COMPARISON OF THE IMMUNE RESPONSE TO TYPHOID FEVER AND INVASIVE NONTYPHOIDAL *SALMONELLA* DISEASE

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Abstract

Nontyphoidal *Salmonella* is strongly associated with HIV infection, whereas there is a negative association between HIV and typhoid fever. To investigate this phenomenon, we explored humoral and cytokine responses in HIV-infected and uninfected blood and sera against *S.* Typhimurium and *S.* Typhi, finding that HIV-infected sera had significantly impaired bactericidal and opsonic activity against S. Typhi compared with HIV-uninfected sera. We observed dysregulated cytokine responses in blood from HIV-infected individuals after *Salmonella* stimulation, with RANTES levels being modulated by HIV status and *Salmonella* serovar. Most *S.* Typhi isolates express Vi capsule and O:9 O-antigen. The roles played by the Vi capsule and by anti- *S.* Typhi antibody in infection are unclear. Effective vaccines against typhoid exist, but the mechanisms of protection afforded by the antibody they elicit is poorly characterised. We investigated the role of Vi capsule and anti-*S.* Typhi antibody in the killing of *Salmonella*. Using isogenic $Vi^{+/-}$ *Salmonella* we show that Vi-expression is associated with reduced antibody and complement deposition on *Salmonella* and increased resistance to serum and phagocyte killing. We characterised the bactericidal and opsonic activity of purified human anti-Vi and anti-O:9 antibodies against *S.* Typhi, finding that both antibodies kill *Salmonella* but anti-O:9 antibody has poor opsonic activity.

Collectively our data suggest cytokine, but not antibody, dysregulation may underlie the dichotomy of *Salmonella* infection in the context of HIV. We demonstrate that both anti-capsular and anti-O-antigen antibodies elicit bactericidal activity against *S.* Typhi but anti-O:9 antibody is a poor opsonin. This has implications in the development of O:9 vaccines against O:9 –expressing *Salmonellae*.

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Abbreviations

Ab	Antibody
ADH	Adipic dihydrazide
ART	Anti-retroviral therapy
BCK	Blood cell killing assay
BD	Becton Dickinson
BRC	Baby rabbit complement
BRS	Baby rabbit serum
BSA	Bovine serum albumin
BSI	Bloodstream infection
C'	Complement
CFU	Colony forming units
CGD	Chronic Granulomatous Disease
ELISA	Enzyme linked immunosorbance assay
EU	ELISA units
FITC	Fluorescein isothiocyanate
FS	Fresh sera
GMFI	Geometric mean fluorescence intensity
H58	Haplotype-58
HI	Heat-inactivated
HiS	Heat-inactivated sera
HIV	Human immunodeficiency virus
iNTS	Invasive nontyphoidal *Salmonella*
IU	International units
LDA	Linear discriminant analysis
MAC	Membrane attack complex
MDR	Multi-drug resistant
NTS	Nontyphoidal *Salmonella*
O-Ag	O-antigen
OMP	Outer membrane protein
OR	Odds ratio
PBS	Phosphate buffered saline
PCA	Principal component analysis
SBA	Serum bactericidal assay
SCV	*Salmonella* containing vacuole
SEM	Scanning electron microscopy
sIgA	Secretory IgA
SPI-1	*Salmonella* pathogenicity island 1
SPI-2	*Salmonella* pathogenicity island 2

STM	*Salmonella* Typhimurium
STY	*Salmonella* Typhi
TLR	Toll-like receptor
TTSS	Type-three secretion system
ViPS	Vi polysaccharide
VL	Viral load

1. Introduction

1.1. Salmonella

Salmonella is a genus of Gram-negative, rod shaped bacteria of the Enterobacteriaceae family (Dougan et al., 2011). They are non-spore forming, facultative anaerobes around 0.7 – 1.5μm in diameter and 2 – 5μm in length and have peritrichous flagellae conferring motility (Chevance and Hughes, 2008). *Salmonella* are chemoautotrophic, using carbon dioxide and hydrogen to derive energy from oxidation and reduction reactions with organic sources. The *Salmonella* genus has two species, six subspecies and thousands of individual serovars. They are broadly pathogenic and have a global distribution, being found in hosts and the environment alike. *Salmonella* were first isolated and described in 1885 in the laboratory of the eponymous Dr. Daniel Salmon (Kass, 1987). Since then, over 2500 serovars have been described, a number of which are important human pathogens with major clinical significance (Popoff et al., 2001).

1.2. *Salmonella* Taxonomy and Nomenclature

As a genus, *Salmonella* is composed of two species; *Salmonella enterica* and *Salmonella bongori* (Porwollik et al., 2004). *S enterica* consists of six subspecies; enterica (I), salamae (II), arizonae (IIIa), diarizonae (IIIb), houtenae (IV) and indica (VI) (Porwollik et al., 2004). As mentioned in section 1.1, more than 2,500 distinct serovars have been described to date. Serovars are classified in accordance with the Kauffman-White scheme which characterises serovars based upon their O (LPS repeating carbohydrate), H (flagellar) and K (capsular) antigens (Grimont, 2007).

Salmonella nomenclature is complex. The complete name of a serovar typically includes both the species and subspecies names for example *S. enterica* (species) subspecies Enterica serovar Typhi. The majority of clinically relevant serovars, including all those used and discussed within this thesis belong to the enterica subspecies. As such, when discussing serovars herein only the genus and serovar name will be used e.g. *Salmonella* Typhi or *S.* Typhi.

1.3. Pathogenic *Salmonella* and disease epidemiology

Though belonging to the same species, different *Salmonella* serovars are able to cause different disease syndromes with varying levels of mortality in the immunocompetent host. Moreover, the immune status of the host affects susceptibility to, and the severity of infection. Broadly speaking, clinically important *Salmonella* serovars can be divided into two groups; typhoidal and nontyphoidal *Salmonella*. The geographical distribution and burden of invasive disease caused by typhoidal (Figure 1.1 A) and nontyphoidal *Salmonella* (Figure 1.1 B) can be seen in Figure 1.1.

1.3.1. Nontyphoidal Salmonella serovars

Nontyphoidal *Salmonella* (NTS) are not host-restricted pathogens and are ubiquitous in animals, humans and the environment. Infection occurs after ingestion of contaminated food, such as eggs, poultry or vegetables, or water (Hawkey et al., 2013, Bayer et al., 2014). These serovars usually cause self-limiting gastroenteritis in immunocompetent individuals, but can cause severe and/or fatal disease depending on the infecting serovar and the immune status of the host. In healthy individuals, mortality is typically less than 1%, and treatment is unnecessary (Majowicz et al., 2010). The two most

common serovars globally are *S.* Typhimurium and *S.* Enteritidis, but the prevalence of NTS serovars varies according to geographical region and other factors (Hendriksen et al., 2011).

Globally, the burden of NTS infection is high, with an estimated 93.9 million cases per annum resulting in approximately 155,000 deaths (Majowicz et al., 2010). In the United States of America, NTS remains the leading cause of foodborne illness (Mead et al., 2000).

In sub-Saharan Africa and parts of South East Asia, NTS infections are common and NTS serovars are some of the most common, if not the leading etiological agents of bacterial bloodstream infections (BSIs) in these areas (Reddy et al., 2010). These atypical, invasive infections are difficult to diagnose and have a high mortality rate (20 – 25%) (Feasey et al., 2012). This contrasts with iNTS infections in developed countries, which are relatively rare (Vugia et al., 2004). In both instances, iNTS is strongly associated immunocompromise or with co-infections which affect immune function, such as HIV or malaria (Huson et al., 2014, Biggs et al., 2014, Gordon, 2008).

1.3.2. S. Typhi and S. Paratyphi

In contrast to NTS, the majority of typhoidal *Salmonella* are human-restricted pathogens which cause severe invasive disease, commonly known as enteric fever. *S.* Typhi alone is estimated to cause over 21 million infections per annum and approximately 200,000 deaths (Crump et al., 2004). Mortality is typically high if left untreated (10 – 30%), but can be reduced to <1% with appropriate antibiotic treatment (Crump et al., 2004). The incidence of typhoidal *Salmonella* has decreased in developed countries with the advent

of adequate sanitation and water treatment facilities (Crump and Mintz, 2010). Autochthonous transmission is all but absent in these countries but cases do occur in returning travellers or foreign visitors (Clark et al., 2010). Typhoidal serovars still pose a serious public health problem in less developed countries, particularly in South East Asia, where the incidence can be over 500 cases per 100,000 population (Bhan et al., 2005). Unlike NTS infection, infection with *S.* Typhi is not associated with immunocompromise (Gordon, 2008).

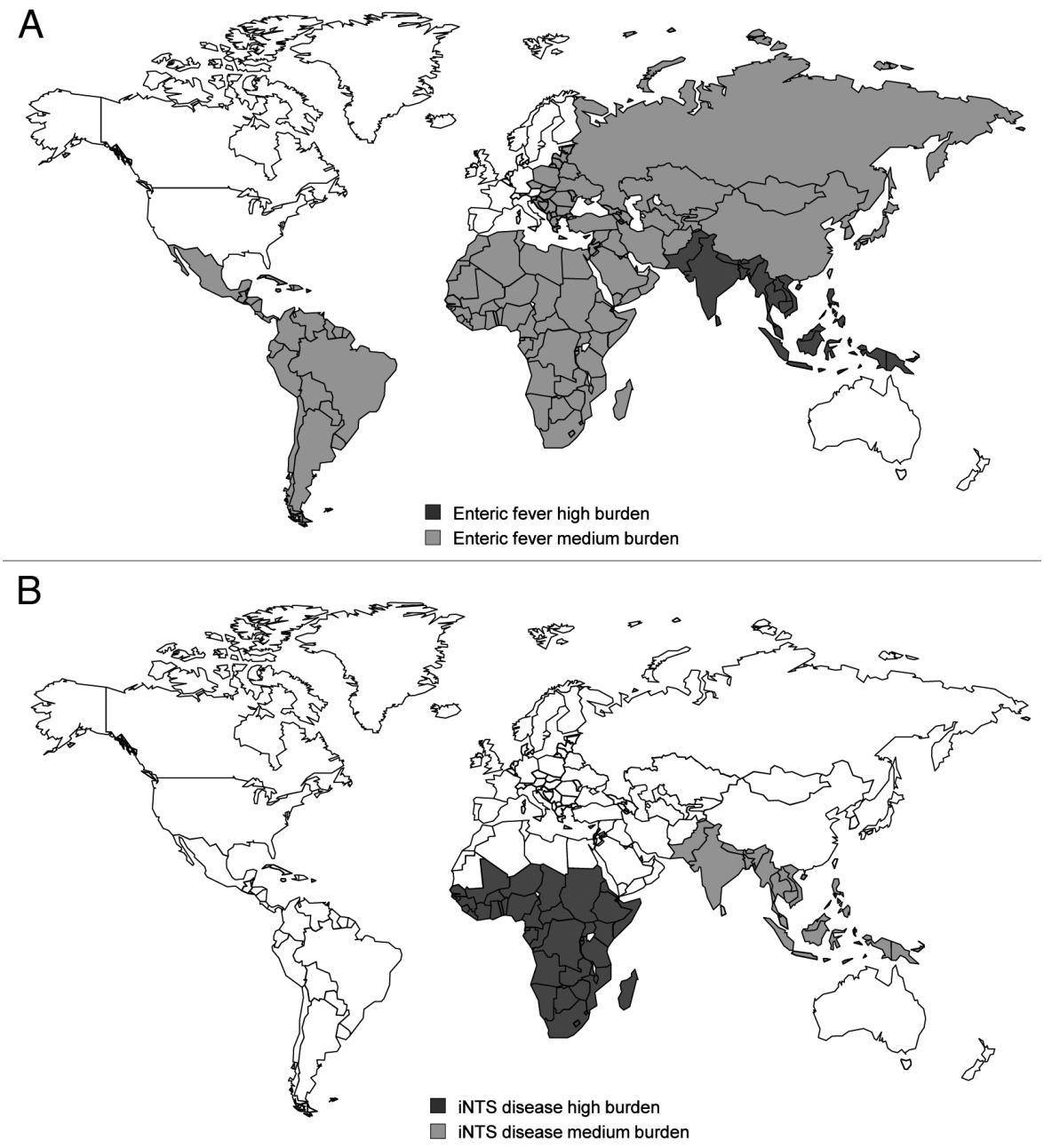


Figure 1.1 Global *Salmonella* burden

The global distribution of (A) enteric fever and (B) iNTS. High disease burden is classified as >100 cases/100,000 population and medium burden is 10 – 100 cases/100,000 population (Figure taken from MacLennan et al., 2014).

1.4. Risk factors for *Salmonella* infection

1.4.1. Age

Young age is a risk factor for iNTS infection. In Africa, NTS bacteraemia peaks in children aged between 6 and 24 months (Berkley et al., 2005, Gordon et al., 2008). Similarly in the USA, the incidence of iNTS is greatest (7 cases / 100,000 population) in infants less than 1 year old (Vugia et al., 2004). This high incidence at a young age may reflect the loss of maternal antibody to *Salmonella* (MacLennan et al., 2008) and/or gastric hypoacidity in infants (Hohmann, 2001). Young age is also a risk factor for *S.* Typhi infection. However, the peak incidence of typhoid fever in endemic areas is often between 5 – 18 years (Sen and Mahakur, 1972, Mahle and Levine, 1993). There is some evidence to suggest *S.* Typhi infection is common in the 1 – 5 year old age group (Lin et al., 2000, Ochiai et al., 2008). Cases of typhoid decrease into adulthood, possibly reflecting the acquisition of protective immunity after multiple exposures to *S.* Typhi during childhood and adolescence (Lin et al., 2000).

1.4.2. Malnutrition

Malnutrition is strongly associated with increased susceptibility to NTS and subsequent mortality in less-developed countries in Africa and South America (Rosanova et al., 2002, Brent et al., 2006). Malnutrition is also considered a risk factor for iNTS disease in parts of Africa where over 50% of isolates from malnourished children have been found to be NTS (Feasey et al., 2012, Gordon et al., 2008). A recent Tanzanian study documented severe malnutrition in over a quarter (26.7 %) of children with typhoidal

bacteraemia (OR = 5.74, p=0.003) (Biggs et al., 2014), but no association between typhoid fever and malnutrition has been reported on a wider scale.

1.4.3. Malaria infection

A strong association exists between NTS infection and malaria (Mabey et al., 1987, Mackenzie et al., 2010, Bronzan et al., 2007). In murine studies, haemolysis of erythrocytes by the plasmodium parasite results in elevated free heme levels. Free heme is cytotoxic, and its presence induces expression of the cytoprotective enzyme heme-oxygenase 1 (HO-1) by myeloid cells (Cunnington et al., 2012). However, HO-1 reduces the production of phagocytic reactive oxygen species (ROS), impairing the oxidative burst and intracellular killing of *Salmonella*. This reduction in ROS, combined with increased iron availability are two mechanisms which increase *Salmonella* survival in malarial infection and increase the susceptibility of the host to NTS infection (Cunnington et al., 2012, van Santen et al., 2013). In murine co-infection models, increased IL-10 production in response to malaria infection is associated with defective myeloid cell function and increased bacterial burden (Lokken et al., 2014). Reduced levels of IL-12 in response to *S.* Typhimurium challenge were observed in the context of Plasmodium infection (Roux et al., 2010).

Multiple studies have documented a negative association between Malaria and *S.* Typhi infection (Biggs et al., 2014, Tabu et al., 2012). However, any causality or mechanism for this association has yet to be established. Due to the strong association between malaria and NTS infection, competition between NTS and *S.* Typhi may be a reason for the lack of malarial-associated typhoid but there is no evidence to suggest this is the case. Other, as yet unidentified, immunological changes in malarial infection may be responsible for

differences in *Salmonella* infection, but further research is necessary to clarify the nature of the relationship.

1.4.4. Immunodeficiencies

S. Typhi is not associated with any primary or acquired immunodeficiencies and the reasons why are not understood (Gordon, 2008). In contrast, NTS infection is strongly linked to immunosuppression and/or immunodeficiencies. NTS bacteraemia is common in patients with both chronic granulomatous disease (CGD) and those with defects in the IL-12/IL-23/IFNγ axis (Winkelstein et al., 2000, MacLennan et al., 2004). Moreover, increased susceptibility to, and/or atypical, NTS infection have been observed in cancer patients undergoing chemotherapy, diabetics, and patients with autoimmune or liver disease (Hames et al., 2008, Abramson et al., 1985, Gordon, 2008).

1.4.5. HIV infection

The relationship between HIV and *Salmonella* infection is complex. At the advent of the global HIV epidemic in the early 1980's, NTS bacteraemia in adults became associated with, and soon became highly indicative of advanced HIV infection, especially in homosexual males in the USA (Profeta et al., 1985, Jacobs et al., 1985). Since then, HIV infection has become a well-established risk factor for iNTS disease, particularly in sub-Saharan Africa where NTS is the most common cause of BSIs in HIV-infected individuals (Gordon et al., 2002, Reddy et al., 2010). There are three mechanisms by which HIV infection may account for increased susceptibility to NTS infection. Firstly, dysregulated cytokine production by primary macrophages in the context of HIV infection is thought to impair the immune response to NTS (Gordon et al., 2007). Secondly, high titre anti-

LPS antibodies in a subset of HIV-infected Africans have been shown to impair bactericidal activity against NTS (MacLennan et al., 2010). Finally, increased NTS dissemination due to loss of intestinal mucosal integrity as a result of reduced IL-17 has been observed in macaques infected with SIV (Raffatellu et al., 2008).

Why NTS serovars should be so closely linked to HIV infection when typhoidal *Salmonella* is not, remains a mystery. Only one report has described significantly increased susceptibility of HIV-infected adults to *S.* Typhi infection (Gotuzzo et al., 1991). This suffers from a small sample size and its observations have not been corroborated by other studies. While *S.* Typhi-HIV co-infection has been reported in both Africa and developed countries, disease in this context is typically no different than in HIV-uninfected individuals (Vugia et al., 1993, Gilks et al., 1990, Manfredi et al., 1998).

Recent studies and meta-analyses have identified a negative association between HIV infection and *S.* Typhi infection insofar as to suggest that HIV infection is protective against typhoid fever (Reddy et al., 2010, Crump et al., 2011, Huson et al., 2014). Odds ratios for HIV-*S.* Typhi co-infection in these studies are consistently around 0.1 (range: 0.07 - 0.12, $p<0.0001$) but this can vary according to geographical location. This indicates that the incidence of *S.* Typhi infection in HIV-infected individuals is approximately ten times less than that for HIV-uninfected individuals. The effect seems to be persistent, as recent studies have documented resurgences in the incidence of typhoid fever associated with declining HIV incidence at sites in both Tanzania and Malawi (Biggs et al., 2014, Feasey et al., 2013).

The mechanism underlying the paradox in HIV-NTS/*S.* Typhi infection remains unclear and causality has yet to be established. There exists some argument as to whether the susceptibility of HIV-infected individuals to *S.* Typhi is reduced; or rather whether the disease syndrome of *S.* Typhi infection in the context of HIV infection is atypical and/or subclinical.

It has been suggested that antibiotic prophylaxis or a hospital selection bias may be responsible for the observations (Levine and Farag, 2011, Crump et al., 2011). If the effects of antibiotic prophylaxis were responsible for this discrepancy, then one would expect to observe a similar effect of prophylaxis on NTS infections as both NTS and *S.* Typhi have similar drug resistance levels in many locations (Gordon et al., 2008, Rowe et al., 1997) but this is not the case. Similarly, if a hospital selection bias was responsible, one would expect case reports of the two-infections to indicate that *S.* Typhi infection in the context of HIV is severe, fulminant and lethal disease, but reports indicate it is no different than typhoid fever in HIV-uninfected individuals (Manfredi et al., 1998).

More complex hypotheses have been advanced which suggest *S.* Typhi infection relies on an intact cell-mediated immune response. When this is dysfunctional or absent, such as in the case of advanced HIV infection and AIDS, it is thought that this could leave typhoid bacilli sequestered within the intracellular niche resulting in diminished virulence (Levine and Farag, 2011). However, this hypothesis is counterintuitive as one would expect intracellular *S.* Typhi to be killed by the oxidative burst. Even in the event of a defective oxidative burst, *Salmonella* are able to escape the intracellular niche via caspase-mediated apoptosis of phagocytes (Chanana et al., 2007, Hersh et al., 1999) Moreover, no resurgence of *S.* Typhi has been reported following the successful role out

of anti-retroviral therapy (ART) in areas with a high incidence of both HIV and typhoid (Feasey et al., 2014). Further research is required to fully characterise and examine this phenomenon and this is something we investigate within this thesis.

1.5. Clinical presentation of *Salmonella* infection

1.5.1. Salmonellosis

In immunocompetent individuals, NTS infection typically presents as acute, self-limiting gastroenteritis. Onset of illness after ingestion of *Salmonella* is usually between 6 – 72 hours and illness usually lasts for between 4 – 7 days (Khuribulos et al., 1994). Symptoms include, but are not limited to; nausea, vomiting, diarrhoea and abdominal pain. In rare cases, complications such as bacteraemia may occur which can lead to meningitis or septic arthritis (Hohmann, 2001). Hospitalisation is usually around 5% with mortality less than 1% but this varies according to the infecting serovar and the immune status of the host (Todd et al., 2007, Hohmann, 2001).

1.5.2. iNTS disease

In immunodeficient individuals, NTS infection often presents as severe invasive disease with high recrudescence and mortality. In areas of Africa, mortality of HIV-infected individuals with iNTS can be as high as 47% (Gordon et al., 2002). The non-specific symptoms of iNTS, such as fever and myalgia, and the absence of gastro-intestinal involvement, render diagnosis difficult (Kotloff et al., 2013, Graham et al., 2000).

1.5.3. Enteric fever

In contrast to NTS infection, infection with *S.* Typhi in otherwise healthy adults is typically severe, causing febrile illness with flu-like symptoms such as myalgia, cough and headache (Parry et al., 2002). Diarrhoea is variable as a symptom, only presenting in one third to one half of patients (Roy et al., 1985). The time from exposure to onset of symptoms is longer than NTS infection and is thought to be between 7 – 14 days (Parry et al., 2002). Mortality is high and varies between 10 – 30% but this can be reduced to <1 – 4% following treatment with appropriate antibiotics (Crump et al., 2004). Serious side effects, such as necrosis of Peyer's patches and meningitis often occur (Bhan et al., 2005).

1.6. Management of *Salmonella* infection

1.6.1. Diagnosis

Salmonella are typically identified via blood or stool culture where applicable. However both of these methods have limitations and further testing after positive identification is often required to identify the infecting serovar (Wain and Hosoglu, 2008). The Widal test for typhoid fever is still used in endemic areas despite having low specificity and sensitivity, especially if patients have had previous *S.* Typhi infection (Olopoenia and King, 2000). Next generation methods involving PCR, mass spectroscopy or microwave-accelerated metal-enhanced fluorescence (MAMEF) show promise, but have yet to become widely established in a clinical setting (Tennant et al., 2011b, Tennant et al., 2010, Akyar and Can, 2013). Issues concerning the specificity of PCR and the high cost of newer methods means bacterial culture and Widal tests continue to be used in less-

developed settings (Sanchez-Jimenez and Cardona-Castro, 2004, Wain and Hosoglu, 2008). Due to the non-specific nature of the symptoms of both enteric fever and iNTS, there is a requirement for cheaper and more accurate diagnostic tests.

1.6.2. Antibiotics

As with many other bacterial diseases, antibiotic resistance poses a problem in the effective treatment of *Salmonella.* Use of antimicrobials is not indicated in uncomplicated gastroenteritis associated with NTS infection. However, antibiotics are essential for treating iNTS infection and typhoid fever. First-line antibiotics such as ampicillin and chloramphenicol were originally widely used to treat *Salmonella* infection (Woodward et al., 1948, Whitby, 1964). High relapse rates associated with chloramphenicol and resistance to both drugs soon became an issue leading to the use of third generation cephalosporins and fluoroquinolones as the drugs of choice (Gunell et al., 2010, Guerrant et al., 2001, Cao et al., 1999).

The spread of *S.* Typhi isolates from the haplotype H58 lineage throughout Asia and Africa is associated with the acquisition of fluoroquinolone resistance (Roumagnac et al., 2006). The dominant iNTS pathovar in eastern Africa, ST313, also carries multiple resistance genes (Kingsley et al., 2009). In some areas of Africa, such as Malawi, approximately 90% of iNTS isolates are classed as multi-drug resistant (MDR) (Gordon et al., 2008). Similarly, around 90% of *S.* Typhi isolates in Pakistan are thought to be MDR (Mirza et al., 1995). Resistance has also been documented in developed countries resulting in the non-conventional use of antibiotics such as azithromycin in the treatment of *Salmonella* infection (Sjolund-Karlsson et al., 2011).

1.7. *Salmonella* pathogenesis

The pathogenesis of *Salmonella* varies greatly depending on the infecting strain and/or serovar. *S.* Typhi infection is systemic and the severity differs between hosts. In contrast, NTS infection in immunocompetent hosts is typically acute, self-limiting gastroenteritis. *Salmonella* infection typically begins after ingestion of *Salmonella* bacteria via contaminated water or food sources. Bacteria must survive the low pH of the stomach before passing into the small intestine.

1.7.1. NTS gastroenteritis

NTS serovars cause typhoid-like illness in mice, and as such, murine models of NTS gastroenteritis are unsuitable. Therefore, the majority of work investigating NTS pathogenesis has been conducted using the bovine ileal loop infection model, or infection of intestinal epithelial cell monolayers (Coombes et al., 2005). Once in the intestine, NTS utilises the *Salmonella* pathogenicity island 1 (SPI-1) encoded type-three secretion system (TTSS) to inject effector proteins such as SopB and SopE1 into intestinal epithelial cells and M cells (Andrews-Polymenis et al., 2010). These T3SS effector proteins initiate intracellular signalling cascades which result in actin polymerisation and cytoskeletal rearrangements (Kaur and Jain, 2012). This "trigger" mechanism, leads to the protrusion of the cell membrane in ruffles around the *Salmonella* and internalisation of *Salmonella* by a process known as micropinocytosis (Francis et al., 1993, Clark et al., 2011). Recently a "zipper" mechanism of *Salmonella* internalisation has also been described in non-phagocytic cells (Rosselin et al., 2010).

This is mediated by the outer membrane protein (OMP) Rck. Like the trigger mechanism, the zipper mechanism causes the internalisation of *Salmonella* as a result of intracellular signalling and actin polymerisation (Velge et al., 2012). However, the primary difference between the two mechanisms is that the initiation signal for the zipper mechanism is extracellular (Rck binding to its receptor), whereas the initiation of the trigger mechanism occurs intracellularly as a result of the injection of TTSS effector proteins. Schematics of the two invasion mechanisms can be seen in

Figure 1.4.

