and does not proceed via alternative or mannose-binding lectin pathways (12), the lower titers of C3 deposition on *Salmonella* seen with serum from immunized mice compared with human serum indicate that classical pathway activity is reduced in relation to that in human serum. The lack of killing of the D23580 *galE*$^-$ mutant also indicates limited alternative pathway activity and/or terminal pathway activity.

Nevertheless, functional classical pathway complement activity was detected against sensitized rabbit erythrocytes in the hemoglobin release assay used. We employed this assay to ensure that the lack of bactericidal complement function against *Salmonella* was not merely an artifact of suboptimal preparation and storage of sera. These findings suggest that differences exist between the susceptibility of erythrocytes and *Salmonella* to the lytic/bactericidal effects of mouse complement, with *Salmonella* being more resistant than erythrocytes. Complement that does not insert into the *Salmonella* outer membrane can be shed from the surface of *S.* Minnesota (26, 27), and various molecules on the *Salmonella* surface, including those encoded by *rck* (28), *traT* (29), and *pgtE* (30), confer some degree of resistance to complement-mediated killing.

Such mechanisms are likely to raise the threshold of complement deposition required for bactericidal activity against *Salmonella* to proceed. Previously, we found that the *rck* gene is encoded in the virulence plasmid of D23580 and that C9 is necessary for serum bactericidal activity against D23580 (12). In contrast, several studies on complement-mediated hemolysis have demonstrated that this can proceed in the absence of C9 by limited perturbation of the membrane by C8 (31–33).

With mouse complement lacking bactericidal activity against *Salmonella*, it is likely that the protective effect of anti-*Salmonella* Ab in C57BL/6, BALB/c, and C3H mice is mediated by opsonization facilitating phagocytosis, respiratory burst, and intracellular killing of *Salmonella* by neutrophils, monocytes, and macrophages. The importance of anti-*Salmonella* Ab for these modalities of cell-mediated immunity has been previously demonstrated using serum and cells from C57BL/6 mice (21). Other work in men and mice has indicated that opsonization of *Salmonella* with C3 is required for optimal intracellular killing (13, 34–36), and work with C1q-deficient mice has shown a role for the classical pathway of complement in protecting against *S.* Typhimurium (37). If C3 deposition on *Salmonella* is a requirement for such killing, this suggests that the threshold level of C3 deposition required for opsonization leading to intracellular killing of *Salmonella* is lower than the level required for bactericidal terminal pathway complement activity.

Although *Salmonellae* are facultative intracellular bacteria, we have previously identified that the high case-fatality rate among young African children is associated with extracellular bacterial growth unchecked by bactericidal Ab (12). Because our findings indicate a lack of bactericidal activity in mouse serum against *Salmonella*, this could contribute to the sensitivity of C57BL/6, BALB/c, and C3H mice to infection with *S.* Typhimurium. Different mouse strains differ in their ability to handle infection with *Salmonella*, and this may be due to a variety of reasons, including differences in innate *Nramp* polymorphism-mediated genetic susceptibility of each strain to *Salmonella* and complement function. Variations in complement function between strains can be complex. For example, A/J mice are more effective at depositing C3b on *Salmonella* than C57BL/6 mice but are deficient in the C5 component of complement, hence preventing complement-mediated bactericidal activity against *Salmonella* in such mice (36).

A positive outcome from the current study is the clear demonstration of the bactericidal potential of Ab produced in mice following immunization with *Salmonella* provided suitable exogenous complement is provided. We have been able to use this finding to demonstrate that porins Omp F, C, and D are targets of bactericidal Ab by immunizing mice with these porins and performing bactericidal assays supplemented with human complement (38). Furthermore, this study suggests that the mouse model underestimates the potency of Ab in protecting against *Salmonella* infections. A consequence of this is that if immunizations with an Ag offer Ab-mediated protection in the mouse, they may be more likely to offer protection in humans.

In conclusion, Ab-mediated immunity against NTS in C57BL/6, BALB/c, and C3H mice and African children involves both distinct and shared immunological mechanisms. Bactericidal Ab is able to mediate cell-free complement-mediated killing of extracellular bacteria in children, whereas Ab in mice appears incapable of this modality of immunity. In contrast, both human and mouse Ab are able to protect against *Salmonella* via opsonization in conjunction with cell-mediated mechanisms.

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Disclosures

The authors have no financial conflicts of interest.

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Corrections

Siggins, M. K., A. F. Cunningham, J. L. Marshall, J. L. Chamberlain, I. R. Henderson, and C. A. MacLennan. 2011. Absent bactericidal activity of mouse serum against invasive African nontyphoidal *Salmonella* results from impaired complement function but not a lack of antibody. *J. Immunol*. 186: 2365–2371.

In the second to last sentence of the *Abstract*, the word "affect" should be "effect." The correct sentence is: "These findings indicate that mouse serum cannot effect cell-free complement-dependent killing of *Salmonella*, because of the reduced mouse complement ability to kill these bacteria compared with human complement."

In the *Results* section, in the second paragraph, line 11, the word "affected" should be "effected". The correct sentence is: "In contrast, African adult serum effected a 2 $\log_{10}$ kill over 3 h (Fig. 2*D*)."

In the *Discussion* section, in the first paragraph, line 10, "affected" should be "effected". The correct sentence is: "This contrasts with the 1–3 $\log_{10}$ killing of D23580, which is effected by African adult and child serum containing anti-*Salmonella* Ab."

In the *Discussion* section, in the third paragraph, line 9, "affect" should be "effect." The correct sentence is: "Indeed, our finding that serum from immunized mice supplemented with human complement can effect cell-free killing indicates that the Ab generated following immunization with D23580, SL1344, and SL3261 is capable of directing complement-deposition to the *Salmonella* surface at locations that enable membrane attack complex to kill the bacteria (12)."

All instances of "*Salmonella typhimurium*" and "*S. typhimurium*" in the article should be changed to "*Salmonella* Typhimurium" and "*S.* Typhimurium." This change was requested by the authors at proof stage and was not made in production, with the exception of the first page of the printed article (page 2365).

All of the above errors have been corrected in the online version, which now differs from the print version as originally published.

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