Once *Salmonella* has crossed the enterocytes and/or M cells, infection is contained within inflammatory foci in the lamina propria. Invasion of enterocytes causes the production of pro-inflammatory cytokines such as IL-1 and IL-18 which have strong chemotactic properties (Eckmann and Kagnoff, 2001). This results in a rapid neutrophil influx (within 1 – 3 hours after infection) (Tsolis et al., 1999) which is associated with damage and necrosis of the intestinal mucosa. This is accompanied by leakage of a protein rich exudate into the intestinal lumen which causes the characteristic diarrhoea associated with *Salmonella* infection (Tsolis et al., 1999). This occurs approximately 8 – 72 hours post-infection. In immunocompetent hosts, infection resolves within 5 – 7 days after exposure (Coburn et al., 2005).

1.7.2. Systemic infection

S. Typhi also employs TTSSs and effector proteins like NTS does, as discussed in section 1.7.1. However, unlike NTS infection, infection and invasion of the intestinal mucosa by

S. Typhi is not accompanied by a neutrophil influx and inflammatory exudates, instead there is a steady monocyte/ macrophage influx (Kraus et al., 1999, Harris et al., 1972). This may also explain why diarrhoea only occurs in between one third to one half of patients with typhoid fever (Roy et al., 1985). This difference in the initial inflammatory response is believed to be due to inhibition of LPS and flagellin recognition by TLR4 and TLR5 respectively (Raffatellu et al., 2006). Moreover, expression of Vi capsule by *S.* Typhi has been demonstrated to reduce expression of IL-8, a potent neutrophil chemokine, by macrophages (Raffatellu et al., 2005) and inhibit complement component C5 mediated neutrophil chemotaxis (Wangdi et al., 2014).

Once across the intestinal epithelium, *S.* Typhi may be phagocytosed or spread as planktonic cells in the bloodstream. This allows *S.* Typhi to colonise secondary lymphoid tissues such as the spleen and bone marrow (Kaur and Jain, 2012). Expression of the Vi capsule increases the resistance of *S.* Typhi to the bactericidal activity of serum and is also thought to reduce killing by blood phagocytes (Looney and Steigbigel, 1986, Wilson et al., 2011) permitting bacteraemia to occur. Within phagocytes *S.* Typhi employs the SPI-2 TTSS and its effector proteins to manipulate the conditions in phago-lysosome to those favourable to the survival and replication of the bacteria (Waterman and Holden, 2003) thus creating the *Salmonella* containing vacuole (SCV). This involves the secretion of various effector proteins which are discussed in section 1.8.4. Once a threshold level of bacterial numbers is reached, *Salmonella* induces caspase-1 or caspase-3 mediated apoptosis of macrophages (Chanana et al., 2007, Hersh et al., 1999) thus liberating the bacteria from their intracellular niche and permitting extracellular spread. This results in secondary bacteraemia and allows *S.* Typhi to colonise other organs such as the liver, and gallbladder (Kaur and Jain, 2012). Bacteria can persist in the mesenteric lymph

nodes, bone marrow and gall bladder for life, resulting in the development of a chronic carrier state and sporadic shedding of viable typhoid bacilli in the faeces (Gonzalez-Escobedo et al., 2011).

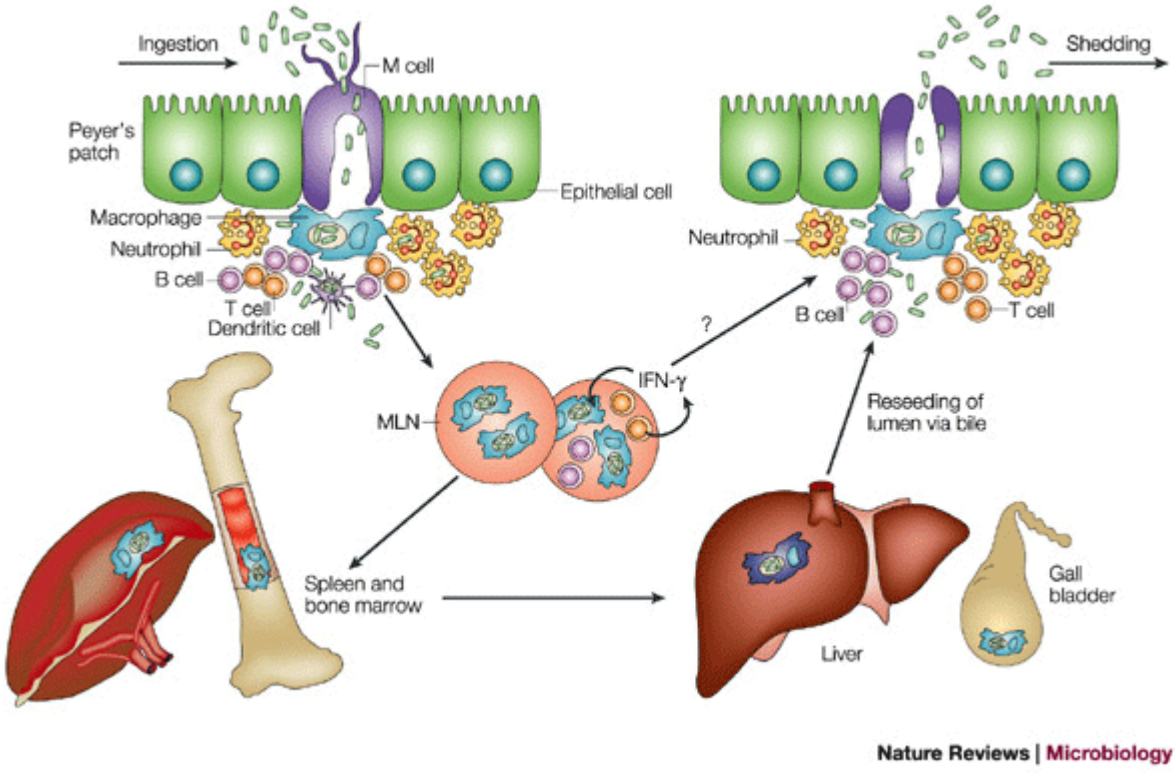


Figure 1.2 Pathogenesis of *S.* Typhi infection

Salmonella reach the Peyer's patches of the small intestine by invading enterocytes or M cells. A mild, localised inflammatory response occurs and bacteria are phagocytosed by macrophages. *Salmonella* survive and replicate within macrophages which travel through the bloodstream to the mesenteric lymph nodes, spleen and bone marrow. *Salmonella* multiply within the *Salmonella* containing vacuole (SCV) and are released into the bloodstream causing secondary bacteraemia and colonisation of other organs such as the liver and gall bladder. *Salmonella* can persist in these sites for life. Periodic reseeding of the mucosal surface by *Salmonella* from the gall bladder can lead to faecal shedding of *Salmonella* and subsequent infection of new hosts (Figure taken from Monack et al., 2004).

1.8. Salmonella virulence factors

1.8.1. Lipopolysaccharide

The lipopolysaccharide (LPS) of *Salmonella* is an important virulence factor and differences in LPS regulation, expression, distribution and O-antigen chain length can all affect the resistance of *Salmonella* to the immune response and impact the course of infection (Bravo et al., 2008, Burns and Hull, 1998, Grossman et al., 1987).

LPS can be divided into three constituent components; lipid A, the core region and the O-antigen. Lipid A is also known as endotoxin and this portion of the LPS molecule is thought to contribute to the severity and lethality of *Salmonella* infection by stimulating the release of pro-inflammatory cytokines which culminate in endotoxic shock (Khan et al., 1998).

LPS contributes to serum resistance by diverting complement away from the bacterial cell membrane (Grossman et al., 1987, Bravo et al., 2008). Rough mutants of *Salmonella*, which either lack or have truncated LPS are attenuated in mice (Ilg et al., 2009), exhibit greater levels of complement deposition (Rautemaa and Meri, 1999) and are more sensitive to the lytic action of bactericidal antibody and complement than LPS-intact strains (Rowley, 1968). Moreover the length of the O-antigen chain also influences serum resistance with very-long chains providing greater protection than shorter variations (Bravo et al., 2008). This is likely to contribute to the inherent differences in susceptibility to serum killing between NTS and *S.* Typhi. It is also thought that LPS contributes to the survival of *Salmonella* by diverting the membrane attack complex

(MAC) away from the cell membrane to a site where it cannot cause cellular damage (Joiner et al., 1989b).

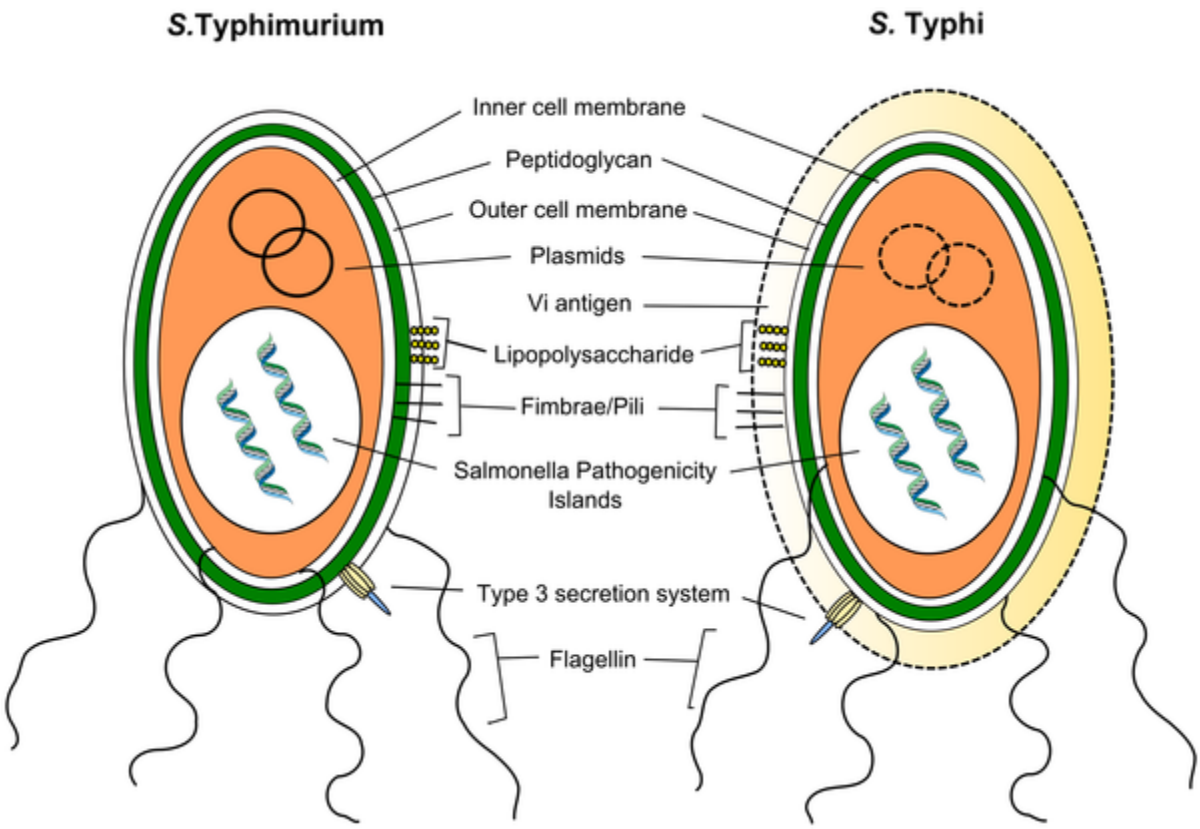


Figure 1.3. *Salmonella* virulence factors

Both *S.* Typhimurium and *S.* Typhi share multiple virulence factors such as LPS, TTSSs and SPIs. *S.* Typhi also expresses a unique virulence factor, the Vi polysaccharide capsule, which is only expressed by a small number of *Salmonella* serovars (*S.* Typhi, *S.* Paratyphi C and *S.* Dublin) (Figure taken from de Jong et al., 2012).

1.8.2. PgtE and Rck mediated complement resistance

In addition to LPS, *Salmonella* expresses multiple surface proteins which enhance resistance to complement mediated killing. The outer membrane protease *PgtE* proteolytically cleaves complement components C3b, C4 and C5, thereby interrupting the complement cascade and increasing the serum resistance of *Salmonella* (Ramu et al., 2007). The *Rck* gene encodes an outer membrane protein (OMP) which binds the complement regulatory protein; factor H (Ho et al., 2010). Factor H usually binds to host cells where it contributes to the cleavage of C3b and the inhibition the alternative complement pathway (Pangburn, 2000). In binding factor H, rck contributes to the inhibition of activation of the alternative complement pathway on the *Salmonella* surface. Rck also prevents tubular MAC formation thereby inhibiting the lytic action of complement (Heffernan et al., 1992). It has also been shown to promote the invasion of non-phagocytic cells by promoting adhesion and inducing invasion via a Trigger-like mechanism (Rosselin et al., 2010).

1.8.3. Salmonella pathogenicity islands (SPIs)

Salmonella pathogenicity islands are genomic islands with a G+C content that differs to the rest of the genome. These islands usually encode a number of virulence related genes and often play a crucial part in *Salmonella* infection (Marcus et al., 2000). SPI-1 encoded genes include a TTSS and its effector proteins which play an important role in invasion (Zhou and Galan, 2001). SPI-2 also encodes for a TTSS and effector proteins such as necessary for the intracellular survival of *Salmonella* (Waterman and Holden, 2003) (For more details on the SPI-1 and SPI-2 TTSS and their effector protein see section 1.8.4).

Salmonella is thought to have multiple pathogenicity islands, however the identification of each and the extent to which they contribute to virulence has yet to be fully elucidated (Groisman and Ochman, 1996, Marcus et al., 2000). Another pathogenicity island important for virulence that is relatively well studied is SPI-7 (Seth-Smith, 2008). SPI-7 is a pathogenicity island found in *S.* Typhi, *S.* Paratyphi C and *S.* Dublin and contains approximately 150 genes (Morris et al., 2003, Liu et al., 2007, Seth-Smith, 2008). Very few *S.* Typhi isolates lack SPI-7 indicating its importance in virulence. The pathogenicity island is unstable however, and may be lost during storage or passage of *Salmonella* (Wain et al., 2005). Importantly, SPI-7 houses the *viaB* locus, one of two loci necessary for the biosynthesis of the Vi polysaccharide (ViPS) capsule, an important virulence factor of *S*. Typhi (Liu and Sanderson, 1995).

1.8.4. TTSS

Salmonella contains genes which encode for two type three secretion systems (TTSS). The SPI-1 encoded TTSS, enhances uptake of *Salmonella* by enterocytes and plays a crucial role in invasion of the intestinal epithelia (Andrews-Polymenis et al., 2010). This TTSS injects effector proteins such as SipA and SopE which cause actin filamentation and cytoskeletal rearrangement resulting in the internalisation of *Salmonella*. Another effector protein SigD then seals invaginated regions of the cell membrane, ultimately leading to the formation of the *Salmonella* containing vacuole (SCV) (Andrews-Polymenis et al., 2010, Hamid and Jain, 2007, Kaur and Jain, 2012). The SPI-2 encoded TTSS is important for the delivery of effector proteins to maintain the integrity of the SCV and manipulate SCV conditions to those favourable for bacterial survival (Waterman and Holden, 2003). A SPI-2 TTSS effector protein, SpiC inhibits endo-

lysosomal fusion thereby enhancing intracellular bacterial survival (Uchiya et al., 1999). SifA, another SPI-2 TTSS effector protein, is required to maintain SCV integrity and SifA mutants exhibit defective intracellular replication within macrophages (Beuzon et al., 2000).

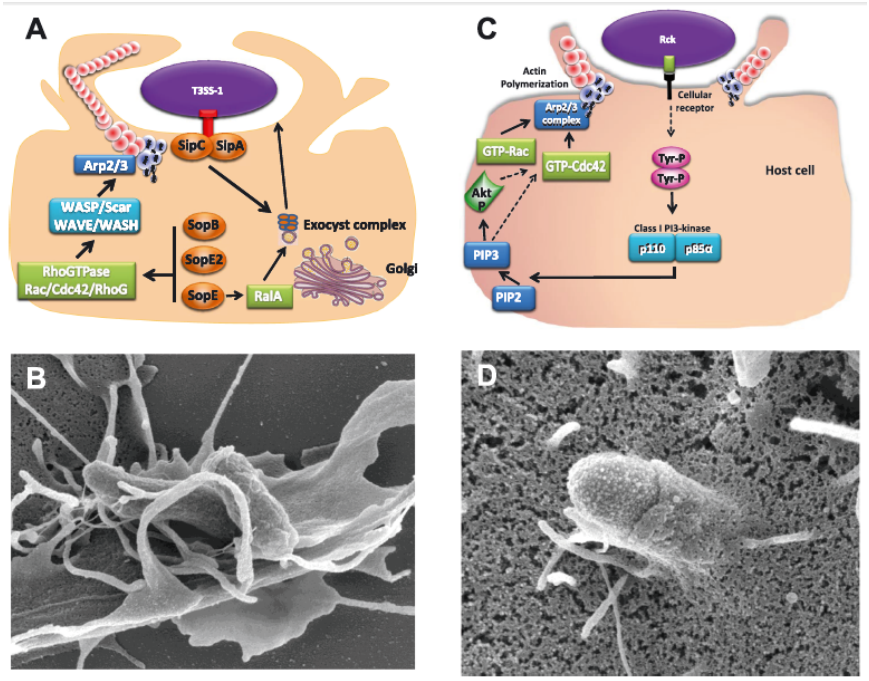


Figure 1.4. *Salmonella* employs zipper and trigger mechanisms to invade non-phagocytic cells

(A) Schematic representation of *Salmonella* induced trigger mechanism involving SPI-1 TTSS effector proteins such as SopB and SopE. SPI-1 TTSS effector proteins induce the remodelling of the actin cytoskeleton and subsequent internalisation of the *Salmonella*. (B) Scanning electron microscopy (SEM) photograph of the characteristic membrane protrusions or "ruffles" at the *Salmonella* entry site. (C) Diagram of the Rck-mediated zipper mechanism of invasion. Binding of Rck to its unknown cellular receptor induces tyrosine kinase mediation actin polymerisation around the *Salmonella.* (D) SEM image of the localised membrane protrusions induced by *Salmonella* using the zipper mechanism (Figure taken from Velge et al., 2012)

1.8.5. Vi capsule

The *viaB* locus contained within SPI-7 contains genes important to the biosynthesis and export of Vi capsule (Seth-Smith, 2008). Another regulatory locus, *viaA* is not present on SPI-7 and is found in most *Salmonella* strains (Virlogeux et al., 1996). In conjunction with *viaB*, these two genetic loci contain genes important for the biosynthesis, regulation and export of the Vi polysaccharide (ViPS) capsule (Santander et al., 2008). Vi capsule is an important virulence factor found in *S.* Typhi, *S.* Paratyphi C and *S.* Dublin. Virtually all clinical isolates of *S.* Typhi contain *viaB* and express Vi capsule, underscoring its importance in survival of these *Salmonella* (Wain et al., 2005, Pulickal et al., 2013) *.* However, Vi-negative, virulent *S.* Typhi strains have been isolated, suggesting Vi capsule is not essential for infection (Baker et al., 2005). Moreover some strains of *S.* Paratyphi cause enteric fever without expressing Vi capsule (Maskey et al., 2006).

It is thought expression of Vi capsule by *S.* Typhi limits intestinal inflammation by reducing the production of inflammatory mediators such as GROα and IL-17, thereby promoting a "stealth" mode of early pathogenesis (Sharma and Qadri, 2004, Raffatellu et al., 2007). Vi is also thought to contribute to the survival of *S.* Typhi by promoting resistance to complement-mediated killing and phagocytosis of *S.* Typhi (Looney and Steigbigel, 1986, Wilson et al., 2011). However, the full extent of its contribution in these processes is unclear and how Vi negative strains cause disease in the absence of Vi capsule has yet to be established.

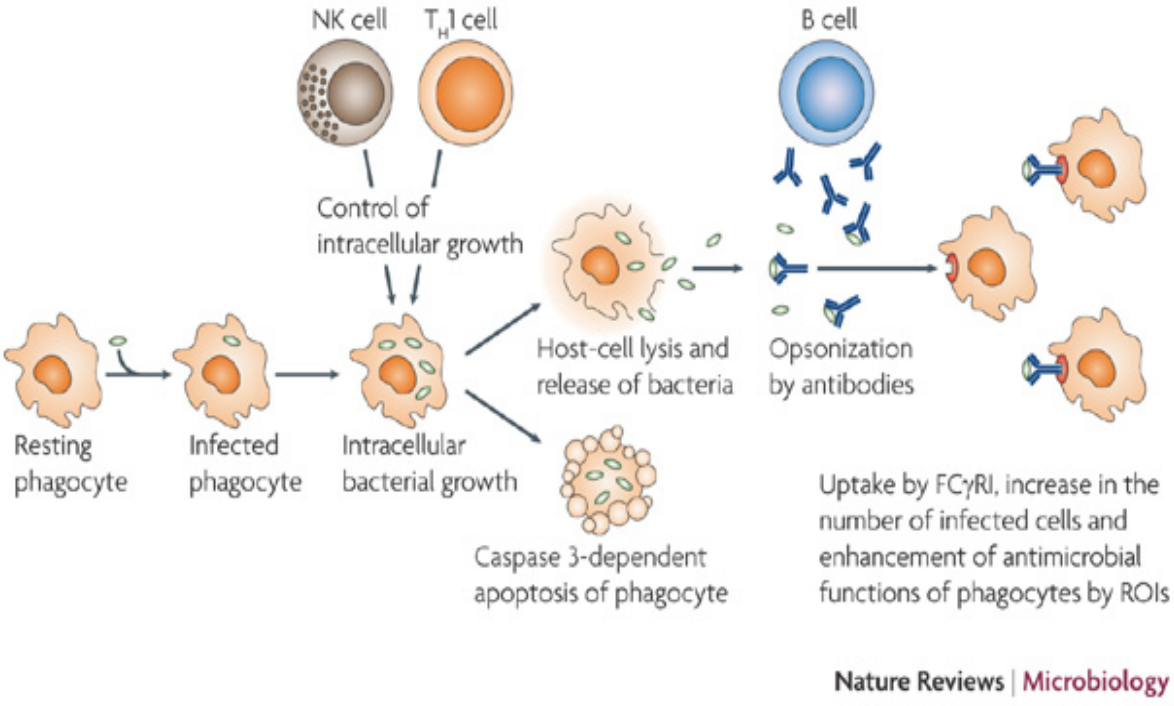


Figure 1.5. Components of the Immune system which control intracellular and extracellular *Salmonella* infection

Growth of *Salmonella* within phagocytes is controlled by cell mediated immunity which activates phagocytes and enhances their antibacterial activity. Lysis of phagocytes by host or bacterial mechanisms results in the release of *Salmonella* into the bloodstream. In the bloodstream antibody binds to *Salmonella* facilitating bactericidal killing and/or opsonophagocytosis contributing to the control of *Salmonella* by blood phagocytes (Figure taken from Mastroeni et al., 2009).

1.9. Immunity to Salmonella

Immunity to *Salmonella* is complex and involves both innate and adaptive arms of the immune system. The immune response and subsequent level of immunity varies according to the infecting *Salmonella* serovar, the immune status of the host and the presence of co-morbidities.

1.9.1. Secretory IgA (sIgA)

During *Salmonella* infection, upon reaching the small intestine, *Salmonella* must overcome the action of mucosal IgA which binds to the *Salmonella* surface and inhibits adhesion to enterocytes (Iankov et al., 2002). In a naïve host, it is likely that natural mucosal IgA, produced in response to gut commensals, inhibits invasion of epithelial cells by *Salmonella* (Wijburg et al., 2006). In murine models, mice deficient in sIgA were more sensitive to *Salmonella* infection and shed more bacteria in their faeces than wild-type mice (Wijburg et al., 2006). Incubation of *S.* Typhimurium with sub-agglutinating levels of anti-LPS IgA leads to increased *Salmonella* membrane stress, reduced epithelial invasion and reduced T3SS activity (Forbes et al., 2012). In humans, immunisation with the live attenuated *S.* Typhi vaccine, Ty21a, elicits the production of LPS specific IgA antibody secreting cells (ASCs). These were found to express the gut homing molecules CD27 and integrin α4β7, suggesting they may effect mucosal, LPS-specific IgA responses (Wahid et al., 2012).

1.9.2. Complement

Functional complement activity is crucial to the early response and control of bacteria within the bloodstream (Rautemaa and Meri, 1999). Complement can act directly on the

bacterial cell through the formation of the membrane attack complex (MAC), which leads to cell lysis. C3b, a complement component is also a potent opsonin, binding to both the bacteria and complement receptors on phagocytes and inducing phagocytosis (Walport, 2001).

Complement is activated in three different ways; the classical, alternative and lectin pathways. The classical pathway requires the initial formation of antigen-antibody complexes to which the first complement component, C1q, binds and in doing so triggers the rest of the cascade (Cooper, 1985). The alternative pathway does not require antibody binding and results from the deposition of spontaneously activated C3 to the bacterial surface (Xu et al., 2001). The lectin pathway involves the interaction of carbohydrate binding proteins to the various carbohydrate moieties on the surface of bacterial cells and viruses (Dodds, 2002). *Salmonella* are able to activate all three pathways (Tateishi and Matsushita, 2011, Grossman et al., 1990). However, the bactericidal activity of human serum against certain *Salmonella* isolates is severely diminished in the absence of antibody, or when the host lacks *Salmonella*-specific antibody (MacLennan et al., 2008). This suggests that the classical pathway is the major contributor to the innate immune response against *Salmonella*. C1q deficient mice are more susceptible to *S.* Typhimurium infection (Warren et al., 2002), illustrating the importance of the classical pathway. In human sera, complement, in addition to antibody, is required for effective phagocytosis of *Salmonella* and production of the oxidative burst (Gondwe et al., 2010, Chateau and Caravano, 1997).

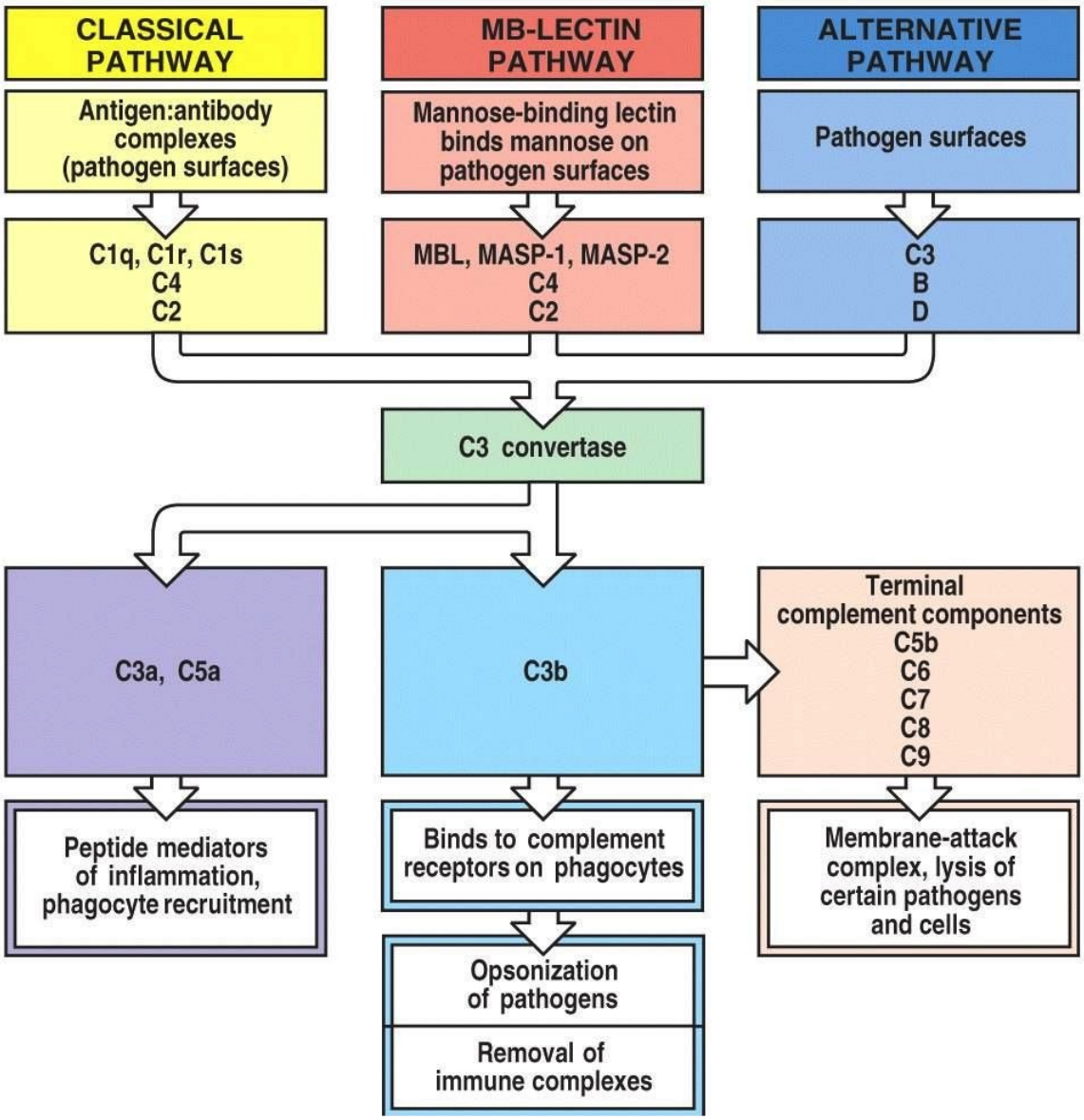


Figure 1.6. Overview of activation and function of the three complement pathways

The three complement pathways are initiated by different mechanisms but converge with the formation of C3 convertase. This cleaves C3 into C3a and C3b and culminates in either opsonisation of the pathogen, formation of MAC and bacterial lysis, or the recruitment of phagocytes (Figure taken from Immunobiology, 5th edition, Garland 2001, Charles A.Janeway Jr, 2001).

1.9.3. Antibody

Salmonella infection involves both an intracellular phase and extracellular dissemination through the bloodstream. This multiphasic pathogenesis leaves *Salmonella* vulnerable to the action of both bactericidal and opsonic serum antibodies (MacLennan et al., 2008, Gondwe et al., 2010). This susceptibility to antibody is highlighted by the effective use of vaccines in the protection against *S.* Typhi infection and the development of O-antigen based vaccines against NTS infection (Maclennan et al., 2014).

Bactericidal antibody contributes to the control of *Salmonella* infection by binding to its respective antigen on the bacterial cell surface. As discussed in section 1.9.2, this activates the classical complement pathway by enabling complement component C1q to bind to antibody-antigen complexes. C1q binding activates the classical pathway and this culminates in the formation of the MAC which is inserted into the *Salmonella* cell membrane and leads to cell lysis and death (Walport, 2001). Both IgG and IgM are able to activate the classical complement pathway with IgM being the more potent activator of the two due to its pentameric structure (Cooper, 1985). IgA has very poor complement activation activity, being unable to activate the classical complement pathway (Hiemstra et al., 1987).

In addition to having bactericidal activity, antibody also binds to the surface of pathogens and acts as an opsonin. *Gondwe et al., 2010* found that antibody was essential for effective phagocytosis and the induction of the neutrophil oxidative burst against *S.*

Typhimurium. Anti-*Salmonella* IgG and IgM titres in sera were significantly associated with the level of the neutrophil oxidative burst induced against *S.* Typhimurium (Gondwe et al., 2010). Fc receptors (FcR) on the surface of phagocytes bind to the Fc portion of antibody and this results in the activation of phagocytic effector functions and subsequent internalisation of the bacteria (Vidarsson and van de Winkel, 1998). IgM was not thought to contribute to phagocytosis due to a lack of IgM specific FcRs on phagocytes. However, recently an IgM specific FcR (Fcμ) has been identified, suggesting IgM could have opsonic potential. Moreover, IgM is a potent activator of the classical complement pathway. As the complement component C3 is opsonic, IgM may contribute to phagocytosis in a complement-mediated manner. The full extent of the involvement of IgM in phagocytosis has yet to be characterised (Kubagawa et al., 2009). IgG is a potent opsonin, but its activity varies depending on the subclass and the Fc receptor available. IgG3, G1 and G4 have high affinity for the FcRI whereas IgG2 has a higher affinity for FcyRIIa (Goh et al., 2011, Nimmerjahn and Ravetch, 2008)

Antibody plays an important role in immunity to and protection against *Salmonella* infection but clear correlates of protection for either NTS or *S.* Typhi have yet to be established in humans. Baseline anti-H antigen (flagellar antigen) titres in unvaccinated individuals have been associated with protection against reinfection with *S.* Typhi. Individuals with anti-H antigen titres >20 had a 22% attack rate of typhoid compared with 48% with anti-H antigen titres lower than this (Levine et al., 1976). Weak associations between anti-O-antigen titres and serum bactericidal activity against *S.* Typhimurium have been observed but SBA activity has yet to be correlated to protection against disease (Nyirenda et al., 2014). Older studies found O-antigen titres are not associated with protection against typhoid, however (Hornick et al., 1970b). Work by

Pulickal et al noted an inverse associated between the incidence of typhoid and bactericidal titre of sera but this could not be attributed to any particular antibody (Pulickal et al., 2009). Moreover post-vaccination sera from immunisations with a live-attenuated Vi^+ *S.* Typhi vaccine effected greater bactericidal activity than pre-vaccination sera (Lindow et al., 2011). Opsonisation with post-vaccination sera also resulted in greater phagocytosis and intracellular killing (Lindow et al., 2011). However, again the contribution of individual antibodies in this study was not established, thus the role of individual antibodies in immunity to typhoid fever remains unclear.

Antibody also contributes to protection against NTS infection. In murine studies, immunisation with heat-killed *Salmonella* induced protection which correlated with the anti-*Salmonella* antibody titre of the sera (Xu et al., 1993). Antibody has also been shown to reduce bacteraemia and limit the hematogenous spread of NTS in mice (Cunningham et al., 2007). In humans, a lack of anti-NTS antibody in African infants has been shown to increase susceptibility to NTS infection (MacLennan et al., 2008). Opsonising antibody, in addition to complement , is necessary for optimal phagocytosis and oxidative burst-mediated intracellular killing of *S.* Typhimurium (Gondwe et al., 2010). Patients with primary hypogammaglobulinaemias or an impaired ability to produce antibody can suffer from recurring and persistent *Salmonella* enterocolitis (Bergbreiter and Salzer, 2009)

1.9.4. Phagocytes

In the early stages of infection, after crossing the intestinal epithelia, *Salmonella* enter the bloodstream and hematogenous spread occurs (Monack et al., 2004). At this point, along with bactericidal antibody and complement, phagocytes such as monocytes and

neutrophils play a key role in controlling *Salmonella* numbers and limiting the spread of bacteria (Dougan et al., 2011, Mastroeni et al., 2009).

Phagocytes express a host of receptors which contribute to the identification and uptake of pathogens such as *Salmonella.* As mentioned in section 1.9.3, they express Fc receptors which recognise the Fc region of opsonic antibody (Vidarsson and van de Winkel, 1998). Phagocytes also express complement receptors such as CR1 (Joiner et al., 1989a). Binding of both the FcR and Cr1 to opsonic antibody and complement on *Salmonella* facilitates the phagocytosis of the bacteria (Ishibashi and Arai, 1990). In addition to these receptors, phagocytes also express Toll-like receptor 4 (TLR4) which is an important pattern recognition receptor (PRR) and recognises the LPS of Gram-negative bacteria such as *Salmonella (Royle et al., 2003).* Toll-like receptor 5 (TLR5) is another important PRR, which is expressed by phagocytes and recognises flagellin (Hayashi et al., 2001).

Monocytes and neutrophils play a key role in the control of *Salmonella* early in infection. They are able to recognise and internalise opsonised *Salmonella*, leading to a reduction in bacterial numbers within the blood in mice (Biozzi et al., 1960). Both neutrophils and macrophages are crucial for the survival of mice in NTS infection (Vassiloyanakopoulos et al., 1998, Friedman and Moon, 1977) and neutropenic humans have a higher risk of iNTS infection (Tumbarello et al., 1995). NTS infections causes a rapid neutrophil influx to intestine and this contributes to the pathophysiology of disease (Tukel et al., 2006). This is absent in *S.*Typhi, due to a Vi-mediated reduction in TLR-dependent IL-8 expression (Raffatellu et al., 2005). Single cell experiments using *S.* Typhimurium and Vi$^-$ *S.* Typhi have shown complement component 5a-dependent neutrophil chemotaxis

which is absent in the presence of Vi$^+$ *S.* Typhi (Wangdi et al., 2014). The effects of Vi-expression by *S.* Typhi could account for some of the differences seen between the pathogenesis of NTS and typhoid infection.

Once internalised, phagocytes produce lysosomes, antimicrobial peptides and reactive oxygen and nitrogen species in an effort to kill or control the intracellular replication of *Salmonella* (Vazquez-Torres and Fang, 2001). NADPH oxidase produces oxygen radicals by catalysing the reduction of molecular oxygen to produce toxic superoxide ions and hydrogen peroxide in a process known as the respiratory burst. This is an important killing mechanism employed by phagocytes against intracellular pathogens (Segal, 2005). The importance of the respiratory burst in the intracellular control and killing of *Salmonella* is highlighted by individuals who suffer from chronic granulomatous disease (CGD). CGD is an inherited immunodeficiency where the genes encoding the NADPH oxidase subunits contain mutations and are unable to produce reactive oxygen species (Segal et al., 2000). This leaves sufferers susceptible to recurrent and atypical *Salmonella* infections which usually require antibiotic treatment (Finocchi et al., 2014, Holland, 2010).

1.9.5. T cell responses

Development of CD4$^+$ T cells in response to *Salmonella* infection and associated IFNγ production are important for the resolution of primary *Salmonella* infection in the murine model. Production of IFNγ activates macrophages, enhancing the phagocytosis and killing of *Salmonella* (Hess et al., 1996, VanCott et al., 1998). T-bet knockout mice

and mice with problems in Th1 development, production and/or responsiveness to IFNγ, all suffer from increased susceptibility to *Salmonella* infection, illustrating the importance of this T cell subset in resolving primary infection (Ravindran et al., 2005, Ravindran and McSorley, 2005) .

The role of $CD8^+$ T cells in the clearance of primary *Salmonella* infection is unclear. Mice deficient in β2-microglobulin were not more susceptible to *Salmonella* infection compared to control littermates suggesting $CD8^+$ T Cells do not contribute to immunity in primary infection (Hess et al., 1996). However, recent studies using MHC class 1a gene, perforin and granzyme knockout mice showed delayed resolution of primary *Salmonella* infection in these mice compared to wild-type mice. This suggests that $CD8^+$ T Cells contribute to the resolution of primary infection (Lee et al., 2012).

Both $CD4^+$ and $CD8^+$ T Cell subsets are thought to be important for the contribution of protective immunity against *Salmonella* (Nauciel, 1990, Mastroeni et al., 1992). However, adoptive transfer of splenocytes from immune to naïve mice was not sufficient for protection against secondary infection. Transfer of both splenocytes and serum from immune mice however, did offer protection (Mastroeni et al., 1993). This indicates that a combination of T Cells and antibody is important for protective immunity. Interruption of the Th1 response early in infection by antibiotic treatment impairs the contribution of $CD4^+$ T cells to protective immunity in mice (Griffin et al., 2009). Antibiotic treatment has been associated with relapses in Chlamydia infection (Brunham and Rekart, 2008) and this may explain why patients with typhoid fever undergoing antibiotic treatment often experience relapses of disease (Griffin et al., 2011).

1.9.6. B cell responses

B cell responses are not thought to be involved in the resolution of primary *Salmonella* infection and mice which are deficient in B cells resolve primary infection in a similar fashion to wild-type mice (McSorley and Jenkins, 2000, Mastroeni et al., 2000). In contrast, B cells are essential to the development of protective immunity against *Salmonella,* and B cell deficient mice are unable to resolve secondary infection with virulent *Salmonella* (Mastroeni et al., 2000). B cells contribute to protective immunity in both antibody-dependent and independent manners. The transfer of immune serum to B cell deficient mice leads to the resolution of secondary infection in these mice and illustrate the contribution of antibody to protective immunity (McSorley and Jenkins, 2000). B cells are also able to present antigen to *Salmonella* specific Th1 cells which augments the production of pro-inflammatory cytokines such as IL-12 and IFNγ (Nanton et al., 2012, Mastroeni et al., 2000).

1.9.7. Cytokines

1.9.7.1 Enterocyte cytokine responses

Infection of enterocytes with either NTS or *S.* Typhi results in increased expression and secretion of a number of cytokines and chemokines (Eckmann and Kagnoff, 2001). *Salmonella* infection increases the expression of IL-8 and GROα, important neutrophil chemoattractants (Katancik et al., 2000, Eckmann et al., 1993) and also MCP-1, a monocyte/ macrophage chemoattractant (Izadpanah et al., 2001). Enterocytes also secrete G-CSF and GM-CSF in response to infection which can activate phagocytic cells (Saarinen et al., 2002).

1.9.7.2 Phagocyte cytokine responses

Monocytes and macrophages express and secrete a wide range of pro-inflammatory cytokines and chemokines such as IL-1, TNFα, IL-6, MIP-1α and MIP-2α in response to *Salmonella* stimulation (Galdiero et al., 1993, Rosenberger et al., 2000, Yamamoto et al., 1996). These cells also express IL-12 and IL-18 in response to *Salmonella* infection whereas enterocytes do not (Bost and Clements, 1997, Mastroeni et al., 1999). IL-12 and IL-18 are important for the induction of IFNγ responses which are central to the control of *Salmonella* infection (Nakahira et al., 2002, Dybing et al., 1999). Monocytes and macrophages are also able to secrete pro-inflammatory cytokines in response to stimulation with bacterial products such as LPS and flagellin (Wyant et al., 1999, Rosenberger et al., 2000). This is in contrast to enterocytes which are typically hyporesponsive to certain bacterial products such as LPS, or only respond to intracellular flagellin (Eckmann et al., 1993, Huang, 2012, Gewirtz et al., 2001). This is most likely due to the requirement for immune tolerance resulting from continual exposure to intestinal flora. Neutrophils also express and secrete chemokines such as IL-8 and MIP-1α in response to *Salmonella* infection (Hachicha et al., 1998). These act to potentiate neutrophil chemotaxis and activation, and stimulate the phagocytosis of microbial pathogens.

1.9.7.3 IFNγ

The role of IFNγ in *Salmonella* infection is well studied and this one of the most important cytokines involved in defence against *Salmonella.* It is produced primarily by T cells and natural killer cells (Schoenborn and Wilson, 2007). During infection, expression of IFNγ is upregulated by these cells in the intestinal mucosa, M cells, Peyer's

patches, liver and spleen (Eckmann and Kagnoff, 2001). Mice depleted of IFNγ by antibody, or IFNγ knockouts have increased *Salmonella* numbers in the liver and spleen and experience decreased survival after *Salmonella* infection, highlighting the importance of this cytokine in control of *Salmonella* (Bao et al., 2000, Nauciel and Espinassemaes, 1992). Production of IL-12 and IL-18 by macrophages stimulates increased production of IFNγ by T cells, and in a self-potentiating manner, IFNγ is thought to activate monocytes and macrophages (Eckmann and Kagnoff, 2001). The contribution of IL-12 and IL-18 is illustrated using murine depletion studies. Depletion of either IL-12 or IL-18 leads to an increase in numbers of splenic *Salmonella* and decreased survival of mice (Kincy-Cain et al., 1996, Dybing et al., 1999). Depletion was associated with reduced serum IFNγ levels and in the case of IL-12 depletion, IFNγ treatment reversed the effects of IL-12 neutralisation. Treatment of *Salmonella* infection in mice deficient in the IFNγ receptor with exogenous IL-18 had no effect, whereas it increased the survival of, and reduced bacterial numbers in, control littermates (Mastroeni et al., 1999). This indicates that the effects of IL-12 and IL-18 on survival of *Salmonella* infection are dependent on IFNγ.

1.9.7.4 TNFα and IL-1

Both TNFα and IL-1 are pro-inflammatory cytokines which are important to the control of *Salmonella* infection (Eckmann and Kagnoff, 2001). Neutralisation of TNFα by genetic or pharmacological methods in mice increases the severity of infection and decreases host survival (Gulig et al., 1997, Nauciel and Espinassemaes, 1992). Accordingly, treatment with either IL-1a or TNFa increases host survival after *Salmonella* infection (Morrissey et al., 1995).

1.10. Salmonella vaccines

As discussed in section 1.3.1, NTS infection, especially iNTS is a major cause of morbidity and mortality in sub-Saharan Africa (Reddy et al., 2010) and is one of the commonest causes of bloodstream infections in Africa. *S.* Typhi remains a problem in Southeast Asia but the incidence of typhoid fever is also increasing in parts of Africa and areas of the Middle East affected by conflict (Feasey et al., 2013, World Health Organisation, 2014). Multi-drug resistance is common for both typhoidal and NTS serovars (Kariuki et al., 2010, Kingsley et al., 2009) and diagnostic facilities are often poorly equipped or unable to identify infecting *Salmonella* serovars (Morpeth et al., 2009). Thus *Salmonella v*accination has great potential for the improvement of public health in areas where infection is common.

Multiple vaccines are currently licensed for use against *S.* Typhi but none exist against other clinically important serovars such as *S.* Paratyphi, *S.* Typhimurium or *S.* Enteritidis (Maclennan et al., 2014). Current typhoid vaccines are either the live attenuated Ty21a oral vaccine (Germanier and Fuer, 1975), which is a Vi^- mutant strain derived from virulent Ty2, or vaccines employing the *S.* Typhi Vi polysaccharide (ViPS) as their protective antigen (Maclennan et al., 2014). A whole cell heat-inactivated vaccine was formerly licensed and induced good three year efficacy of 73% (Fraser et al., 2007). However, this vaccine suffered from high reactogenicity limiting its use to military personnel. Current Vi-based vaccines either contain purified Vi polysaccharide (ViPS) or ViPS conjugated to a carrier protein such as tetanus toxoid (TT), *P. aeruginosa* exoprotein A (rEPA), diphtheria toxin (DT), or a nontoxic mutant of DT, CRM_{197} (Maclennan et al., 2014). As ViPS alone is a T-independent antigen, conjugation to a

carrier protein should enlist T helper cell recognition of the antigen, thus shifting the peak antibody response to a younger age and stimulating the production of memory B cells specific for ViPS (Stein, 1992). Vi-conjugate vaccines such as Vi-rEPA and Vi-TT are licensed for use in China and India respectively (Szu, 2013). Despite the availability of effective vaccines, typhoid remains prevalent in endemic areas (Bajracharya et al., 2014).

The isolation of Vi^-, virulent, *S.* Typhi isolates in Pakistan has caused concern that widespread use of a Vi-vaccines may select for virulent Vi^- *S.* Typhi (Baker et al., 2005). Scope exists to improve existing typhoid vaccines for use in infants under the age of 2 years, and the immunogenicity and vaccine potential of other *S.* Typhi antigens should be considered. *S.* Typhi O-antigen conjugate vaccines have been trialled in mice and exhibit good immunogenicity and lead to increased IgG titres compared to immunisation with O-antigen alone (Ali et al., 2012). However, the efficacy of anti-O-antigen antibodies against Vi-expressing *S.* Typhi is something which requires further study.

No vaccines against NTS serovars are currently licensed. NTS O-antigen conjugated to flagellin or porins have shown promise in mice, and conjugation to O-antigen increases protection afforded by immunisation with flagellin or porins alone (Simon et al., 2011, Svenson et al., 1979). The expression of the same O-antigen by multiple *Salmonella* serovars in the same serogroup also provides scope for a multivalent, cross-protective O-antigen vaccines (for example the O:9 O-chain antigen is expressed by both *S.* Enteritidis, *S.* Dublin and *S.* Typhi). New live attenuated NTS vaccines have been developed. A live attenuated *S.* Typhimurium vaccine strain, containing mutations in guanine synthesis and protease regulation genes, has been tested in mice and protected

against challenge with virulent *S.* Typhimurium (Tennant et al., 2011a). A live attenuated *S.* Typhimurium strain has also been tested in humans and prolonged stool shedding of the vaccine strain was observed (Hindle et al., 2002).

A potential problem with live attenuated vaccines is that strains are often able to causes fulminant disease in the context of immunocompromise and this has been demonstrated with live attenuated NTS vaccines in immunodeficient mice (Hess et al., 1996). This is an important consideration given that faecal shedding of the trialled live attenuated vaccine in humans was observed and this could lead to environmental spread of live-attenuated vaccine strains. Moreover, the relative abundance of co-morbidities and subsequent immune dysfunction in potential vaccination areas such as Africa means that for a live attenuated vaccine to be effective in these areas it must be safe in HIV-infected individuals.

Protein based subunit vaccines are attractive candidates for several reason. Some, such as members of the Omp family, are conserved across multiple *Salmonella* serovars, thereby offering increased potential for cross protection. The proteinaceous nature of these vaccines should augment their immunogenicity and elicit T Cell help, inducing greater protection than polysaccharide vaccines alone. *Salmonella* porins have shown good immunogenicity in mouse immunisations, and protection against challenge has been attributed to anti-OmpD antibody (Gil-Cruz et al., 2009). Immunisation with OmpC and OmpF induced long-lasting antibody responses in mice and was found to be safe and immunogenic in phase 1 human trials (Secundino et al., 2006, Salazar-Gonzalez et al., 2004). The complex structure of these antigens however, poses challenges for their effective production and delivery. Flagellin has been investigated as a vaccine candidate

and induces protection in mice when used alone or as a carrier protein for O-antigen polysaccharide (Simon et al., 2011, Simon et al., 2013). Indeed conjugation offers the advantage of combining multiple protective antigens in the one vaccine. No NTS or *S.* Paratyphi vaccines are currently licensed, but conjugates such as an O:2-TT conjugate are in clinical trials (Maclennan et al., 2014).

1.11. Aims and objectives

One of the principal aims of this study was to investigate the contribution of the Vi capsule to the protection of *Salmonella* against the innate immune response. We also wanted to characterise the role of human anti-Vi antibody and antibody to the *S.* Typhi and *S.* Enteritidis O:9 O-Antigen in the bactericidal and opsonic killing of wild-type *S.* Typhi. We hypothesised that Vi capsule should protect against bactericidal and opsonic antibody regardless of the expressing serovar, with protection being dependent on the presence on specific antibody. With antibody dysregulation thought to contribute to an increased susceptibility to iNTS, we investigated the HIV-NTS/*S.* Typhi infection paradox in an *in vitro* setting. We examined both the role of antibody and cytokines to further understand this phenomenon and address whether dysregulation in these arms of the immune system may account for the differences in NTS and typhoid infection in the context of HIV-infection.

2. Materials & Methods

2.1. Ethics

Ethical approval for the collection of blood and blood products in the UK was granted by the Life and Health Sciences Ethical Review Committee of the University of Birmingham. Collection and use of blood from the Queen Elizabeth Hospital HIV clinic, Birmingham, was granted by the North Staffordshire Research Ethics Committee.

The collection of blood and blood products from HIV-infected and uninfected adults and children in Malawi was approved by the College of Medicine Research and Ethics Committee, The University of Malawi. Approval for collection of Tanzanian samples came from the ethics committees of the London School of Hygiene and Tropical Medicine, United Kingdom and the National Institute for Medical Research, Tanzania.

2.2. *Salmonella*

Nontyphoidal *Salmonella* serovars used in this study were *Salmonella enterica* serovars Typhimurium and Enteritidis. Both *S.* Typhimurium D23580 and *S.* Enteritidis D24954 are representative, invasive, African strains isolated from bacteraemic children admitted to the Queen Elizabeth Central Hospital (QECH), Malawi, in 2003 and 2004. Other wild-type nontyphoidal African strains were isolated from HIV-infected and uninfected adults and children at Teule hospital and the Kilimanjaro Christian Medical Centre in Muheza and Moshi, Tanzania respectively. *S*. Typhimurium SGB1 and its isogenic derivative, C5.507 Vi^+, which harbours the *viaB* locus and expresses the Vi polysaccharide capsule were used for functional studies into the role of the Vi capsule (Jansen et al., 2011).

Typhoidal *Salmonella* serovars used in this study were all *Salmonella enterica* serovar Typhi. *S.* Typhi ISP-04-06979, EO3-9804 and EO3-4983 were isolated from Central Africa, Nepal and Indonesia respectively and are all virulent, wild-type strains which belong to the haplotype 58 (H58) lineage of *S.* Typhi, typically characterised by multidrug-resistance (Holt et al., 2008, Kariuki et al., 2010). Other African wild-type *S.* Typhi strains used were isolated alongside nontyphoidal serovars in Moshi and Muheza, Tanzania. *S*. Typhi BRD948, an *aroC*, *aroD* and *htrA* mutant derived from Ty2, but which still expresses the Vi polysaccharide capsule, and *S*. Typhi BRD948 *tviB*-, which is isogenic to *S*. Typhi BRD948 but lacks the *tviB* gene required for biosynthesis of the Vi polysaccharide capsule, were used for functional studies into the role of Vi capsule on *S.* Typhi in a containment level-2 setting (Dougan et al., 1987). Media for *S*. Typhi BRD948 and *S*. Typhi BRD948 *tviB*- were supplemented with tyrosine, tryptophan and phenylalanine at a final concentration of 40µg/L and para-aminobenzoic acid (PABA) and di-hydroxybenzoic acid at a final concentration of 10µg/L (Sigma). *S.* Typhimurium C5.507 and SGB1 and *S.* Typhi BRD948 and BRD948 *tviB*- were provided by the Wellcome Trust Sanger Institute (WTSI), Cambridge, UK. For a list of the principal *Salmonella* strains used in this study, please refer to Table 2.1.

Table 2.1. Principal *Salmonella* strains used in this study

Strain	Serovar	Vi Status	Location Isolated/ Donor
D23580	*S.* Typhimurium	-	Blantyre, Malawi
C5.507	*S.* Typhimurium	+	WTSI*, Cambridge
SGB1	*S.* Typhimurium	-	WTSI*, Cambridge
D24954	*S.* Enteritidis	-	Blantyre, Malawi
BRD948	*S.* Typhi	+	WTSI, Cambridge
BRD948 -tviB	*S.* Typhi	-	WTSI, Cambridge
ISP-04-06979	*S.* Typhi	+	Kenya
EO3-9804	*S.* Typhi	+	Nepal
EO3-4983	*S.* Typhi	+	Indonesia
129-0238-M	*S.* Typhi	+	Moshi, Tanzania
63714	*S.* Typhi	+	Muheza, Tanzania

*Wellcome Trust Sanger Institute, Cambridge, UK

2.3. *Preparation of Stationary and Logarithmic Phase Salmonella Culture*

2.3.1. Stationary phase

A sterile loop was used to take a single colony of *Salmonella* grown on LB agar (Sigma) and inoculate 10ml LB broth. This was incubated overnight at 37°C in a loosely capped universal tube providing stationary phase inoculum the following morning.

2.3.2. Logarithmic phase

100µl stationary phase *Salmonella* was used to inoculate 10ml pre-warmed (37°C) LB broth and incubated, in a tightly capped universal tube at 37°C whilst on a rocker plate at 20rpm. The total length of the logarithmic phase incubation was dependent on the final concentration of *Salmonella* required and pre-determined by strain specific growth curves. Once ready, a known quantity of inoculum was centrifuged at 3300 x g for 5 minutes. The supernatant was discarded and pellet suspended in 1ml of Phosphate Buffered Saline (PBS) (Sigma). This washing step was repeated thrice with the pellet suspended in a predetermined quantity of PBS after the final wash to give the required concentration of *Salmonella*. The final concentration was determined using the Miles and Misra method (Miles et al., 1938).

2.4. *Preparation of Serum*

2.4.1. Human Sera

Venesection was performed on consenting adults. Blood was either aliquoted directly into a sterile, 50ml tube (Sarstedt) or drawn into a clotting activator tube (CAT, BD). Blood aliquoted into tubes was left to clot at room temperature for 4 hours then

centrifuged at 4000 x g for 20 minutes. The serum fraction was aliquoted into microfuge tubes and immediately frozen at -80°C. The CAT Vacutainers containing freshly drawn blood were inverted 10 times and left to clot for 30 minutes at room temperature. Once clotted, CATs were centrifuged at 2000 x g for 10 minutes. All tubes were centrifuged in a Megafuge (Heraeus). Heat inactivated serum was prepared by heating serum in a water bath at 56°C for 30 minutes to 1 hour depending upon the *Salmonella* strain being tested. C6 deficient serum, which is unable to form the membrane attack complex (MAC), was from a patient with congenital C6 deficiency.

2.4.2. Animal sera

Animal anti-Vi-sera were provided by Novartis Vaccines Institute for Global Health (NVGH), Siena, Italy. Cd1 mice and New Zealand white rabbits were immunised with a conjugate Vi vaccine Vi-CRM_{197} (Micoli et al., 2011) at day 0, 14 and 28. Terminal bleeds were done at day 42 and blood left to clot at room temperature. Once clotted, serum was prepared by centrifugation for 10 minutes at 2000 x g. Sera were stored at -80°C until use.

2.5. *Preparation of Washed Blood*

Blood was taken from consenting, healthy adults directly into a 50ml tube containing preservative free Sodium Heparin (Wockhardt) at a final concentration of 4 international units (IU) per ml of blood. Blood was washed twice using RPMI-1640 (Sigma) in a 1:10 ratio to remove exogenous antibody and complement and centrifuged

in a Megafuge (Heraeus) at 250 x g for 20 minutes. The supernatant was discarded and the washed blood re-suspended in RPMI-1640 to the original volume.

2.6. *Serum Bactericidal Assay (SBA)*

2.6.1. Human sera SBAs

5µl *Salmonella* in log growth at a concentration of 1 x 10^7 CFU/ml (prepared as described in section 2.3.2.) was added to 45µl 100% human serum in a 96 well, round bottom plate (Sarstedt) to give a final concentration of 1 x 10^6 CFU/ml. Samples were incubated at 37°C for 180 min on a rocker plate at 20 rpm. At 45, 90 and 180 min, 10µl sample was taken and the concentration of viable *Salmonella* determined by serial dilution and plating onto LB agar in triplicate. Agar plates were incubated overnight at 37°C and colonies counted the following day. By calculating the number of *Salmonella* at the start of the assay and at each time point, we measure the growth or killing of *Salmonella* in relation to the initial concentration. Data was $\log_{10}$ transformed allowing the relative growth or kill to be plotted as depicted in Figure 2.1.

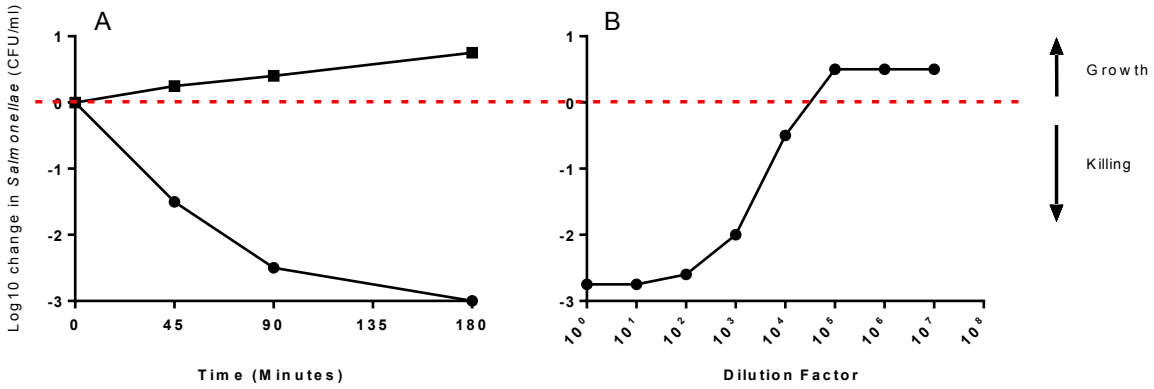


Figure 2.1. Representation of bactericidal assay data

Data from SBAs or BCKs can be depicted in either a chronological manner (A) or the results of a dilution series at one time point (B). Lines represent the $\log_{10}$ change in bacterial numbers in relation to the initial starting concentration of *Salmonella*. Bacterial growth is characterised by a line above 0 (---) while killing is depicted as a line below this level.

2.6.2. SBAs with animal sera

Bactericidal assays using animal sera were performed essentially as described in section 1.6.1. Sera from mice and rabbits immunized with a Vi conjugate vaccine, Vi-CRM_{197} (Micoli et al., 2011), were heat-inactivated and combined with human sera depleted of antibody against Vi^+ *S.* Typhimurium or Vi^+ *S.* Typhi as a source of exogenous C'. 11.25µl of animal serum (25% final volume) in a 1:10 dilution series was added to 33.75µl of *Salmonella*-antibody-depleted human serum (75% final volume) prior to incubation with *Salmonella*.

2.6.3. SBAs with purified human Ab

SBAs using purified human antibody (hAb) against the Vi and O:9 antigens of *Salmonella* were conducted as detailed in section 1.6.1. 11.25µl concentrated anti-Vi and anti-O:9 antibody (25% final volume) corresponding to 750 and 4268 IgG EU (Quantified by ELISA as described in section 2.16) per ml respectively were combined, in a 1:4 dilution series, with 33.75µl *Salmonella*-antibody-depleted human serum (75% final volume) and incubated with *Salmonella* at 37°C for 180 minutes.

2.7. *Peripheral Blood Cell Killing Assay (BCK)*

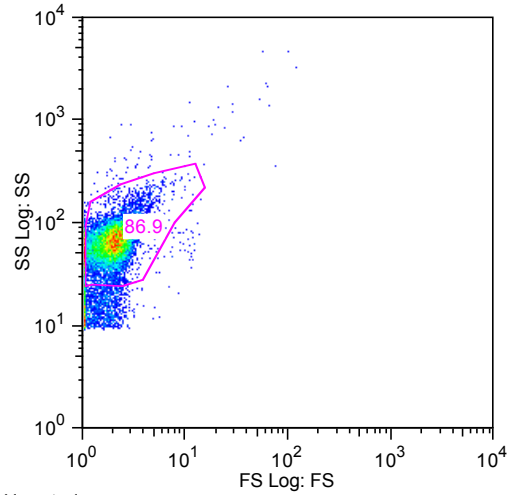
To test the killing of opsonised *Salmonella* by washed peripheral blood cells, Log phase *Salmonella* at a concentration of 1 x 10^7 CFU/ml were prepared as described in section 2.3.2. 5µl washed *Salmonella* was opsonised in microfuge tubes with 45µl antibody and complement to give a final concentration of 1 x 10^6 CFU/ml. For the opsonin 50-75% heat-inactivated human sera or purified hAb were used as an antibody source and

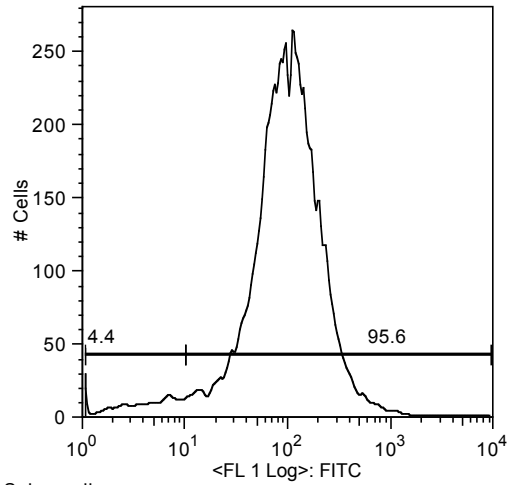
combined with 25-50% C6-deficient serum or *Salmonella*-antibody-depleted human serum as a source of complement prior to incubation for 20 minutes at room temperature. The ratio of antibody to complement was dependent on the *Salmonella* strain used and its sensitivity to C'. 5µl opsonised *Salmonella* were added to 45µl washed blood (as prepared in section 2.5.) and incubated at 37°C for 180 min on a rocker plate at 20 rpm. Samples were taken at 45, 90 and 180 minutes and 10µl sample serially diluted with 90µl PBS in a 96 well plate. To ensure killing in the assay was due to peripheral blood phagocytes and not serum bactericidal activity, *Salmonella* were opsonised with serum and added to RPMI 1640 (Sigma). To check endogenous antibody and complement were removed on washing of the blood *Salmonella* were opsonised with PBS and added to washed blood. Serial dilutions were plated onto LB agar in triplicate and incubated overnight at 37°C. Bacterial colonies were counted the following day and the number of viable bacteria calculated using the Miles and Misra method. Viable counts were $\log_{10}$ transformed allowing the relative growth or kill to be plotted against the initial concentration of *Salmonella* (See Figure 2.1).

2.8. *Antibody Binding Assay*

5µl Log phase *Salmonella* at a concentration of 1 x 10^9 CFU/ml (prepared as detailed in section 2.3.2.) was added to 45µl human serum or 45µl purified hAb (both diluted 1:10 with PBS). 45µl PBS was used as a negative control. Samples were gently vortexed and incubated for 20 minutes at room temperature. 1ml PBS was added and samples were washed, centrifuging at 3300 x g. The supernatant was discarded and an additional 1ml PBS added for the second wash. After the final wash, the supernatant was aspirated to

leave 50µl to which 2µl neat, polyclonal, FITC-conjugated anti-human IgG, IgA or IgM (Sigma) was added. The sample was mixed by pipetting and incubated in the dark at room temperature for 20 minutes. 1ml PBS was added and the sample was washed twice, again centrifuging at 3300 x g for 5 minutes. The supernatant was discarded and the sample fixed with the addition of 200µl 1% formaldehyde PBS. Samples were analysed by flow cytometry using a FACSCalibur (BD Biosciences) or a Cyan (Dako) and samples were analysed using Cellquest Pro or Summit software respectively. *Salmonella* populations were identified according to their forward and side scatter characteristics (Figure 2.2) and the geometric mean fluorescence intensity (GMFI) in the FL1 channel taken as the relative antibody binding titre.





Ancestry Subset Value Type For	Salmonella/FITC+ Freq. of Parent	Salmonella/FITC+ Geom. Mean <FL 1 Log> FITC	Salmonella/FITC- Freq. of Parent	Salmonella/FITC- Geom. Mean <FL 1 Log> FITC
Sample_26.fcs	95.6	102	4.4	3.67

Figure 2.2. Antibody binding and complement deposition assay gating scheme for identification of *Salmonella* and quantification of corresponding fluorescence.

Ab binding to and complement deposition onto *Salmonella* was measured by flow cytometry. *Salmonella* were identified according to their forward and side scatter characteristics. The GMFI in the FL-1 channel was taken as the level of antibody binding or complement deposition onto *Salmonella.*

2.9. *Complement Deposition Assay*

5µl Log phase *Salmonella* (section 2.3.2.) was added to 45µl undiluted human serum or to 45µl 25% purified hAb, 75% *Salmonella*-antibody-depleted human serum (final bacterial concentration 1 x 10^9 CFU/ml) and incubated for 20 minutes at room temperature. 1ml of PBS was added prior to washing at 3300 x g for 5 minutes. The supernatant was discarded and a further 1ml PBS added and washing repeated. The supernatant was aspirated to leave 50µl to which FITC-conjugated mouse anti-C3 IgG or mouse anti-C5-9 IgG (Dako) for the detection of C3 or MAC respectively. Samples were incubated for 20 minutes protected from light, following which they were washed twice as before and those labelled with anti-C3 IgG were fixed using 1% formaldehyde PBS. Samples labeled with anti-C5-9 antibody had their supernatant aspirated to leave 50µl to which 2µl FITC-conjugated rabbit anti-mouse IgG (Dako) was added. Samples were again incubated in the dark for 20 minutes at room temperature then washed and fixed as before using 1ml PBS and 1% formaldehyde PBS respectively. Acquisition and analysis of the levels of complement deposition on the surface of *Salmonella* was conducted as detailed in section 2.8.

2.10. *Depletion of Anti-Salmonella antibody from Human Sera*

Stationary phase *Salmonella* culture was prepared as in section 2.3.1. *Salmonella* were washed three times with PBS at 3300 x g for 5 minutes and resuspended in PBS to give a final concentration of 1 x 10^{11} CFU/ml. Human serum was thawed at 4°C and the concentrated bacterial culture pre-cooled to 4°C. 100µl cooled *Salmonella* was incubated with 900µl chilled serum at 4°C for 1 hour at 20 rpm. Where chilled mechanical mixing apparatus was unavailable samples were inverted 10 times at 15 minute intervals. After

1 hour, samples were centrifuged at 3300 x g for 5 minutes at 4°C. Serum was aliquoted into a new microfuge tube and the pellet discarded. 100µl chilled *Salmonella* was added to the serum and this cycle repeated twice. After a total of three cycles, the sample was centrifuged as above and the serum filter sterilized with a 0.22µm syringe driven filter (Millex). Antibody depleted serum was stored at -80°C. Antibody titres pre and post-depletion were assayed by ELISA (See section 2.16) and the functionality of the antibody-depleted sera assessed in SBAs prior to use.

2.11. *Phagocytosis Assay*

2.11.1. FITC labelling of Salmonella

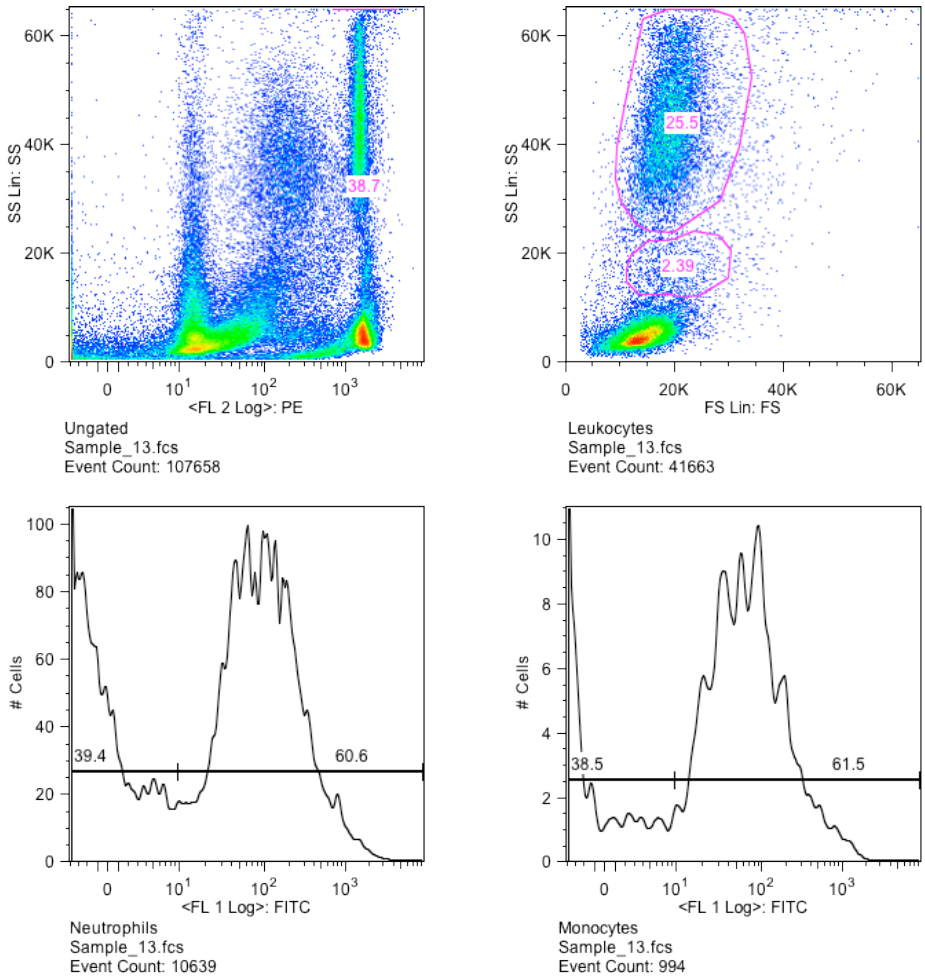
Log phase *Salmonella* were grown as detailed in section 2.3.2. Nontyphoidal serovars were grown for a total of 3 hours and *S*. Typhi for 5 hours. *Salmonella* were resuspended in PBS at a final concentration of 1 x 10^{10} CFU/ml prior to killing with 1% formaldehyde PBS for 30 minutes. 10mg Fluorescein isothiocyanate (Sigma) was added to 10ml PBS to give a final concentration of 1mg/ml. This was incubated at room temperature, in the dark for 10 minutes before filter sterilisation using a 0.22µm syringe driven filter (Millex). 3.75µl FITC solution was added to 150µl killed *Salmonella* in a microfuge tube and vortexed. The mixture was then incubated, protected from light, at 37°C on a rocker plate at 20 rpm for 30 minutes. After labelling, samples were washed twice with 1ml PBS and centrifugation at 3300 x g for 5 minutes. After washing, FITC labelled *Salmonella* were stored in the dark at 4°C until ready to use.

2.11.2. Quantification of phagocytic activity

50µl washed blood (as prepared in section 2.5) was added to 5ml FACS tubes (BD) and tubes were incubated on ice for 10 minutes. 5µl FITC-labelled *Salmonella* were added to either 45µl fresh, human sera or 45µl antibody and complement in a 25:75 % ratio respectively. Antibody was either heat-inactivated sera (as detailed in section 2.4.1) or purified human antibody (Section 2.16). Complement was antibody-depleted serum as prepared in section 2.10. Samples were incubated in the dark for 20 minutes at room temperature. After 20 minutes, 10µl opsonised, FITC-labelled *Salmonella* were added to the pre-cooled blood tubes. Half the samples were immediately placed in a water bath at 37°C whilst the remaining control samples were kept on ice. Samples at 37°C were incubated for 10-20 minutes depending on the strain of *Salmonella* and the time course required.

After incubation, samples were immediately transferred to an ice bath and 50µl, ice cold, 0.16% Trypan blue (Sigma) added to each tube to quench extracellular fluorescence. Samples were vortexed and 1.5ml, ice cold, PBS added to each tube. After further vortexing, samples were centrifuged at 250 x g, the supernatant discarded and samples washed again with 1.5ml cold PBS. After washing, 1ml, room temperature, FACS lysing solution (BD) (diluted 1:10 with diH_20) was added to each tube and samples incubated at room temperature for 20 minutes. Samples were washed twice as before and 100µl propidium iodide at a final concentration of 20µg/ml was added to each tube. Samples were incubated on ice for 10 minutes before acquisition on a FACSCalibur or Cyan. Intact cells were identified by their side scatter characteristics and high fluorescence in the PE or FL-2 channels (Cyan and Calibur respectively). From this population, neutrophils and monocytes were identified according to their forward and side scatter characteristics.

Levels of internalized bacteria were assessed using the GMFI in the FL-1 or FITC channel (Cyan and Calibur respectively) and the percentage of cells positive for FITC signal determined (See Figure 2.3). Samples were analysed using FlowJo software (TreeStar Inc.)



Ancestry Subset Value Type For	Leukocytes/Monocytes/FITC+ Freq. of Parent	Leukocytes/Monocytes/FITC+ Geom. Mean <FL 1 Log> FITC	Leukocytes/Neutrophils/FITC+ Freq. of Parent	Leukocytes/Neutrophils/FITC+ Geom. Mean <FL 1 Log> FITC
Sample_13.fcs	61.5	69.6	60.6	96

Figure 2.3. Gating schematic for the assessment and quantification of *ex vivo* phagocytosis of *Salmonella* by peripheral blood neutrophils and monocytes.

Peripheral blood leukocytes were identified by their side scatter and high fluorescence in the PE/FL-2 channel (Channel chosen dependent on FACS machine). Monocytes and neutrophils were discriminated from the leukocyte population according to their forward and side scatter characteristics. The GMFI in the FL-1 channel was taken as the level of internalised *Salmonella.* With the percentage of FITC+ cells of the parent population representing phagocytosing cells. Incubation of washed blood cells with opsonised *Salmonella* at 4°C was used as the negative control.

2.12. *Characterisation of Vi Capsule Expression*

Anti-Vi binding to *Salmonella* was measured as a proxy for Vi capsule expression. Log phase *Salmonella* were prepared as described in section 1.3.2. but *Salmonella* were grown to OD_{600} of 0.5. 1ml of log phase *Salmonella* was washed twice in LB broth (Sigma) by centrifuging at 3300 x g for 5 minutes and discarding the supernatant between washes. Washed *Salmonella* were resuspended in 1ml PBS. 5µl mouse anti-Vi antisera (NVGH) was added to 50µl washed *Salmonella* at a 1:800 final dilution. Samples were incubated on ice for 1 hour then washed twice with PBS, centrifuging at 3300 x g for 5 minutes. *Salmonella* were resuspended in 20µl APC-conjugated goat anti-mouse IgG (Sigma) at a 1:800 final dilution and incubated for 1 hour on ice. Samples were washed twice as before with PBS and fixed using 200µl 1% formaldehyde in PBS. Samples were analysed using a FACSCanto (BD) and FlowJo software (Treestar Inc.). The *Salmonella* population was identified according to forward and side scatter characteristics and the level of Vi expression determined by the GMFI in the FL-4 channel.

2.13. *Whole Blood Cytokine Stimulation*

Fresh whole peripheral blood was taken from consenting HIV-infected and uninfected adults into sodium heparin vacutainers (BD) containing 110 international units (IU) of heparin (11IU/ml blood) (BD). Log phase *Salmonella*, as prepared in section 1.3.2. was added to the blood to give a final concentration of between $1 \times 10^1 - 1 \times 10^6$ CFU/ml.

Inoculated blood was incubated at 37°C whilst shaking at 20 rpm. For pilot stimulations, plasma was prepared from stimulated blood at 1, 2, 4, 8, 16, 24, 32, 48, 72, 96, and 120 hours post-inoculation. In final experiments, blood was stimulated using a final concentration of *Salmonella* at 1 x 10^6 CFU/ml for 24 hours. As a negative control, blood was mock-stimulated with an equal volume of PBS as *Salmonella*. Plasma was prepared from stimulated blood by centrifugation at 2000 x g for 10 minutes. The plasma fraction of centrifuged blood was aliquoted into microfuge tubes and immediately stored at -80°C.

2.14. *Multiplex Immunoassay*

Levels of cytokines and chemokines in plasma from stimulated whole blood were quantified using multiplex immunoassay kits (eBioscience). Ag standards were reconstituted using 250µl of plasma specific standard buffer (eBioscience) before incubation on ice for 10 minutes. A 4-fold dilution series of the reconstituted standard was made by adding 50µl of standard to 150µl plasma specific standard buffer and repeating this process a total of 8 times. 50µl of antibody coated beads were added to each well of a 96 well filter plate (Nunc). Liquid was removed from the wells using a vacuum plate washer (Millipore). 150µl wash buffer (eBioscience) was added to each well and plates vacuum filtered after 30 seconds. 25µl plasma specific buffer was added to each well of the plate followed by 25ul standard to the standard wells and 25µl sample added to the sample wells. For blank wells 25µl plasma buffer was added.

Plates were sealed and incubated in the dark, at room temperature, shaking (Labsystems) at 700rpm for 2 hours. Plates were washed a total of 3 times as before. 25µl premixed detection antibody was added to each well and the plate was sealed as before and incubated for 30 minutes in the dark at room temperature, shaking at 700 rpm. Liquid was removed from the plate using the vacuum plate washer and plates washed three times again using 150µl wash buffer. 50µl Streptavidin-conjugated R-phycoerythrin (SAPE) solution was added to each well and plates incubated as before for 30 minutes at room temperature. The plate was washed three times again as before and 120µl reading buffer added to each well. Plates were read on a Luminex-200 machine (BioRad), using a sample size of 50µl, timeout of 45 seconds and bead limit of >50 beads per analyte. Results were analysed using Bio-plex manager software (Bio-Rad) (Figure 2.4). Unless stated otherwise, all reagents came from the eBioscience multiplex kit.

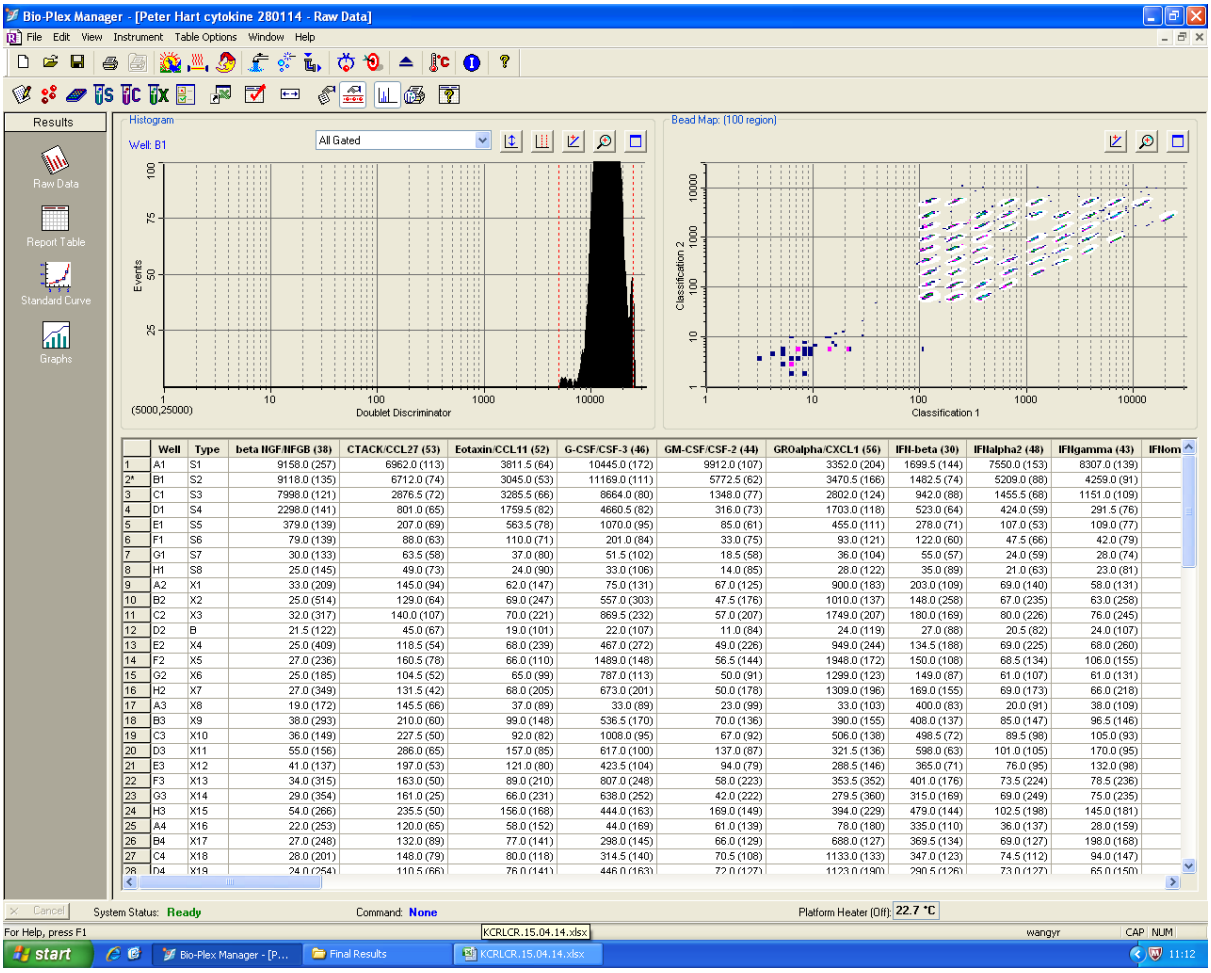


Figure 2.4. Gating schematic for multiplex immunoassay quantification of cytokine levels in *Salmonella* stimulated blood plasma

Levels of multiple cytokines in plasma from stimulated whole blood were quantified by multiplex immunoassay. Each cytokine is measured with a distinct antibody conjugated bead population with a unique bead number. Each bead population has its own gating characteristics and the fluorescence of each gate is measured to provide quantification of the levels of cytokine bound to a specific bead population. A reading limit of > 50 beads was used with a sample size of 50µl

2.15. *Transmission Electron Microscopy*

Log phase *Salmonella* were grown as in section 1.3.2. to give 1 x 10^9 CFU/ml. Washed *Salmonella* was added to either 100% human serum or PBS at a final concentration of 1 x 10^8 CFU/ml and incubated at 37°C for 10 minutes. After incubation, samples were washed three times with PBS then centrifuged at 3300 x g for 5 minutes.

2.15.1. Ultrastructure Imaging

The washed *Salmonella* pellet was fixed by resuspension for 20 minutes at room temperature in 750µl fixative containing one part 5% glutaraldehyde (GA, TAAB), 1.4% ruthenium hexamine trichloride (RHT, Sigma) in 0.1M Sodium cacodylate buffer (pH 7.42) and one part 100mM L-lysine monohydrochloride (Sigma) in PBS. Samples were rinsed once using cacodylate buffer and centrifuging at 3300 x g for 5 minutes. A second fix of 2.5% GA and 0.75% RHT was added for 40 minutes, again at room temperature. Samples were washed three times in cacodylate buffer as before and fixed further using 1% Osmium tetroxide (OsO_4, Sigma), 0.05M potassium ferrocyanide (Sigma) and 0.6% RHT in cacodylate buffer for 30 minutes at room temperature.

Fixed cells were resuspended in 2% GA and 4% paraformaldehyde (Sigma) in PBS for 1 hour at room temperature prior to centrifugation at 9,000 x g for 2 minutes. Cells were then post-fixed using 1% OsO_4 in cacodylate buffer for 1 hour at room temperature.

2.15.2. Ethanol Dehydration

Samples were dehydrated using an ethanol dehydration series. A sufficient amount of 20% ethanol in distilled water was added to submerge the pellet and this was incubated for 15 minutes at room temperature. The solution was then removed and followed by the addition of 30% ethanol, again for 15 minutes. The 30% ethanol was removed and replaced with a solution containing 1 part neat ethanol, one part distilled water and one part 3% uranyl acetate. This was incubated in the dark for 15 minutes before removal. Further sequential dehydration steps were conducted using 50, 75, 90 and 100% ethanol. Neat propylene oxide (TAAB) was added to the sample and incubated for 30 minutes.

2.15.3. Embedding and Imaging

Samples were embedded in 1ml neat resin made using an Agar 100 resin kit (Agar Scientific) and left to polymerise at 60°C for 48 hours. Cured, embedded samples were cut to 70 nM using a diamond knife on a Leica UltraCut UC6 ultramicrotome. Sections were examined using a FEI Spirit Biotwin 120 KV transmission electron microscope and images captured using a Tietz F415 CCD camera.

* Sections 1.15.2 and 1.15.3 were performed by Dr. David Goulding at the Wellcome Trust Sanger Institute, Cambridge, UK

2.16. *Enzyme Linked Immunosorbance Assay (ELISA)*

50µl of either 1mg/ml lipopolysaccharide (LPS) purified from *S*. Typhimurium or *S*. Enteritidis (Alexa), 1mg/ml purified outer membrane proteins (OMPs) from *S*. Typhimurium (produced in house) or 2.7µl of 3.7mg/ml Vi polysaccharide (ViPS)

(NVGH) were diluted in 10 ml carbonate coating buffer (0.015M Na_2CO_3, 0.035M $NaHCO_3$, pH9.6, to give a final concentration of 5µg/ml or 1µg/ml for LPS and OMPs or ViPS respectively. 100µl Ag containing coating solution was added to each well of a 96-well round bottom maxisorp plate (Nunc) and plates were incubated overnight at 4°C. After coating, plates were emptied and washed three times with wash buffer (PBS 0.05% Tween). Non-specific binding sites were blocked by adding 200µl block buffer (PBS, 0.1% BSA (Sigma)) and incubating at 37°C for 1 hour. Plates were washed three more times with wash buffer. 100µl of sample diluent (PBS, 1% BSA, 0.05% Tween) was added to each well. For the first well of a dilution series a further 50µl sample was added to give an initial 1:3 dilution of the sample. Samples were serially diluted using a 3-fold dilution series, taking 50µl of the first sample and transferring it to 100µl sample diluent and so on sequentially for a total of 8 wells. Plates were incubated at 37°C for 1 hour before washing three times with wash buffer. Alkaline-phosphatase (AP) conjugated, goat anti-human IgG, A or M (Southern Biotech) were diluted 1:2000 with sample diluent and 100µl added to each well before incubating at 37°C for 1 hour. Plates were washed three times before the addition of 100µl p-nitrophenyl phosphate substrate solution (Sigma). Plates were developed for 1 hour at 37°C and the OD at 405 nm was read using an ELISA plate reader (Emax) and Softmax pro software (Softmax).

2.17. *Cytokine ELISA*

Quantification of cytokine levels in plasma from stimulated human blood was done using Ready-Set-Go ELISA kits (eBioscience). 96-well, round bottom, maxisorp plates (Nunc) were coated with 100µl per well of diluted capture antibody in coating buffer (0.015M Na_2CO_3, 0.035M $NaHCO_3$, pH9.6) before sealing the plate and incubating overnight at

4°C. After coating plates were washed three times with wash buffer (PBS 0.05% Tween) to remove unbound antibody. Wells were blocked using 200µl 1 x assay diluent and incubated at room temperature for 1 hour before washing once with wash buffer. Standards were diluted with assay diluent in accordance with manufacturer's instructions and 100µl of the highest standard concentration was added to the first of eight wells. A two-fold dilution series of the standard was carried out by adding 50µl standard to 50µl assay diluent and repeating the process sequentially a total of eight times. This was done twice for each plate. 100µl neat sample was added to sample wells and 100µl assay diluent to blank wells.

Plates were sealed and incubated at room temperature for 2 hours before washing three times with wash buffer. 100µl detection antibody diluted in assay diluent was added to each well and the plates sealed and incubated at room temperature for 1 hour. Plates were washed 3 times as before and 100µl diluted avidin-horseradish peroxidase conjugate was added to each well prior to incubation for 30 minutes at room temperature.

Finally, plates were washed 5 times before the addition of 100µl substrate solution to each well. Plates were developed at room temperature for 15 minutes before 50µl stop solution was added to each well and the plates read on an ELISA plate reader (Emax) at 450 nm using Softmax Pro software (Softmax). Unless otherwise stated, all reagents were included in the eBioscience Ready-Set-Go ELISA kit.

2.18. *Purification of Human Anti-O:9 and Anti-Vi Antibody*

2.18.1. Affinity Chromatography Columns

Anti-Vi polysaccharide (ViPS) antibodies were extracted from human sera using two different affinity chromatography columns. The first Vi column was prepared using fragmented ViPS which had an average molecular weight of 40 – 50 KDa. ViPS fragments were randomly activated with adipic acid hydride (ADH) and linked to a n-hydroxysuccinide (NHS) column (Hitrap). This column contained 1ml of resin to which 2.35 mg of ViPS were attached. The second Vi column contained ViPS in its native confirmation (200 kDA) which was activated with NHS and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) using the carboxyl groups on the ViPS. For this column 1ml of resin was packed with 2.52 mg ViPS. Antibodies to the *Salmonella* O:9 O-chain antigen were extracted from human sera using an affinity chromatography column activated with O:9 derived from S. Enteritidis D24359. O:9 was activated with ADH at the terminal 3-Deoxy-D-manno-oct-2-ulosonic acid (KDO) of the core O-antigen oligosaccharide. In this instance 1mg of activated O:9 antigen was attached to 1ml of resin.

* Affinity purification columns described in sections 2.18 were prepared and calibrated by Dr. Francesca Micoli at Novartis Vaccines Institute for Global Health, Siena, Italy.

2.18.2. Affinity Purification of Human Antibody

All columns were first equilibrated by washing with 6ml PBS prior to use with any flow through being discarded. Human sera from 3 individuals known to contain both anti-ViPS and anti-O:9 antibody were pooled in a 1:1:1 ratio and this pool was diluted 1:2 with PBS before filtering with a 0.22 μm syringe driven filter (Millex). Pooled serum was passed through the column a total of 6 times and the flow through collected. Loaded columns were incubated overnight at 4°C before washing with 6ml PBS the following day to remove any unbound antibody. Wash fractions were collected for later testing by ELISA.

To establish which elution buffers afforded the optimal return on antibody yields. Initial antibody extractions used experimental sequential elution buffers. 6ml 0.1M glycine, 0.1M NaCl, pH 2.4 was first applied to the column before rinsing with 6ml Tris-buffered saline (TBS). 6ml 4M $MgCl_2$ in 10 mM Tris, pH 7 was added followed by a further rinse using 6ml TBS. 6 ml 20% ethanol in diH_2O was then added followed by a 6ml PBS rinse and a final elution using 6ml 8M urea, pH 7. All eluates were collected in 500ml aliquots to be tested for anti-*Salmonella* antibody levels by ELISA. For eluates using glycine buffer, 25μl 2 M Tris pH 9 was added to each collection tube prior to elution to ensure the immediate neutralization of the eluate. After initial optimisation, glycine was chosen as the optimal elution buffer for anti-O:9 antibody extraction and $MgCl_2$ for anti-ViPS antibody and only these buffers were used thereafter.

After extractions, columns were rinsed with 6ml PBS and stored in 2ml 50 mM Na2HPO4, 0.1% NaN3 pH 7.0 at 4°C until next use. For all columns, samples, buffers and

washes were applied at a flow rate of no greater than 300µl/ minute to ensure minimal disruption of column-bound antigen.

2.18.3. Dialysis of Eluates

Eluates containing anti-*Salmonella* antibody (as detected by ELISA) were pooled and added to pre-soaked (> 1 hour diH_2O) dialysis membrane (Sigma) with a molecular weight cut off of 6 – 8 kDa. Tubing was filled with 4ml of pooled eluate before sealing and dialyzing against PBS for 24 hours at 4°C. PBS was discarded and replaced with fresh PBS after 30 minutes and then a further 3 times over the 24 hour period. After 24 hours, tubing was emptied and the antibody preparation stored at 4°C until use.

2.19. *Statistical Analysis*

For all parametric data a two tailed, unpaired t-test was used to identify statistical differences between groups of data. For non-parametric data a Mann-Whitney U test was performed. All tests were two-tailed unless otherwise stated.

For large datasets of multiplex assay results such as in the HIV cytokine study data, a combination of principal component analysis (PCA), linear discriminant analysis (LDA) and Wilcoxon-Rank t-test were used. Statistical analysis was performed with STATA v11.0 (StataCorp 2009). Where multiple comparisons of variables were conducted, P-values were adjusted using the Holm's correction.

Linear regression models of normalised cytokine levels were fitted with variables representing HIV status/control and *Salmonella* serovar, with and without a *Salmonella* serovar: HIV status/control interaction term. The goodness of fit for each paired model, for each cytokine, was compared using a likelihood ratio test, with $P < 0.05$ considered significant. Statistical analysis was performed in R (http://www.r-project.org/).

* PCA, LDA, and linear regression modelling were done by Dr James Gilchrist of the Wellcome Trust Centre for Human Genomics, Oxford, UK.

3. Role of the *Salmonella* Vi polysaccharide capsule in *S.* Typhi and *S.* Typhimurium

3.1. *Introduction*

Expression of the Vi capsule by *S*. Typhi was first observed in the 1930s and the capsule thus named due to the increased virulence, in mice, of Vi-expressing *S.* Typhi (Vi^+ *S*. Typhi) isolates compared to non-Vi-expressing strains (Felix and Pitt, 1934). The majority of clinical *S.* Typhi isolates express a Vi polysaccharide capsule and expression is associated with increased virulence in humans (Wain et al., 2005, Robbins and Robbins, 1984, Hornick et al., 1970a). However, virulent, non-Vi-expressing (Vi^- *S.* Typhi) isolates have been reported (Baker et al., 2005) .

Vi capsule expression by *S.* Typhi has a number of effects on the immune response to typhoid infection. Vi reduces the inflammatory response in intestinal epithelial cells (Sharma and Qadri, 2004) and its expression by *S.* Typhi and *S*. Typhimurium, genetically-engineered to express the Vi capsule, is associated with reduced inflammatory markers. These include growth related oncogene α (GROα) and IL-17. Fluid influx and neutrophil recruitment in the bovine ileal loop model of *Salmonella* infection are also reduced compared with an isogenic *viaB*$^-$ strain lacking Vi (Raffatellu et al., 2007). Vi expression has therefore been proposed to promote a stealth mode of pathogenesis in which evasion of detection by the host innate immune system promotes systemic spread and limits gastroenteritis (Wangdi et al., 2012)

There is uncertainty regarding the role of Vi capsule in the resistance of *S.* Typhi to the innate immune response. Using non-isogenic strains, Vi^+ *S*. Typhi has been shown to be less susceptible than Vi^- *S*. Typhi to serum bactericidal activity, phagocytosis, killing by hydrogen peroxide (oxidative killing) and non-oxidative killing (Looney and Steigbigel, 1986). Reduced serum killing and phagocytosis is associated with reduced C3 deposition on Vi^+ *S*. Typhi, while Vi^- *S*. Typhi can directly activate the alternative pathway of complement (Looney and Steigbigel, 1986). More recently, Vi-expression by *S*. Typhi has been shown to significantly reduce C3 deposition compared with isogenic Vi^- strains, suggesting that a reduction in complement deposition may be a mechanism by which capsule expression increases resistance to antibody-mediated killing (Wilson et al., 2011). However, other studies using isogenic $Vi^{+/-}$ *S.* Typhi have not documented a protective effect of Vi capsule expression against antibody-mediated killing (Bravo et al., 2008), thus leading to disagreement concerning the role of Vi in the survival of *S.* Typhi during infection.

3.2. *Aims*

To clarify the role of Vi-expression in resistance to serum and blood cell killing we used isogenic $Vi^{+/-}$ *Salmonella* to investigate the effect of capsule expression on antibody-mediated killing. We were particularly interested in the effects of Vi-expression in the non-conventional background of *S.* Typhimurium, and how Vi capsule affects antibody binding and complement deposition. Using electron microscopy, we wanted to visualise how the presence of Vi capsule affects cellular damage following serum exposure.

3.3. *Results*

3.3.1. *S.* Typhi BRD948 and *S.* Typhimurium C5.507 both express the Vi capsule while their isogenic counterparts, *S.* Typhi BRD948 *tviB*- and *S.* Typhimurium SGB1, do not.

To examine the effects of Vi-expression on resistance to antibody-mediated killing, we used isogenic $Vi^{+/-}$ *S.* Typhi and *S.* Typhimurium. This ensures any differences observed between strains reflect the presence of Vi-capsule and also allows investigation of whether the effects of Vi-expression are serovar specific. To confirm Vi-expressing *Salmonella* strains were expressing the capsule under experimental conditions we characterised the levels of Vi-expression on *S.* Typhi and *S.* Typhimurium by flow cytometry. Both Vi^{+} *S.* Typhi (BRD948) (dashed line) and Vi^{+} *S.* Typhimurium (C5.507) (dotted line) expressed Vi capsule but expression was approximately 10 fold lower in *S.* Typhimurium than *S.* Typhi. This is consistent with previously reported differential Vi expression levels for these strains (Jansen et al., 2011) Neither the Vi^{-} *S.* Typhi (BRD948 *tviB*-) or *S.* Typhimurium (SGB1) expressed the capsule (solid lines) (Figure 3.1).

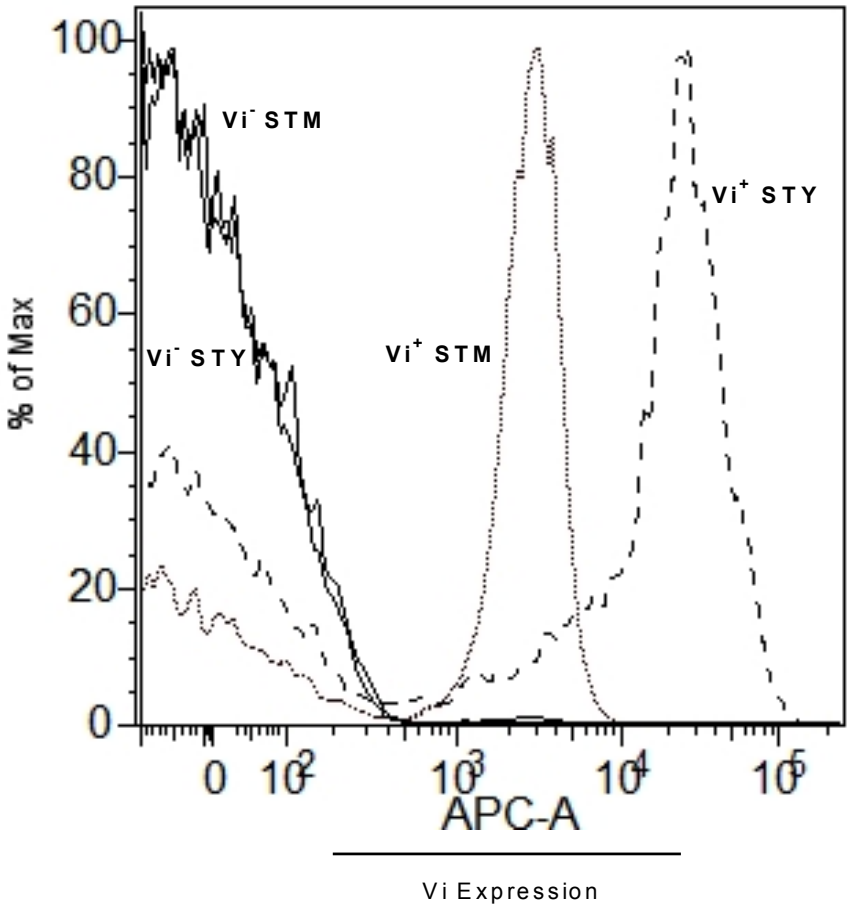


Figure 3.1. Vi expression levels on isogenic Vi$^{+/-}$ *S.* Typhi and *S.* Typhimurium.

Vi capsule expression by Vi^{+} *S.* Typhi (dashed line) and Vi^{+} *S.* Typhimurium (dotted line) and their isogenic partner strains (solid lines) were assessed by flow cytometry using mouse anti-Vi antisera to label the Vi capsule. Data are representative of one experiment.

3.3.2. Levels of anti-*Salmonella* antibody in ten adult sera used in bactericidal and opsonic assays.

To examine the effect of Vi-expression on antibody-mediated bactericidal and opsonic activity of sera, we venesected 10 healthy adults and characterised their anti-*Salmonella* antibod*y* levels. Levels of IgG, IgA and IgM against Vi polysaccharide and the O:4,5 and O:9 O-chain antigens of *Salmonella* LPS were measured by ELISA. Three donors reported having been vaccinated with Vi polysaccharide (ViPS) at least 3 years prior to serum collection, but could not recollect the number of doses received. Two of these had the highest levels of anti-Vi IgG. Anti-O:4,5 and O:9 IgG were detectable in all sera and five out of the ten donors had detectable anti-Vi IgG in their sera (Figure 3.2 A). No IgA against the three Ag was detected except for one serum which had IgA against both O:4,5 and O:9 (Figure 3.2 B). IgM against Vi and O:9 was detected in all the sera whereas anti-O:4,5 IgM was only detectable in eight of the sera (Figure 3.2 C). No donors reported having suspected or confirmed *Salmonella* infection prior to venesection. Donor information is listed in table 3.1.

Table 3.1. Demographic data and *Salmonella* vaccine history for serum donors in this study.

Ten healthy adults were venesected for serum collection. Donors were of mixed age (23 – 46 years), sex (5 male, 5 female) and race (Caucasian and Asian). No donors reported suspected or confirmed prior infection with *Salmonella.* Three donors had received ViPS vaccine against *S.* Typhi more than 3 years prior to serum collection.

Donor	Age	Sex	Race	Vaccination History	Vaccine type
1	43	M	Caucasian	Yes, >3 years	ViPS
2	24	F	Caucasian	No	-
3	23	M	Caucasian	No	-
4	42	F	Asian	Yes, >3 years	ViPS
5	33	M	Caucasian	No	-
6	25	F	Caucasian	No	-
7	46	F	Caucasian	Yes, >3 years	ViPS
8	32	M	Asian	No	-
9	25	M	Caucasian	No	-
10	28	F	Asian	No	-

ViPS, Vi polysaccharide

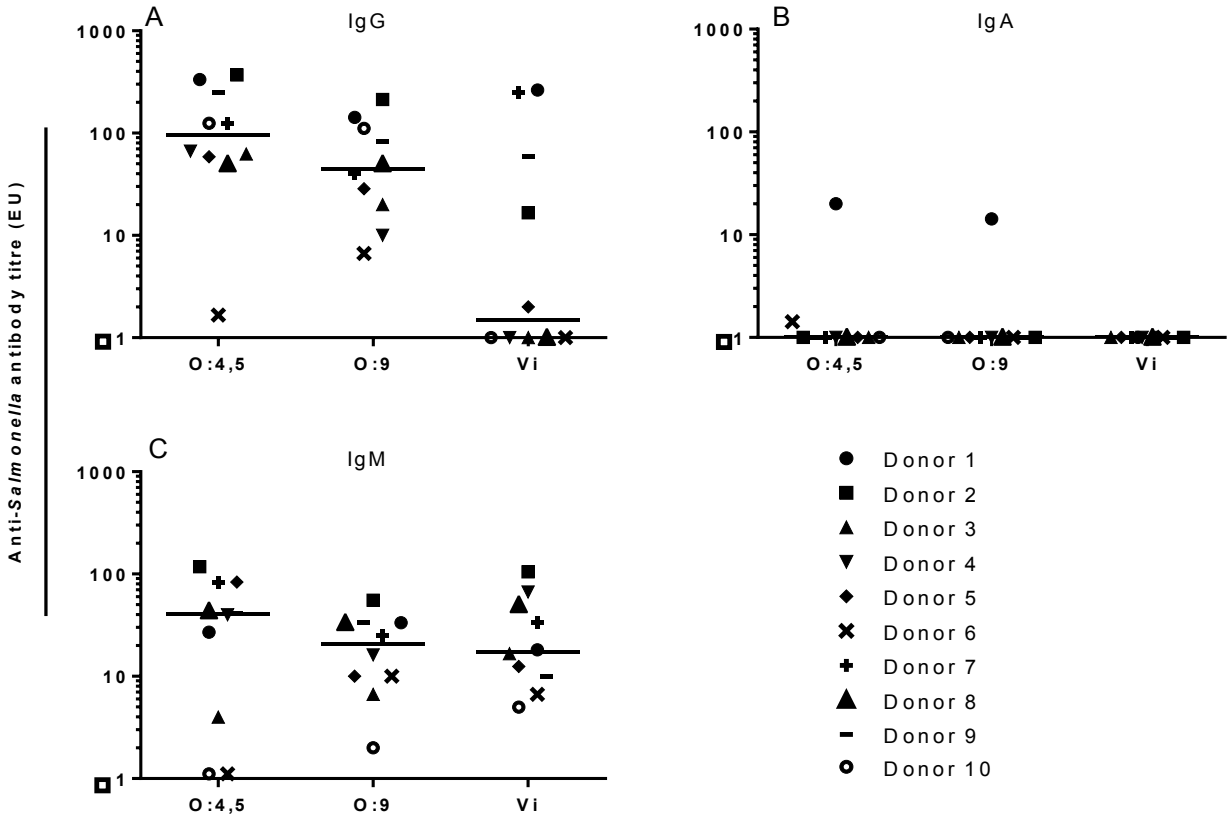


Figure 3.2. Anti-*Salmonella* antibody levels in sera from ten healthy adults

Levels of IgG (A), IgA (B) and IgM (C) against *Salmonella* O:4,5 and O:9 O-chain antigens and Vi polysaccharide in ten human sera from healthy adults were measured by ELISA. ELISA plates were coated with *S.* Typhimurium LPS, *S.* Enteritidis LPS or purified Vi polysaccharide to detect anti-O:4,5, O:9 and Vi antibody respectively. Each symbol represents one donor. Data are representative of one experiment and lines represent the median antibody titre.

3.3.3. Expression of the Vi-capsule by both *S.* Typhimurium and *S.* Typhi is associated with differences in Antibody binding and complement deposition which are dependent on the presence of specific antibody

Many bacterial capsules, such as those of *B. pseudomallei* and *S. aureus* increase bacterial survival by reducing C3 and MAC deposition onto the cell surface, thereby reducing cell lysis (Reckseidler-Zenteno et al., 2005, Cunnion et al., 2001). However, not all capsules protect in this way, and some, such as the K5 and K15 capsules of *E. coli*, have no known role in serum resistance (Burns and Hull, 1998, Schneider et al., 2004).

To examine the effect of Vi-expression on both antibody binding and complement deposition, we incubated the isogenic $Vi^{+/-}$ *Salmonella* strains with human sera from the ten donors and then measured IgG and IgM binding and C3 and MAC deposition onto the surface of *Salmonella* using flow cytometry.

As demonstrated in section 3.3.2, four of the ten human sera had elevated levels of anti-Vi IgG (Figure 3.3 A, red circle) while the remainder had minimal or undetectable Vi-specific IgG (Figure 3.3, blue circle). This division, in terms of the antibody content of the sera, enabled us to examine the effect of Vi capsule expression on antibody binding and complement deposition both in the presence (Figure 3.3, B, D and F, red box), and absence (Figure 3.3, C, E and G, blue box), of Vi-specific IgG.

For sera containing anti-Vi IgG, no differences in total IgG binding to *Salmonella* were detected between the isogenic Vi-expressing and non-expressing strains (Figure 3.3, B). However, when sera lacked Vi-specific IgG, expression of Vi capsule by *S.* Typhi, but not

S. Typhimurium was associated with a significant reduction in total IgG binding to the cell (MW Test, p=0.026) (Figure 3.3, C).

Despite the presence of Vi-specific antibody, expression of Vi capsule by *S.* Typhi, but not *S.* Typhimurium was associated with a significant reduction in C3 deposition after incubation with sera containing anti-Vi IgG (MW Test, p=0.029) (Figure 3.3, D). When sera lacked anti-Vi IgG (Figure 3.3, E), expression of Vi capsule by either *S.* Typhimurium (MW Test, p=0.002) or *S.* Typhi (MW Test, p=0.004) was associated with significant reductions in C3 deposition onto *Salmonella*.

Similar to our observations of IgG binding, Vi expression by either *Salmonella* serovar was not associated with differences in MAC deposition when sera contained Vi-specific IgG (Figure 3.3, F). However, in the absence of anti-Vi IgG, expression of Vi capsule by *S.* Typhimurium (MW Test, p=0.029) or *S.* Typhi (MW Test, p=0.029) was associated with significant reductions in MAC deposition compared to levels on their Vi-negative counterparts (Figure 3.3, G).

All sera contained detectable anti-Vi IgM, so the effect of Vi expression on total IgM binding was characterised for all ten sera against the four strains. Total IgM binding was lower for both Vi^+ *Salmonella* strains compared to their Vi^- partner strains, but this difference was only significant for Vi^+ *S.* Typhi compared to Vi^- *S.* Typhi (MW Test, p=0.015) (Figure 3.3 B).

These data suggest that when a donor lacks Vi-specific IgG, such as in a naïve host, expression of Vi capsule is associated with reductions in total IgG binding, and C3 and MAC deposition. Capsule associated reductions in antibody binding and complement

deposition may represent a virulence factor employed by Vi expressing *Salmonella* to prolong bacterial survival within a host. Interestingly, Vi-associated differences in total IgG binding and MAC deposition were absent, or reduced in the case of C3 deposition when sera contained anti-Vi IgG. This may reflect a mechanism by which anti-Vi IgG protects against infection, by abrogating the effects of Vi expression on antibody binding and/ or complement deposition.

Differences in IgM binding and C3 deposition associated with Vi expression were observed despite the presence of Vi-specific antibody. This suggests that the Vi capsule possesses intrinsic properties which may inhibit the activation of the alternative complement pathway and may reduce IgM binding. IgM may be more susceptible than IgG to the steric effects of Vi capsule expression due to its pentameric structure and larger nature.

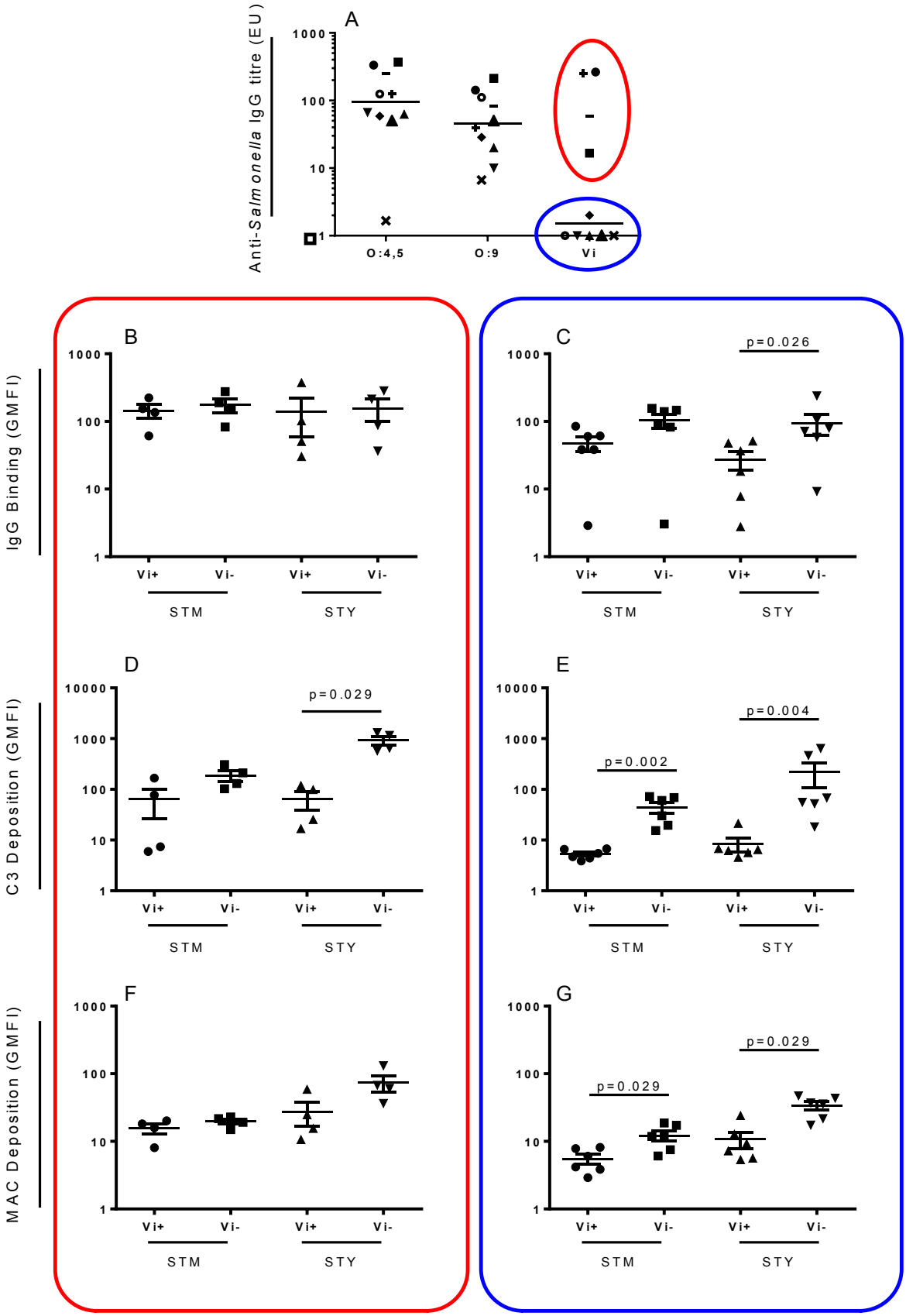

A
Anti-Salmonella IgG titre (EU)
1000
100
10
1
O:4,5
O:9
Vi
IgG Binding (GMFI)
C3 Deposition (GMFI)
MAC Deposition (GMFI)
B
C
D
E
F
G
Vi+
Vi-
STM
STY
p=0.026
p=0.029
p=0.002
p=0.004

Figure 3.3. Vi capsule reduces antibody binding and complement deposition, but the presence of Vi-specific IgG mitigates its effect.

The effect of Vi expression on antibody binding to, and complement deposition, on *Salmonella* was measured by flow cytometry. (A) IgG and (B) IgM binding to, and (C) C3 and (D) C5b-9 MAC deposition on isogenic Vi^+ and Vi^- *S*. Typhimurium (STM) and *S*. Typhi (STY) when exposed to the ten adult sera from table 3.1. IgG binding when exposed to the five sera not containing anti-Vi IgG is also shown (E). Data are the mean values of three separate experiments. Bars represent the median and interquartile range. Statistical significance was calculated using a two-tailed Mann-Whitney U test.

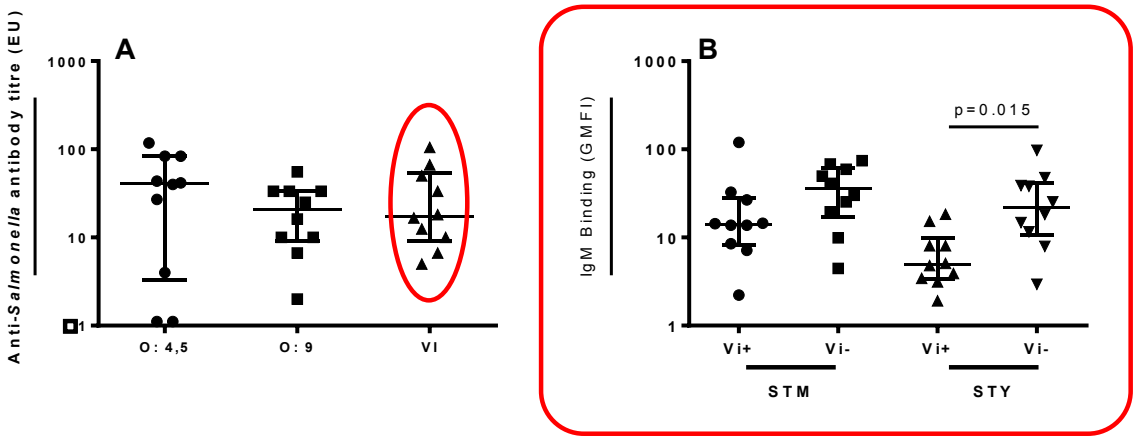


Figure 3.4 Vi capsule reduces total IgM binding, despite the presence of Vi-specific IgM

The effect of Vi expression on IgM binding to *Salmonella* was measured by flow cytometry. (A, red circle) All ten adult sera had detectable anti-Vi IgM as detected by ELISA. (B) IgM binding to isogenic Vi^+ and Vi^- *S.* Typhimurium (STM) and *S.* Typhi (STY) when exposed to the ten adult sera from table 3.1. Data are the mean values of three separate experiments. Bars represent median and interquartile range. Statistical significance was calculated using a two-tailed Mann-Whitney U test.

3.3.4. The Vi capsule increases the resistance of *S.* Typhimurium and *S.* Typhi to antibody-dependent complement-mediated killing

Having documented a reduction in antibody binding and complement deposition onto *Salmonella* in the presence of Vi capsule, we wanted to assess how this affected resistance to antibody-mediated, complement-dependent killing of *Salmonella.*

We incubated *Salmonella* with fresh, undiluted, human serum from each of the ten donors in table 3.1 for 180 minutes at 37°C. We measured numbers of viable *Salmonella* at 45, 90 and 180 minutes and compared these to the initial starting concentration of *Salmonella.*

Expression of Vi capsule by both *S.* Typhimurium (Figure 3.4 A and C) and *S.* Typhi (Figure 3.4 B and D) was associated with enhanced resistance to serum killing and bacterial survival indicating that the protective effect of Vi against C'-mediated killing is not restricted to serovar Typhi. For all ten sera, Vi^+ *S*. Typhi and Typhimurium were killed to a lesser extent than their isogenic Vi^- counterparts, despite differences in amounts of antibody to Vi and O-Ag among the different sera. Vi^+ *S.* Typhimurium was significantly more resistant to serum killing compared to its isogenic Vi^- strain at all time points (unpaired t-test $p<0.0001$) (Figure 3.4 C). Vi^+ *S.* Typhimurium was killed by approximately 1 $\log_{10}$ on average at 180 minutes by human serum whereas Vi^- *S.* Typhimurium were killed by >2.5 $\log_{10}$ at the same time point (Figure 3.4 C). Similarly Vi^+ *S.* Typhi was significantly more resistant to serum killing at 45 and 90 minutes compared to its Vi^- counterpart (unpaired t-test $p<0.001$, both time points). No viable Vi^- *S.* Typhi was detected at 90 minutes (Figure 3.4 D). All *S.* Typhi, irrespective of Vi-expression were killed at 180 minutes and were inherently more sensitive to C'-

mediated killing than their *S*. Typhimurium counterparts. Heat-inactivation of sera rendered it unable to kill *Salmonella.*

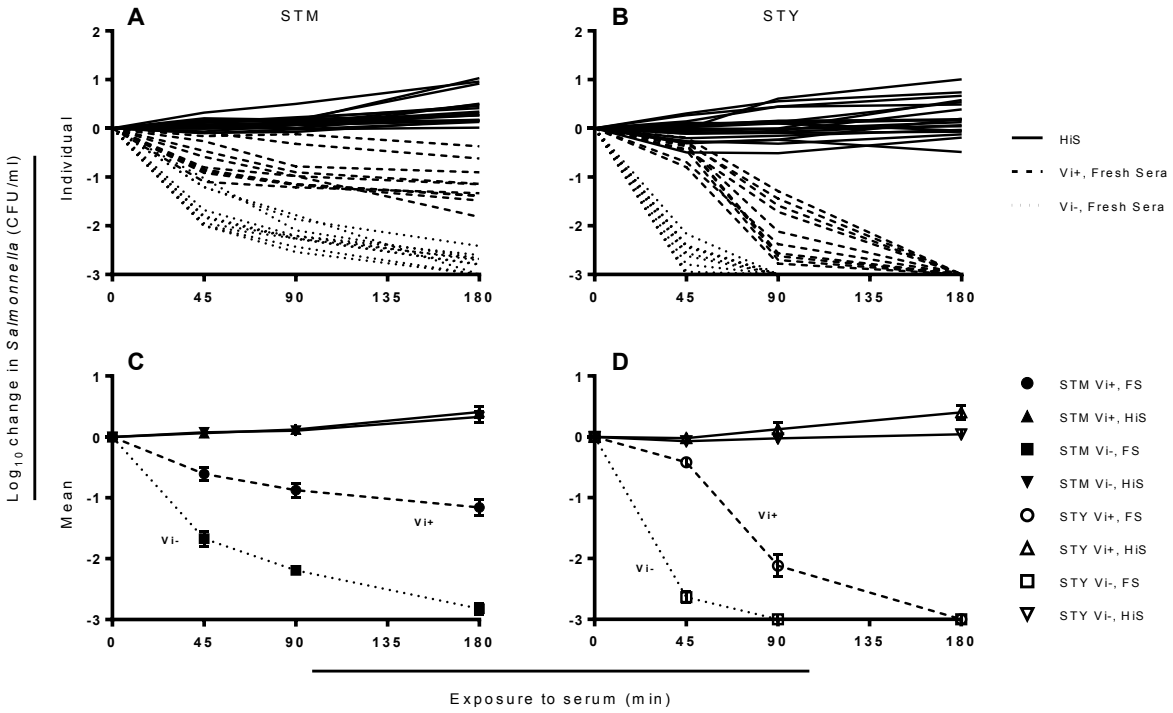


Figure 3.5. Expression of Vi by *Salmonella* is associated with increased resistance to serum killing

Killing of isogenic *S*. Typhimurium (A and C) and *S*. Typhi (B and D) strains either expressing (dashed lines) or not expressing (dotted lines) Vi capsule exposed to the ten adult sera (table 3.1) characterized for anti-*Salmonella* antibody content, in figure 3.2, at 45, 90 and 180 minutes (FS). A and B represent data for each of the individual ten sera, while C and D represent mean values. For all ten sera, Vi-expressing *Salmonella* were significantly more resistant to serum killing than their isogenic Vi^- counterparts (Student's t-test $p<0.0001$, all time points excluding 180 minutes for *S.* Typhi) Solid lines are Vi^+ and Vi^- bacteria incubated with heat-inactivated sera to ensure bacteria were viable (HiS). Data are mean of three separate experiments. Error bars represent SEM.

3.3.5. Vi-expression by *Salmonella* is associated with a reduction in cellular damage following serum exposure

We utilized TEM to visualise the extent of damage to $Vi^{+/-}$ *Salmonella* after exposure to human serum containing both anti-Vi and anti-O:9 antibody for 10 minutes (Donor 1, Table 3.1). We used serum known to contain anti-*Salmonella* antibody to ensure some cellular damage occurred and would be visible by TEM. Incubation with PBS had no effect on cellular integrity and no signs of damage to *Salmonella* were visible (Figure 3.5 A – D).

Visible damage to Vi^+ *S.* Typhimurium following serum exposure was minimal, with the majority of cells remaining intact. A distinct aggregate, which was not visible after PBS exposure, was clearly visible on the surface of Vi^+ *S.* Typhimurium after serum exposure (Figure 3.5 E). In contrast, Vi^- *S.* Typhimurium exhibited clear signs of damage, including enlargement of the periplasmic space and cytoplasmic contraction (Figure 3.5 F). After quantification, a significantly greater percentage of Vi^- *S.* Typhimurium cells showed signs of damage (38%) compared to Vi^+ *S.* Typhimurium (13%) (unpaired t-test $p=0.008$) (Figure 3.5 I).

Vi^+ *S.* Typhi (Figure 3.5 G) showed similar signs and levels of damage to Vi^- *S.* Typhimurium but the percentage of damaged cells (24%) was significantly lower than Vi^- *S.* Typhi (unpaired t-test $p=0.004$, Figure 3.5 H) which displayed signs of extensive damage and where 64% of cells were damaged on average (Figure 3.5 I).

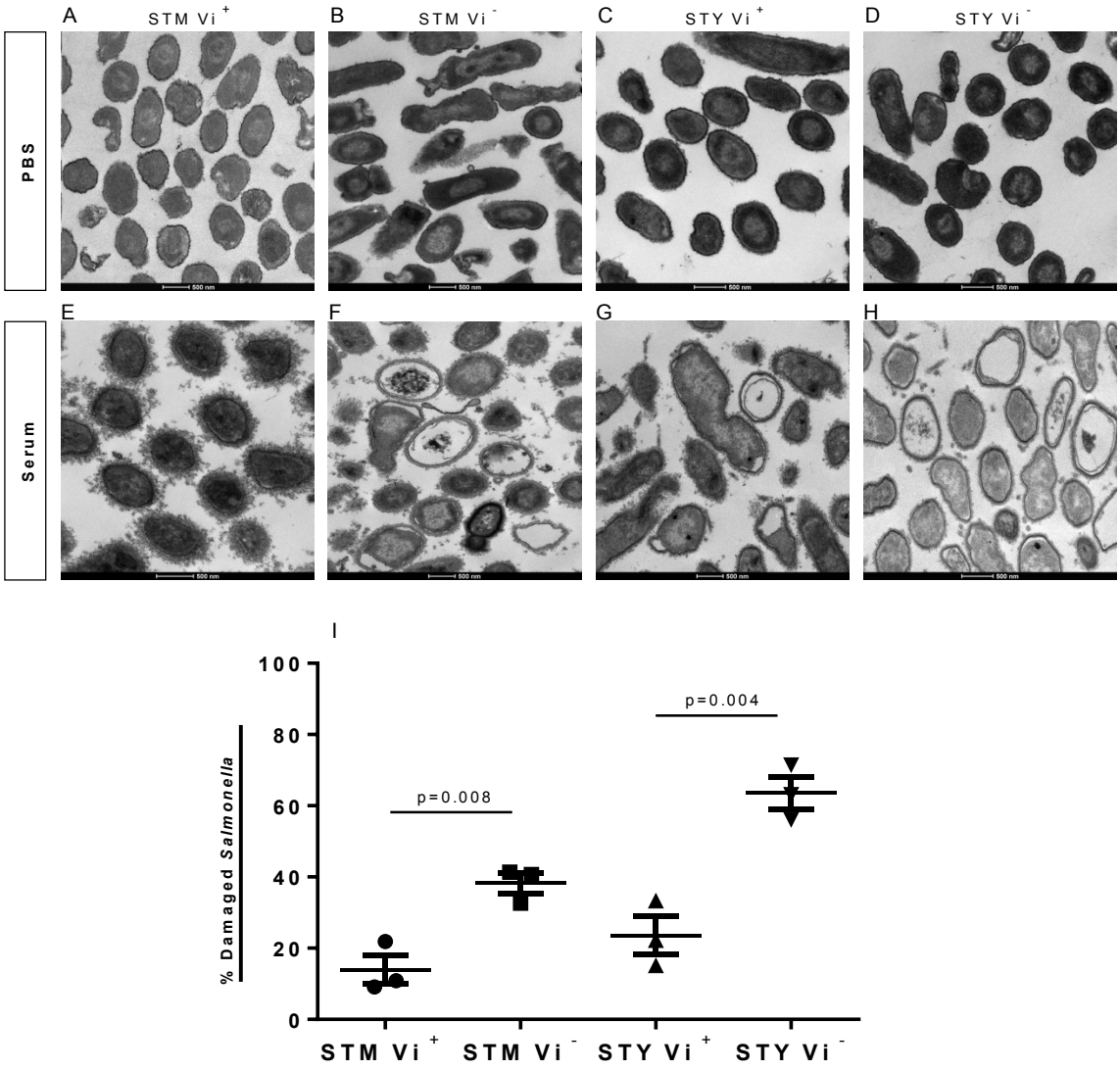


Figure 3.6. Expression of the Vi capsule is associated with reduced *Salmonella* cell damage after exposure to serum

The effects of exposure to PBS or serum on Vi$^{+/-}$ *Salmonella* were visualised by electron microscopy. Exposure to PBS had no effect on the cellular integrity of any *Salmonella* strain (A-D). Vi^{+} *S.* Typhimurium showed minimal damage following exposure to human serum for 10 minutes (E). Both Vi^{-} *S.* Typhimurium (F) and Vi^{+} *S.* Typhi (G) showed moderate levels of damage including periplasmic voiding and cytoplasmic condensation. Vi^{-} *S.* Typhi showed extensive damage with the majority of cells displaying indicators of damage (H). Representative images are shown. Statistical significance was done using a Student's t-test. Scoring was performed on multiple images (n=3) and lines represent mean +/- SEM.

3.3.6. Vi expression is associated with reduced clearance of Salmonella by washed peripheral leukocytes

To examine the effect of Vi expression on the opsonic killing of *Salmonella*, we opsonised $Vi^{+/-}$ *Salmonella* with 50% heat-inactivated sera from each donor as a source of antibody (Table 3.1) and 50% C6-deficient serum. C6-deficient serum was used to enable effective opsonisation, by providing C3b, whilst avoiding MAC deposition and serum bactericidal activity against Vi^{-} *S.* Typhi during the opsonisation period. Following opsonisation, *Salmonella* were incubated with washed peripheral blood. Killing by peripheral blood cells was measured by quantifying the number of viable *Salmonella* at each time point compared to the initial concentration of *Salmonella.*

Whilst less obvious than in the SBA, expression of Vi by *S.* Typhimurium was associated with a small, but significant, increase in resistance to blood cell killing compared to Vi^{-} *S.* Typhimurium at 90 and 180 minutes (unpaired t-test p=0.042 and 0.047 respectively) but not at 45 minutes (Figure 3.6 A and C). In contrast, Vi-expression by *S.* Typhi was associated with a marked increase in resistance to phagocyte killing at 45 and 90 minutes compared to Vi^{-} *S.* Typhi (unpaired t-test $p<0.01$) , but both strains underwent a 3 $\log_{10}$ kill at 180 minutes (Figure 3.6 B and D). These results parallel those of the SBAs and once again *S.* Typhi was inherently more susceptible to killing than *S.* Typhimurium.

Salmonella opsonised with serum then incubated in RPMI, or opsonised with PBS and incubated with blood, did not undergo killing. This confirms that killing observed in the assay is phagocyte-mediated and not the result of residual serum bactericidal activity from opsonisation. The lack of killing of *Salmonella* by blood cells after PBS opsonisation

demonstrates that washing the blood is effective in removing endogenous antibody and complement, both of which are required for phagocytosis to occur.

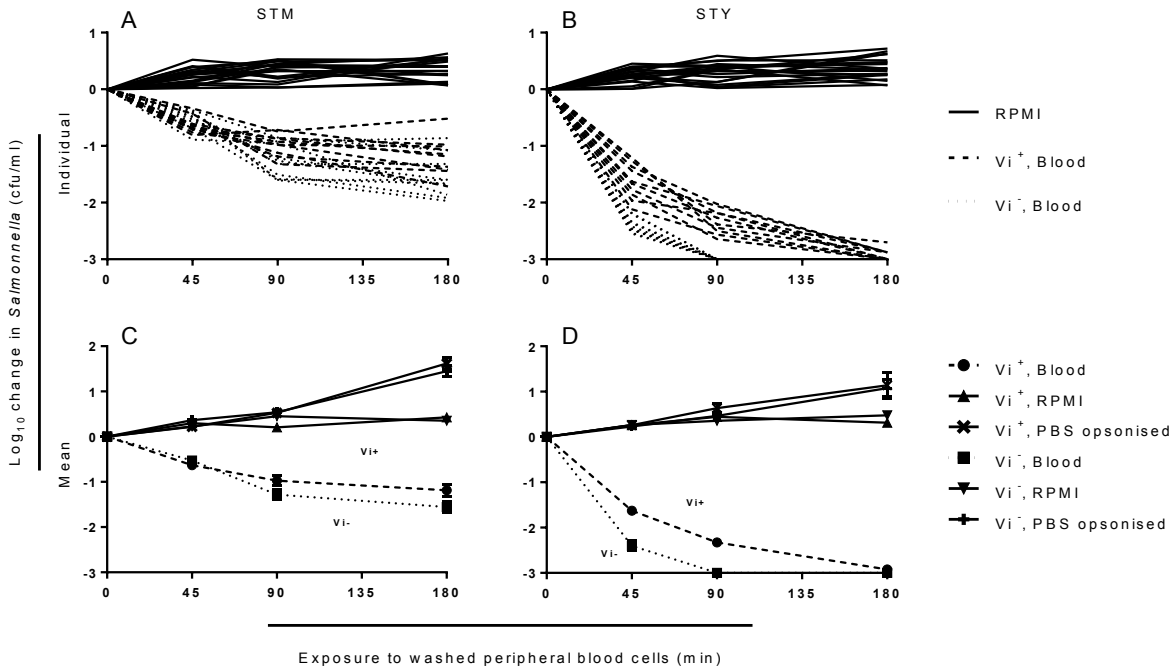


Figure 3.7. Expression of Vi capsule reduces killing of *Salmonella* by peripheral blood phagocytes.

Killing of Vi-expressing (dashed lines) and non-expressing (dotted lines) *Salmonella* by peripheral blood cells. *Salmonella* were opsonised with 50% antibody from ten adult sera and 50% C6-deficient serum then added to washed, peripheral blood cells. *Salmonella* opsonised with sera and added to RPMI, or opsonised with PBS and added to washed blood cells was not killed (solid lines). Data in A and B represent the mean results for each of the ten sera from three separate experiments. Statistical significance was calculated using a Student's t-test. Lines in C and D represent mean values of individual data +/- SEM.

3.3.7. Vi expression by *Salmonella* has no effect on neutrophil or monocyte phagocytosis measured by flow cytometry

To investigate why Vi expression was associated with a reduction in blood cell killing of *Salmonella,* we measured the phagocytosis of FITC-labelled *Salmonella* by peripheral blood monocytes and neutrophils. FITC-labelled *Salmonella* were opsonised with the ten donor sera from table 3.1. Once opsonised, *Salmonella* were incubated with washed peripheral blood for 20 minutes at 37°C after which samples were analysed by flow cytometry.

3.3.7.1 FITC labelled *Salmonella* display similar levels of fluorescence intensity in the FL-1 channel

To ensure any differences observed in the experimental results were not a result of differential levels of FITC-labelling of the *Salmonella,* particularly between encapsulated and acapsulated strains, FITC-labelled *Salmonella* were analysed by flow cytometry for their fluorescence in the FL-1 channel. Vi^+ and Vi^- *S.* Typhimurium displayed similar levels of fluorescence in the FL-1 channel after FITC-labelling (Figure 3.7, orange and green lines respectively), as did Vi^+ and Vi^- *S.* Typhi (red and blue lines respectively). Numbers were too low for statistical analysis, but this observation suggests that FITC-labelling is unaffected by capsular expression on these strains of *Salmonella.* The geometric mean fluorescence intensity was different between the two serovars of *Salmonella.* However, because we are comparing isogenic strains of the same serovar and not between different serovars, this observation should not affect conclusions drawn from the results of experiment utilising these FITC-labelled *Salmonella*.

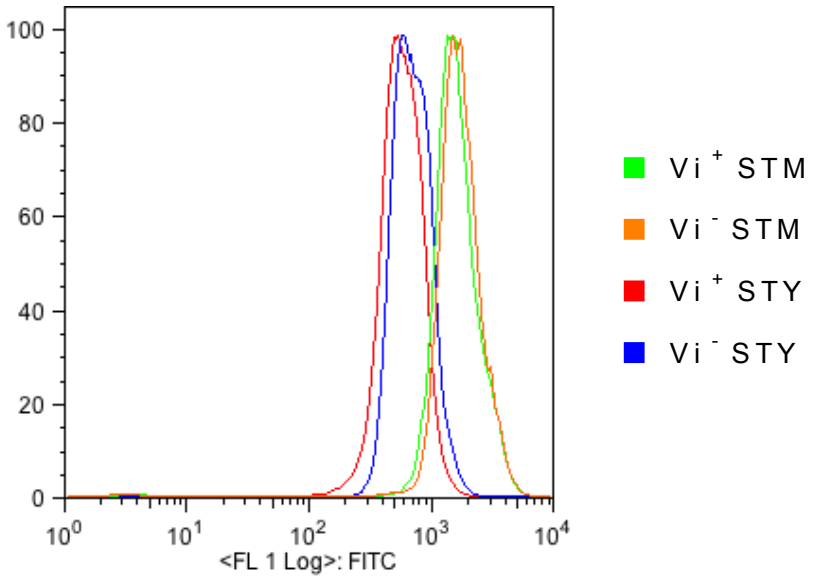


Figure 3.8. Levels of FITC-labelling were similar for *Salmonella* strains but not between serovars

Levels of FITC-fluorescence on FITC-labelled Vi^+ *S.* Typhimurium (green line), Vi^- *S.* Typhimurium (orange line), Vi^+ *S.* Typhi (red line) and Vi^- *S.* Typhi (blue line) were measured by flow cytometry. Each line represents one *Salmonella* strain. Data are representative of one experiment.

3.3.7.2 Vi-expression has no effect on phagocytosis of *Salmonella* by neutrophils or monocytes at 20 minutes

Using opsonised, FITC-labelled *Salmonella,* we measured both the level of intracellular fluorescence as an indicator of internalised bacteria, and the percentage of cells which were FITC+ as an indicator of the percentage of the parent population which were performing phagocytosis. Trypan blue was used to quench extracellular fluorescence, ensuring positive FITC signal detected in the assay was from internalised *Salmonella.*

We did not observe any significant differences between levels of internalised Vi^+ *S.* Typhimurium or Vi^+ *S.* Typhi and their isogenic Vi^- partner strains in either monocytes or neutrophils when comparing the results from *Salmonella* opsonised with the ten adult sera in table 3.1 as a whole (Figure 3.8 A and B). Similarly Vi-expression by either *S.* Typhimurium or *S.* Typhi was not associated with a significant overall change in the percentage of phagocytosing monocytes or neutrophils (Figure 3.8 C and D). These data contrast with published work which has demonstrated a Vi-associated reduction in phagocytosis in non-isogenic *Salmonella* (Looney and Steigbigel, 1986). They also contrast with our observations of *Salmonella* killing in the blood cell killing assay where, albeit a small effect for *S.* Typhimurium, Vi-expression is significantly associated with protection against phagocyte killing.

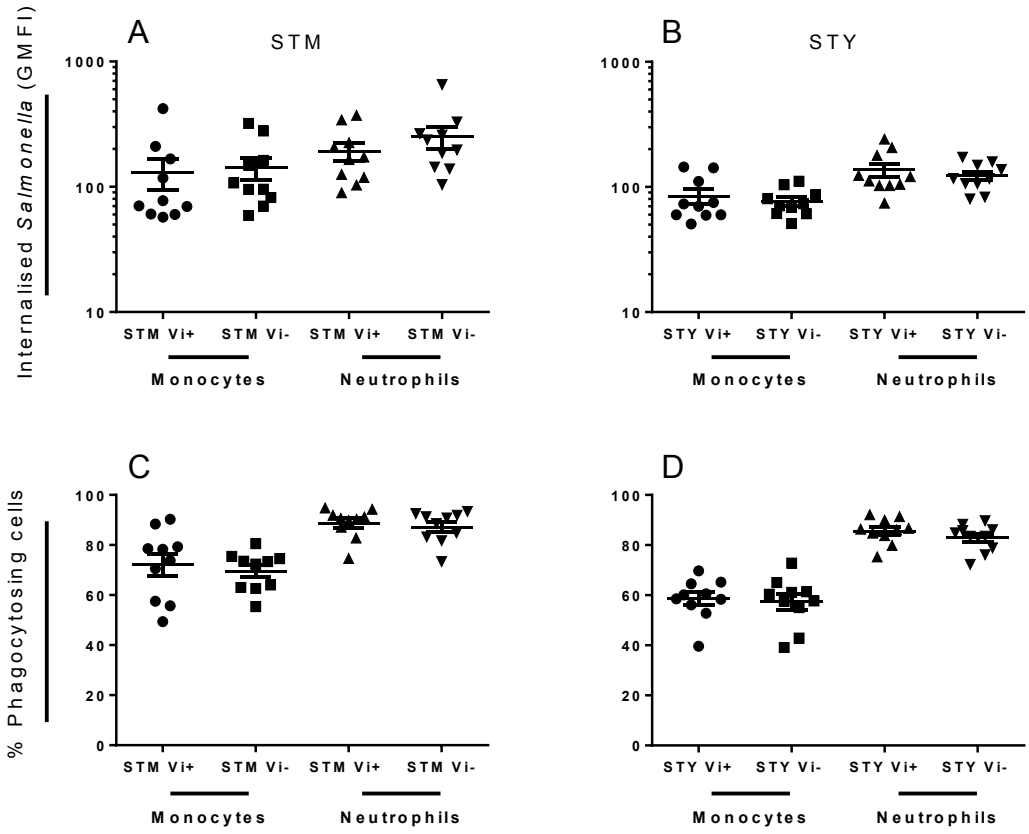


Figure 3.9. Vi-expression is not associated with changes in phagocytosis of *Salmonella* by neutrophils or monocytes after 20 minutes.

Levels of internalised FITC-labelled *Salmonella* and the percentage of monocytes and neutrophils phagocytosing *Salmonella* were assessed by flow cytometry. The geometric mean fluorescence intensity (GMFI) of monocytes and neutrophils phagocytosing $Vi^{+/-}$ *S.* Typhimurium (A) and $Vi^{+/-}$ *S.* Typhi (B) was quantified. The percentage of phagocytosing monocytes and neutrophils after exposure to opsonised $Vi^{+/-}$ *S.* Typhimurium (C) and $Vi^{+/-}$ *S.* Typhi (D) are shown. Each symbol represents results for *Salmonella* opsonised with one adult serum. Statistical analysis was performed using Student's t-test. Lines represent mean of three separate experiments +/- SEM.

3.4. *Discussion*

We used isogenic Vi$^{+/-}$ *Salmonella,* including on the background of *S.* Typhimurium, to characterise the effect of Vi-expression on resistance of *Salmonella* to the immune response. We have demonstrated that expression of Vi capsule by *Salmonella* is associated with a significant increase in resistance to serum killing against sera from 10 healthy adults containing anti-*Salmonella* antibody. These findings support earlier work on the role of Vi capsule (Looney and Steigbigel, 1986, Wilson et al., 2011) and contrast with other work which found a lack of involvement of Vi-expression in serum resistance (Bravo et al., 2008). In the current study we tested human sera from multiple adult donors and used electron microscopy to visualise serum killing of *Salmonella* in the presence or absence of Vi capsule. These data support the concept that Vi expression by *S.* Typhi is a virulence mechanism used to increase bacterial survival during infection.

To examine the effect of Vi-expression on antibody binding to the *Salmonella* surface, we measured total IgG and IgM deposition onto Vi$^{+/-}$ strains by flow cytometry. We were unable to detect any significant differences in IgG deposition associated with Vi-expression. Five of the ten human sera we used contained Vi-specific IgG and all sera contained IgG to the respective, immunodominant *Salmonella* O-chain Ag so this finding is not surprising. When we excluded results from sera containing anti-Vi IgG, Vi-expression was associated with significantly lower IgG deposition for both Vi^{+} *S.* Typhimurium and *S.* Typhi compared to deposition on their Vi^{-} partner strains. This suggests that in a Vi-naïve host, expression of Vi capsule may reduce IgG mediated

activation of the classical complement pathway by reducing total IgG binding. However, the presence of anti-Vi IgG appears to annul this effect.

The observation that all sera had detectable Vi-specific IgM was initially surprising. However, nine of the ten serum donors worked in a vaccine development laboratory which routinely used Vi-expressing *C. freundii* in addition to the Vi-expressing strains used in this study, so this may explain the presence of this antibody. The one donor not working in the vaccine laboratory had received ViPS typhoid vaccination which could account for the presence of anti-Vi IgM in their sera. Despite this, expression of Vi capsule by *S.* Typhi was associated with a significant reduction in total IgM binding to the *Salmonella.* This contrasts to the observations for IgG binding, but may reflect the increased vulnerability of IgM to steric hindrance by Vi capsule due to its pentameric structure.

A key finding of this study is the significant reduction in MAC deposition on the Vi^+ *S.* Typhimurium and Typhi strains compared to their respective isogenic Vi^- counterparts. Expression of the Vi capsule was also associated with a reduction in C3 deposition, an observation in agreement with previous work (Looney and Steigbigel, 1986, Wilson et al., 2011). That Vi-expression is associated with a reduction in IgG binding in the absence of Vi-specific IgG, suggests complement deposition may be reduced due to capsule-mediated inhibition of the classical complement pathway. However, the effect on IgG binding is lost when we compare the groups as a whole, whereas we still see a reduction in complement deposition. Thus suggesting Vi capsule may also inhibit complement activation via the alternative pathway. Taken together, these findings are consistent with the concept that Vi expression impedes C'-mediated killing by directly

reducing the deposition of complement factors (alternative pathway) and restricting binding of antibody to the *Salmonella* surface (classical pathway). A reduction in complement deposition resulting from the modification of surface structures has been shown increase resistance to killing in other bacteria including *E. coli* K1 and *N. meningitidis* (Gemski et al., 1980, Vogel et al., 1997).

In the current study, Vi expression had a similar effect on killing of *Salmonella* by blood phagocytes, as on C'-mediated killing. This builds upon previous work where Vi-expressing strains of *S.* Typhi were phagocytosed less rapidly than non-isogenic Vi^- *S.* Typhi (Looney and Steigbigel, 1986) and isogenic Vi^+ *S.* Typhi had reduced clearance from murine livers compared to Vi^- *S.* Typhi (Wilson et al., 2011). Optimal phagocytosis of *Salmonella* requires opsonisation with both antibody, principally IgG, and complement (Gondwe et al., 2010). Having shown a Vi-associated reduction in binding and deposition of IgM, C3 and MAC, but not IgG, it is likely that Vi expression protects against phagocytosis by reducing both IgM and complement deposition. Whilst IgM itself is not thought to be opsonic, the recent discovery of IgM specific Fc receptors on phagocytes raises the possibility that it may contribute to phagocytosis (Kubagawa et al., 2009). Moreover, IgM is a potent activator of C, which is opsonic. Therefore a reduction in IgM deposition could indirectly have a negative impact on phagocytosis. We show that in the absence of Vi-specific IgG, Vi-expression is associated with a significant reduction in total IgG binding, thus in a naïve host, Vi capsule may act to inhibit phagocytosis by reducing opsonic IgG binding.

The phagocytosis flow cytometry data did not reflect the blood cell killing results and there are a number of reasons why this may be. In the assay we use formaldehyde fixed

bacteria as Vi capsule is heat labile so heat-killing is not a viable method of inactivation. Use of fixed *Salmonella* may not stimulate an adequate phagocytic response and differential phagocytosis of killed compared to live *Salmonella* ,and other bacteria, has been reported (Tomita et al., 1981). However, we still observed fluorescent signal compared to the negative control indicating active phagocytosis and internalisation of *Salmonella.* Alternatively, we did not, in the presence of Vi-specific IgG, see a reduction in total IgG deposition and so Vi-expression may not inhibit phagocytosis of *Salmonella* where uptake is principally mediated through FcR engagement. The blood cell killing assay is a measure of clearance of *Salmonella* by peripheral blood leukocytes, a process which involves both phagocytosis and intracellular killing, thus the results of the blood cell assay may reflect Vi-mediated resistance to the oxidative burst and not phagocytosis, something which has been previously reported (Looney and Steigbigel, 1986). Finally, temporal differences between the lengths of the two assays may be responsible for the discrepancy in results. The effect of Vi-expression on the killing of *Salmonella* by monocytes and neutrophils over time, in particular the contribution of Vi capsule to resistance against phagocytosis and/or intracellular killing, is something which warrants further study.

3.5. *Conclusion*

Vi-expression by *Salmonella,* irrespective of the serovar, was associated with significant reductions in both serum and cell-mediated killing of bacteria. We were able to visualise the protection against serum killing associated with Vi-expression by electron microscopy. We also observed reductions in both antibody binding to, and complement

deposition on *Salmonella* in the presence of Vi capsule. These data support the role of the capsule as a virulence factor which protects *Salmonella* against the innate immune response.

4. Role of anti-Vi and anti-O:9 antibody in immunity to *S.* Typhi

4.1. *Introduction*

Ab plays a protective role against infection with *S.* Typhi and there are multiple vaccines licensed for use against typhoid fever (Maclennan et al., 2014). To date, typhoid vaccines have either been inactivated whole cell, live attenuated or Vi capsular polysaccharide vaccines and their conjugates. Newer live attenuated vaccines or vaccines based on *S.* Typhi outer membrane proteins (OMPs) are in development but are yet to be licensed (Kaur and Jain, 2013, Wahid et al., 2008).

Inactivated whole cell *S.* Typhi vaccines have a 3 year cumulative efficacy of 73% (Engels et al., 1998). However, these vaccines required three doses and are associated with high reactogenicity, thereby limiting their use in the general population (Garmory et al., 2002, Engels et al., 1998). The Ty21a vaccine is a live attenuated whole cell vaccine which is administered orally. It is derived from virulent *S.* Typhi Ty2 but does not express the Vi polysaccharide capsule. The third vaccine and its derivatives use Vi capsular polysaccharide (ViPS) as the protective antigen and are administered intramuscularly. Ty21a and unconjugated ViPS vaccines have similar cumulative efficacies of 51 and 55% 3 years post-vaccination, despite delivering completely different Ag to the immune system. Neither are recommended for use in children under the age of two years and both require refrigeration. Implementation of either vaccine into national public health programs has not occurred, even in areas where typhoid fever is endemic (Fraser et al., 2007, Engels et al., 1998). Effective vaccines with greater 3 year efficacy in adults and

immunogenicity in infants are required thereby driving the development of next generation typhoid vaccines (McGregor et al., 2013, Martin, 2012, DeRoeck et al., 2007).

High levels of multidrug resistance in nontyphoidal *Salmonella* (NTS) and the prevalence of these serovars in sub-Saharan Africa and south east Asia, create a requirement for an effective NTS vaccine, but none are currently licensed (Maclennan et al., 2014). Glycoconjugate vaccines containing NTS O-chain Ag conjugated to carrier proteins are in development, and anti-O-chain antibody has shown potential for protecting against NTS infection for the two most clinically important NTS serovars; *S.* Typhimurium and *S.* Enteritidis (Simon et al., 2011, Simon et al., 2013, Micoli et al., 2013). However concerns exist regarding the use of the LPS O-Ag as the protective Ag in these vaccines following the observation that high titre anti-OAg IgG can inhibit the killing of *Salmonella* in a subset of HIV-infected Africans (MacLennan et al., 2010).

S. Enteritidis is a NTS serovar which is one of the most prevalent globally (Galanis et al., 2006), and is the second most common NTS isolate causing bloodstream infections (BSIs) in Africa (Reddy et al., 2010). Like *S.* Typhi, *S.* Enteritidis also expresses the LPS O:9 O-chain Ag and this is currently being exploited to develop O-antigen conjugate vaccines (Simon et al., 2011). This raises the question of whether anti-O:9 antibodies have bactericidal and/or opsonic activity against *S.* Typhi, and if so, could an O:9 conjugate vaccine protect against the two serovars?

Whilst not deployed on a national scale, ViPS vaccines have seen widespread use in endemic areas. This has led to fears of vaccine selection and subsequent Vi^- *S.* Typhi strain replacement. Clinical isolates of *S.* Typhi which retain virulence but lack the Vi capsule have been documented (Baker et al., 2005). Moreover, other clinically important

serovars such as *S.* Dublin can express both Vi capsule and the O:9 somatic Ag (Levy et al., 2008, Selander et al., 1992). Therefore, investigation into the potential of O:9 conjugate vaccines, particularly in the context of Vi expression, and their protection against *S.* Typhi is warranted.

It is clear that anti-Vi antibody offers protection against infection with *S*. Typhi, as demonstrated by the use of multiple, effective ViPS vaccines. Nevertheless, the mechanism of protection afforded by these antibodies is not well defined. Both rabbit and mouse anti-Vi antibody have been shown to have bactericidal activity against Vi^+ *S.* Typhi, and this effect can be reduced following adsorption of anti-Vi antibody (Osawa and Muschel, 1964). Whilst this has not been described for human anti-Vi Ab, an inverse relationship between the incidence of typhoid fever and serum bactericidal titer against *S*. Typhi has been reported (Pulickal et al., 2009). These data suggest that serum bactericidal activity and/or antibody-dependent phagocyte killing may correlate with protection.

Anti-*Salmonella* antibody in sera from humans vaccinated with the M01ZH09 live attenuated Vi^+ strain of *S*. Typhi were able to increase phagocytosis and the intracellular killing of wild type *S*. Typhi, suggesting that anti-Vi antibody may be responsible for this enhanced phagocyte-mediated immunity (Lindow et al., 2011). These sera were also shown to have greater bactericidal activity compared to unvaccinated sera. However, the specificities of the bactericidal and opsonic antibody in these sera in this study were not reported.

A proven correlate of protection for *S.* Typhi has yet to be established and this is crucial to the development of newer, more effective typhoid vaccines. To do this, it is important to gain a better understanding of the modalities of both anti-Vi and anti-O:9 antibody against *S.* Typhi. To evaluate the cross-protective potential of O:9 and subsequent multivalent OAg vaccines, particularly against Vi^+ *Salmonella,* we first need to understand how capsular expression affects O-Ag antibody binding and subsequent *Salmonella* killing.

4.2. *Aims*

Firstly, we wanted to purify human antibody (hAb) against two important *S.* Typhi Ag; Vi capsular polysaccharide and the LPS O:9 O-chain Ag. Once purified, we assessed the bactericidal and opsonic potential of these antibodies against multiple wild-type Asian and African *S.* Typhi isolates. We also wanted to assess the role of these antibodies in facilitating complement deposition onto *Salmonella* and phagocytosis by washed peripheral blood cells. To mimic the occurrence of natural Vi- *S.* Typhi strains and examine the effect of Vi expression on anti-O:9 activity, we cultured *S.* Typhi at varying sodium chloride concentrations to both augment and down-regulate Vi expression before testing antibody activity.

4.3. Results

4.3.1. Affinity purification of anti-*S.* Typhi antibodies from human serum

We used affinity chromatography columns activated with Vi polysaccharide or O:9 Ag to purify human anti-*S*. Typhi antibody. This technique had been successfully used by our group in the prior extraction of anti-*S*. Typhimurium O-chain antibody (O'Shaughnessy et al., 2013). Three columns were utilised, one containing O:9 O-chain Ag and two containing Vi polysaccharide.

The O:9 column contained 1mg O:9 O-chain Ag derived from *S*. Enteritidis D24359. This was linked to the column using adipic acid dihydrazide (ADH) which reacts with the amine grouping on the purified O-Ag to form a stable bond between the Ag and the ADH.

The two Vi columns contained either 2.52 mg ViPS in its native conformation (avMW 200 kDA) or 2.35mg fragmented ViPS (avMW 40-50 kDa) and will be known as the Vi column A and B respectively hereafter. ViPS was purified from *C. freundii* WR7011 and is immunologically indistinguishable to *S.* Typhi ViPS (Daniels et al., 1989) (For more information on column preparation please refer to section 2.18).

4.3.1.1 Elution of bound antibody from one donor sera with glycine pH2.4 delivers low yields

We initially tested serum from one donor which was known to contain anti-Vi and O:9 IgG. Serum was diluted 1:2 with PBS and passed through the column for a total of 12 column volumes before washing and elution with 0.1M glycine, 0.1M NaCl pH2.4.

Using this method, greater than 95% and 65% of available anti-O:9 IgG and IgM bound to the columns respectively, as calculated by determining the antibody content in the flow-through fraction (Figure 4.1 A and B). 4% of available IgG did not bind to the column and was lost in the flow-through fraction. A further 4% was lost upon first washing of the column. No anti-O:9 IgG was detected in the remaining washes or the first eluate. Elution with glycine yielded 6.1% of bound antibody in elution two and 3.6% in elution 3. No antibody was detected in elutions 4 – 12. This indicates that approximately 83% of available anti-O:9 IgG within the sera remained bound to the column after elution (Figure 4.1 A).

Anti-O:9 IgM levels were low in the donor sera to begin with. 33% of available anti-O:9 IgM did not bind the column, being lost in the flow through and 20% was lost in the first wash. 11% of bound antibody was eluted in the second elution fraction. No antibody was detected in elutions thereafter indicating less than 50% available antibody remained bound to the column (Figure 4.1 B).

Vi column B proved marginally better at binding anti-Vi IgG with only 7% of available antibody being lost in the flow through as opposed to 12% for Vi column A. However, after washing, column B lost 9% of available antibody as opposed to 7% for the column A. As such, after loading and washing, both columns retained approximately equal amounts of anti-Vi IgG. 1.7% of bound antibody was eluted from both Vi columns at the third elution (Figure 4.1 C and D), thus leaving 81 and 78% of available anti-Vi IgG bound to column B and A respectively.

These data show that the columns are functional and the Ag within the columns has retained its structure after activation and column packing. The majority of specific

antibody was able to bind the columns with >85% IgG for all Ag binding to the column prior to washing. However, yields using glycine pH 2.4 as an elution buffer were too low to be viable and left too much antibody bound to the column.

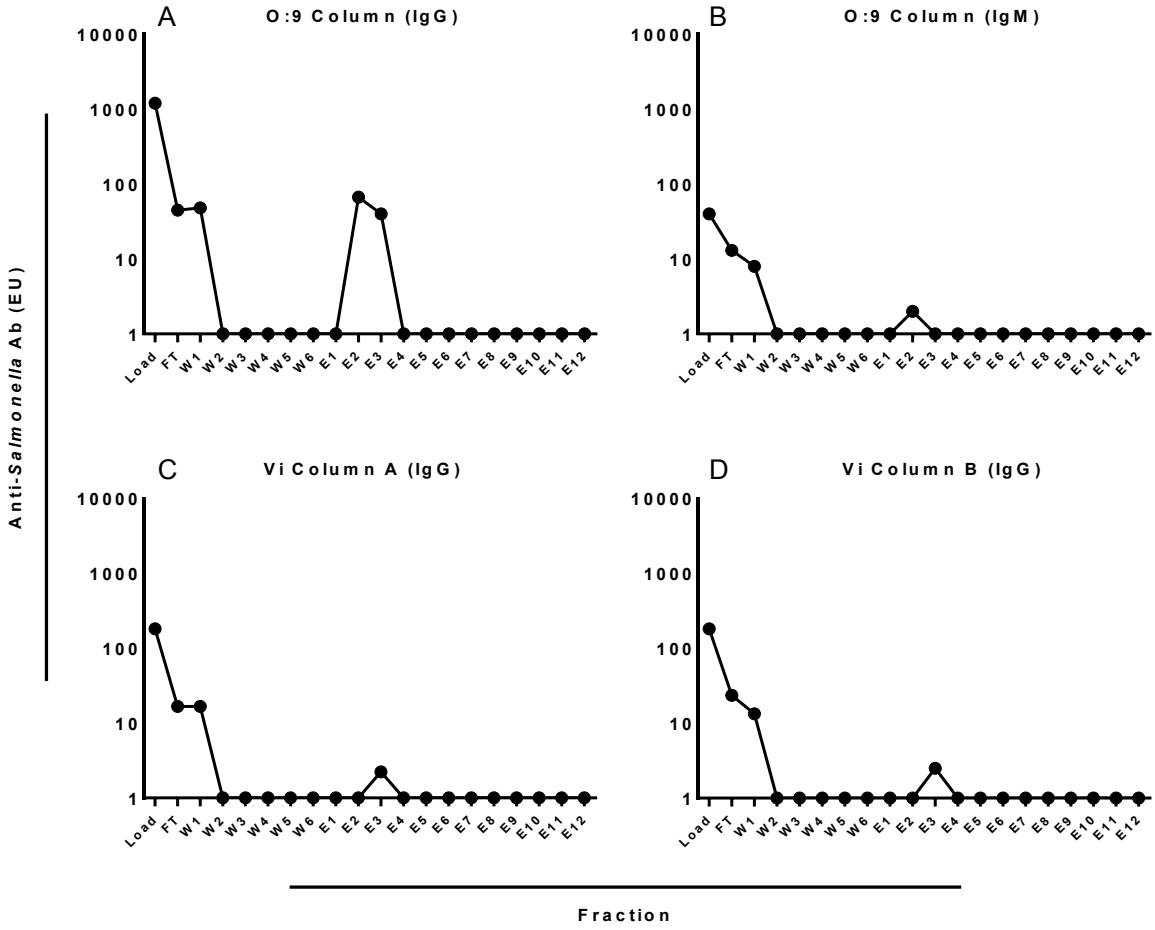


Figure 4.1. Elution of anti-O:9 IgG and M, and anti-Vi IgG from one donor sera using glycine elution buffer.

ELISA data for binding to and elution from affinity chromatography columns for anti-O:9 IgG (A), IgM (B), anti-Vi IgG from the Vi column A (C) and Vi column B (D). 2ml human serum diluted 1:2 with PBS (Equivalent to 1200, 40 and 182 ELISA units of anti-O:9 IgG, IgM and anti-Vi IgG respectively) was loaded onto each column. Unbound antibody was collected in the flow through fraction (FT) and columns were washed with 6ml PBS (W1 – W3) followed by 6ml 50mM NaPi, 500mM NaCl buffer pH 7.2 (W1- W6). Antibody was eluted using 0.1M glycine, 0.1M NaCl pH2.4 into 500µl fractions containing 25µl 2M Tris, pH 9. The antibody content of all fractions, including flow through and washes, were quantified by ELISA. The reciprocal of the concentration of antibody to give an OD1 at λ405nm is plotted. Data are representative of two experiments.

4.3.2. Pooling human serum and using different elution buffers increases recovery of bound antibody more than ammonium sulphate serum protein precipitation

To improve recovery of bound antibody from the columns during elution we made three changes to the technique. Firstly we reasoned that by using polyclonal sera from multiple donors we would be able to saturate the column, thereby increasing recovery yields during the elution steps. To get a greater variety of antibody with different specificities we pooled sera from three individuals with high anti-O:9 and Vi antibody. Secondly, to increase elution of antibody from the column we tried four different elution buffers in a sequential elution series. These were; 0.1M glycine, 0.1M NaCl pH 2.4, 4M $MgCl_2$ in 10mM Tris pH 7, 20% ethanol and 8M urea pH 7. Finally in an effort to concentrate serum proteins and remove lipids and other molecules which could interfere with antibody-antigen interactions, we used ammonium sulphate precipitation to concentrate serum proteins which were then loaded onto the column. Ammonium sulphate concentrated proteins equivalent to 1429 and 612 ELISA units of anti-O:9 IgG and anti-Vi IgG respectively were loaded onto the O:9 column and the Vi column B, while the non-precipitated diluted pool of human serum was applied to the Vi column A.

For all three columns, the percentage of available antibody lost in the flow through was lower than using serum from one donor (0.15, 1.6 and 4.5% for the O:9, Vi column B and column A respectively). This suggests that either the ammonium sulphate precipitation and/or the presence of different specificities of antibody in pooled serum results in a greater proportion of available binding sites being occupied and less subsequent loss of antibody in the flow through and washing steps.

After loading with 300ul concentrated protein solution equivalent to 1429 ELISA units of anti-O:9 IgG, 19 and 15% of bound antibody was eluted using glycine in the second and third elutions. Very low percentages of bound antibody were also eluted in glycine fractions 4, 5 and the third $MgCl_2$ and Urea fractions (2, 1, 1 and 1% respectively) (Figure 4.2 A).

Vi column B was also loaded with concentrated serum protein equivalent to 612 anti-Vi IgG ELISA units. In contrast to elution of O:9, glycine was only able to elute 2 and 1.6% of bound anti-Vi IgG in elutions three and four respectively. MgCl was a more effective elution buffer, enabling recovery of 8.3% of bound antibody. No anti-Vi IgG was eluted using either ethanol or urea as elution buffers (Figure 4.2 B).

Vi column A was loaded with a 1:2 dilution of pooled human sera equivalent to 333 ELISA units of anti-Vi IgG. Again glycine proved ineffective as an elution buffer, yielding a maximum of 4% of bound antibody in the third eluate. MgCl was very effective as an elution buffer enabling recovery of 29 and 53% of bound antibody in the second and third elutions. Again, ethanol and urea were ineffective at eluting anti-Vi IgG (Figure 4.2 C).

These data suggest that using pooled human sera is much more effective at reducing loss of antibody from the column in the flow through and washes, and also increasing the recovery yields. It is clear that that MgCl, an ionic elution buffer, is much more effective at eluting anti-Vi IgG than glycine pH 2.4, whereas glycine pH 2.4 is more effective at eluting anti-O:9 antibody. Differences within the strength and/or nature of the antibody-antigen bonds or the affinity of anti-O:9 and anti-Vi antibody to their respective antigens

could be responsible for the differences in the recovery yields of antibody obtained with different elution buffers.

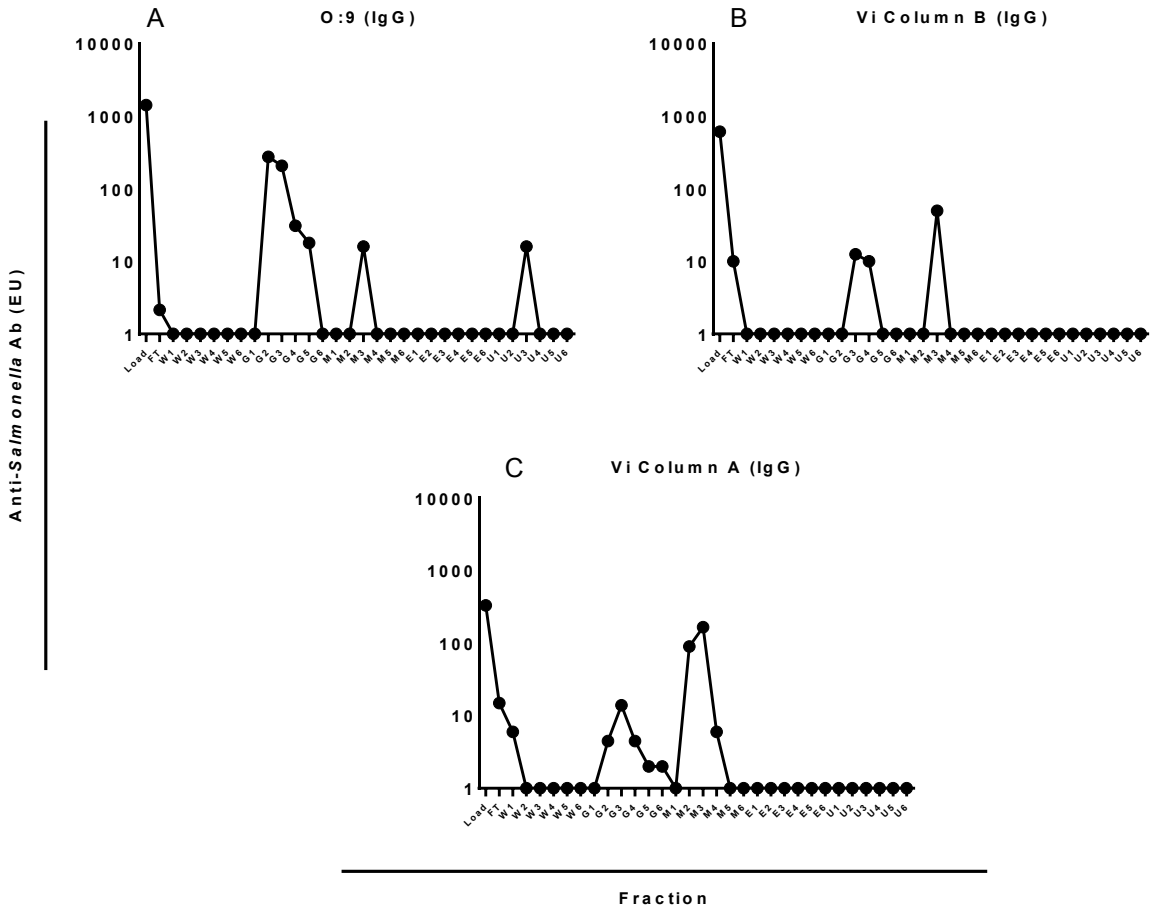


Figure 4.2. Increased recovery yields are achieved with pooled human sera and different elution buffers.

Ammonium sulphate precipitated proteins from pooled human sera were added to O;9 (A) and Vi (B) affinity columns while pooled sera diluted 1:2 PBS was added to the final V column (C). Unbound antibody was collected in the flow through fraction (FT) and columns were washed with 6ml PBS (W1 – W3) and 6ml 50mM NaPi, 500mM NaCl buffer pH 7.2 (W4- W6). Antibody was eluted in a sequential elution series using 0.1M glycine, 0.1M NaCl pH2.4 (G1 – G6), 4M $MgCl_2$ in 10mM Tris pH 7 (M1 – M6), 20% ethanol (E1 – E6) and 8M urea pH 7 (U1 – U6). The antibody content of all fractions, including flow through and washes, were quantified by ELISA. The reciprocal of the concentration of antibody to give an OD1 at λ405nm is plotted. Each panel represents data from one column and is representative of two experiments.

4.3.3. Ammonium sulphate precipitation is not required for high recovery yields

While we did achieve high recovery yields using ammonium sulphate protein precipitation for anti-O:9 Ab, recovery of anti-Vi IgG was lower after precipitation than without it. We wanted to test whether precipitation was required for high recovery yields of anti-O:9 IgG or whether we could use a pool of diluted human sera without precipitation for all columns and still attain high recovery yields. We also chose to use only glycine and MgCl for further elutions due to the poor recovery of bound antibody with ethanol and urea.

Using dilute pooled human sera, 1.7% available anti-O:9 IgG was lost in the flow through with a further 0.6% lost in the first column wash. Elution with glycine yielded 14, 26 and 5.1% recovery of bound antibody for elutions 2, 3 and 4 (G2 – G4) respectively. Again MgCl was ineffective as an elution buffer for anti-O:9 IgG, eluting less than 2% of available antibody between the second and fourth elution (M2- M4) (Figure 4.3 A).

Less than 1.5% of available antibody was lost in the flow through after loading both Vi columns and less than 1% was lost in washing both columns. For the fragmented Vi column, glycine elution only yielded low recovery of less than 2% of bound antibody (G3 and G4). Elution with MgCl however, yielded 10% of bound anti-Vi IgG in the second elution fraction and 98.8% in the third (Figure 4.3 B). The total amount of antibody recovered exceeded that in the load sample. This likely represents elution of antibody which had been unsuccessfully eluted in previous extractions and had remained bound to the column.

For the native conformation Vi column, glycine was ineffective as an elution buffer, yielding less than 2% in eluates two and three (G2 and G3). Elution with MgCl resulted in recovery of 15 and 22% of bound antibody for the second and third eluate (M2 and M3). This level of recovery was lower than the previous round of extractions but was sufficient enough to make the process viable (Figure 4.3 C).

For subsequent extractions, we used pooled human sera without ammonium sulphate precipitation. Glycine was used for anti-O:9 IgG extraction and MgCl for anti-Vi IgG extraction. Yields in further extractions stabilised at approximately 25% and 20-22% for anti-O:9 and anti-Vi IgG respectively (Data not shown).

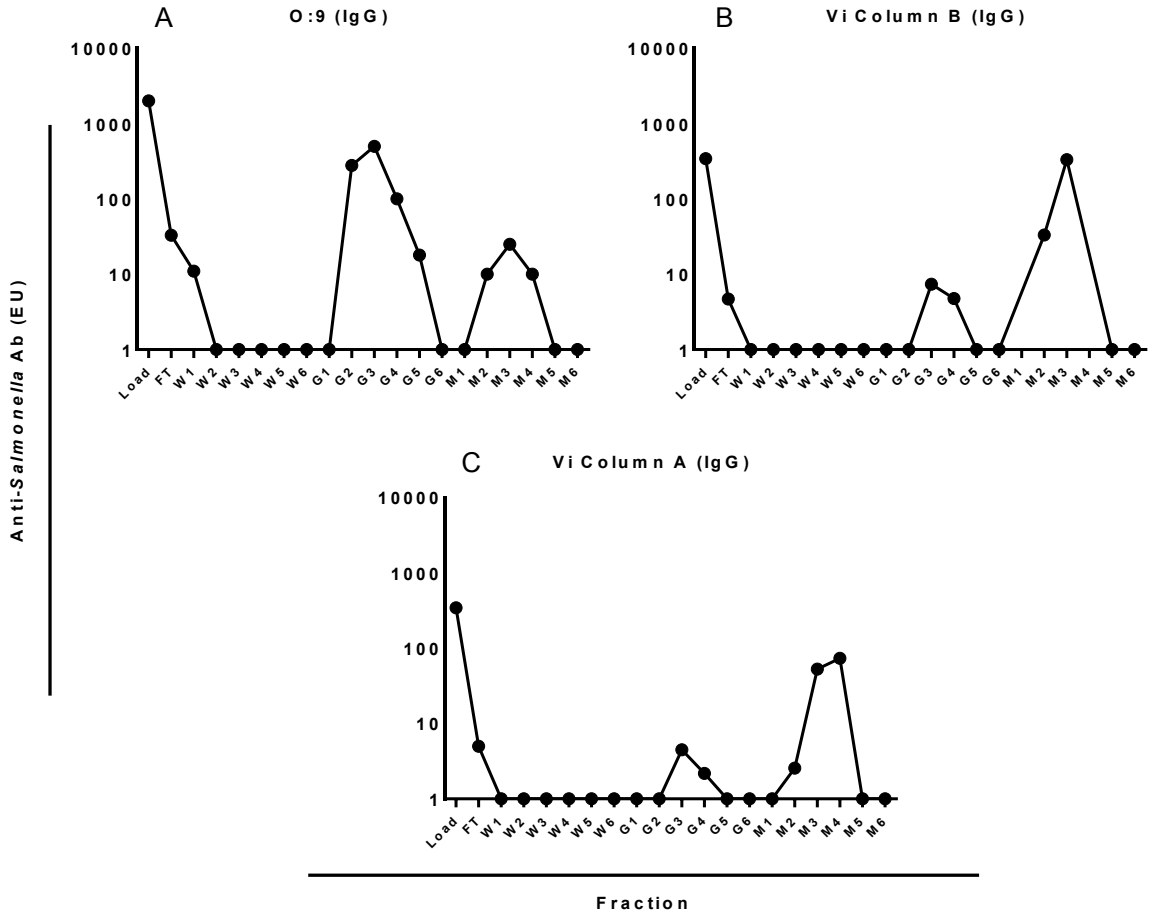


Figure 4.3. High recovery yields in the absence of ammonium sulphate serum protein precipitation

ELISA data for elution of anti-O:9 and anti-Vi IgG from pooled human sea without ammonium sulphate precipitation. Pooled human sera were diluted 1:2 with PBS and loaded onto each column. Flow through (FT) and washes (W1 – W6) containing unbound antibody were collected. Only glycine (G1 – G6) and MgCl (M1 – M6) were used to elute antibody. Glycine was effective at recovering bound anti-O:9 IgG (A), whereas MgCl enabled better recovery of anti-Vi IgG from the fragmented (B) and native conformation columns (C). All fractions were collected and tested for IgG content by ELISA. The reciprocal of the concentration of antibody to give an OD1 at λ405nm is plotted. Each panel represents data from one column and is representative of one experiment.

4.3.4. Purified antibody is not contaminated with other anti-*Salmonella* antibody

To check the purity of the antibody preparations, we tested them in ELISAs using plates coated with other immunogenic *Salmonella* Ag. We tested each antibody preparation using ELISA plates coated with ViPS, *S.* Enteritidis LPS (O:9 OAg), *S.* Typhimurium LPS (O:4,5 OAg) and outer membrane proteins (OMPs) purified from *S.* Typhimurium.

No IgG against any of the other Ag tested was detected in the purified anti-Vi preparation, confirming that only anti-Vi antibody binds to the Vi columns and is extracted. No anti-Vi IgG was detected in the purified anti-O:9 antibody preparation. There were trace amounts of detectable anti-O:4,5 and anti-OMP IgG in the purified anti-O:9 pool, but these accounted for just 0.07% of the total IgG in the preparation. IgG against all four Ag was detectable in fresh human serum (Figure 4.4).

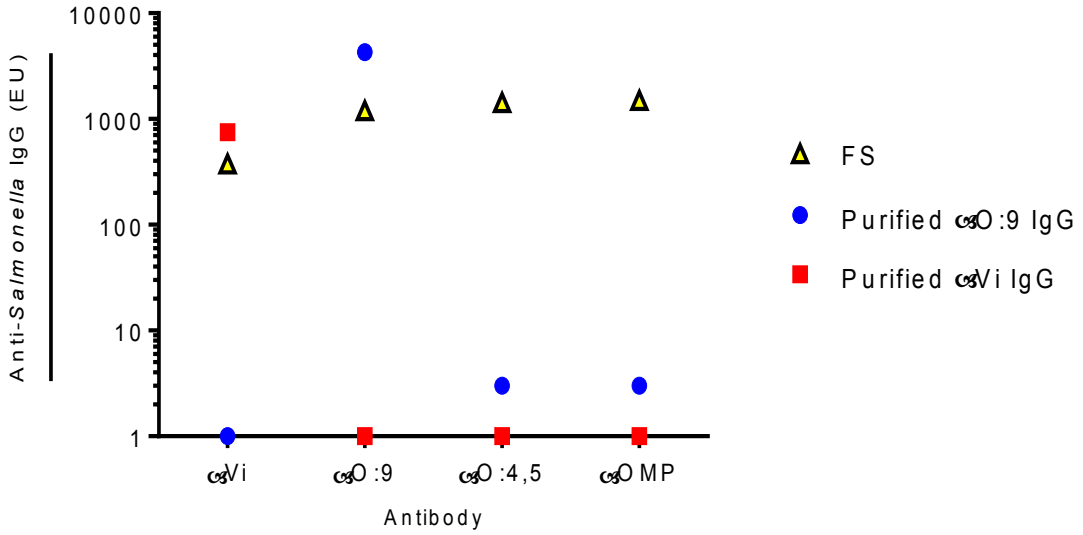


Figure 4.4. Antibody preparations were >99% specific antibody only when tested for the presence of other immunodominant anti-*Salmonella antibody*.

The purified antibody preparations were assessed for purity by testing by ELISA for other antibodies to immunogenic *Salmonella* antigens. Purified anti-Vi antibody (red squares) was tested for the presence of IgG against O:9, O:4,5 or OMPs from *S.* Typhimurium. Purified anti-O:9 antibody (blue circles) was tested for the presence of anti-Vi, O:4,5 or OMP antibody. Levels of IgG against all four *Salmonella* antigens in fresh human serum (FS, yellow triangles) were also assessed. Data are representative of one experiment.

4.3.5. IgG content of purified anti-Vi and anti-O:9 antibody preparations

After repeated extractions using pooled sera from three donors we amassed sufficient quantities of anti-Vi and anti-O:9antibodyto enable us to study their activity in *in vitro* assays.

When characterised by ELISA, the anti-Vi IgG preparation contained 750 ELISA units of anti-Vi IgG. The mean level of anti-Vi IgG in the three donor sera used to extract the anti-Vi antibody from was 387 ELISA units. As such the purified anti-Vi IgG preparation contained 2.7 times the amount of anti-Vi IgG in the pooled human sera. Due to its relative abundance within sera compared to anti-Vi Ab, the purified anti-O:9 IgG preparation contained 4268 ELISA units which corresponded to 4.1 times the average amount of anti-O:9 IgG within the pooled human sera used for the extraction. Detailed information about the antibody preparations is presented in table 4.1.

Table 4.1. Purified Anti-*Salmonella* IgG information.

	Purified antibody preparation:	
	Anti-Vi IgG	Anti-O:9 IgG
ELISA units (EU)	750	4268
Average in pooled sera (EU)	387	1032
Concentration of antibody preparation in relation to pooled average	2.7 X	4.1 X

4.3.6. Role of anti-Vi and anti-O:9 antibodies in bactericidal and opsonic killing of S. Typhi.

While multiple studies have investigated the effect of animal anti-Vi antibody on Vi^+ *S.* Typhi and have examined the protective potential of post-vaccination or immune human sera, nothing has been reported on the specific roles of purified human anti-Vi and anti-O:9 antibodies. We used purified, human anti-Vi and anti-O:9 antibodies in *in vitro* killing assays to investigate the bactericidal and opsonic activity of these antibodies against wild-type *S.* Typhi isolates. To address the question of whether anti-O:9 antibody may protect against Vi^- clinical isolates of *S.* Typhi, we manipulated expression of Vi capsule by varying the osmolarity of the growth medium. This allowed us to examine the activity of anti-O:9 antibody in the presence of minimal Vi expression.

To manipulate Vi-expression without genetically altering the wild-type strains, we cultured *S.* Typhi overnight and to exponential phase in LB broth containing low (9mM), medium (85mM) or high (500mM) levels of sodium chloride (Pickard et al., 1994). Osmolarity affects Vi-expression, resulting in a *RcsB*-mediated decrease in transcription of Vi biosynthesis genes such as *tviA* and *tviB* at high extracellular NaCl concentrations (Arricau et al., 1998). Unlike Vi capsule, OAg expression in *Salmonella* is not affected by media osmolarity (Rojas et al., 2001). Thus we are able to modulate Vi expression whilst leaving O-Ag expression unaffected.

To use purified hAb we also needed a functional complement source. BRC is not suitable as a complement source for *S.* Typhi due to its inherent toxicity at relatively low concentrations (see section 5.3.9), and so we prepared anti-*S.* Typhi antibody-depleted human serum to serve this purpose. The depletion of antibody from sera requires large

concentrations ($1x10^{11}$ CFU/ml of *Salmonella).* As wild-type *S.* Typhi is a category-level 3 organism, growing cultures of 14 wild-type *S.* Typhi strains to this concentration is impractical and unsafe. We used one representative *S.* Typhi strain of the haplotype-58 lineage (ISP-04-06979) and depleted several batches of sera of *S.* Typhi-specific antibody using this strain. Antibody depleted serum was then tested alone and in conjunction with heat-inactivated human serum, as an antibody source, to see if it effected killing of wild-type *S.* Typhi isolates. 14 of 19 wild-type *S.* Typhi isolates were not killed by this "master" serum alone, but were killed when it was combined with antibody (data not shown). We then selected these 14 *S.* Typhi strains for use in *in vitro* assays to examine the role of the purified antibody.

4.3.6.1 Media osmolarity affects both anti-Vi and anti-O:9 antibody binding to, *S.* Typhi

We chose 5 of the 14 *S.* Typhi strains (one from each geographical location of isolation) and assessed levels of anti-Vi and anti-O:9 antibody binding to the surface of these strains after they were grown in LB broth containing 9mM, 85mM or 500mM NaCl. The level of binding of either anti-Vi or O:9 IgG to the cell surface was measured by flow cytometry.

When grown at low concentrations of NaCl (9mM), high levels of anti-Vi antibody binding to *S.* Typhi were detected, with a median GMFI of 371. Increasing the concentration of NaCl to 85mM reduced the median GMFI to 151, and further increasing the concentration to 500mM reduced anti-Vi binding to *S.* Typhi altogether, with levels no different to background levels seen using PBS as a negative control (Figure 4.5 A).

Surprisingly, we observed moderate levels of anti-O:9 antibody binding to *S.* Typhi when *Salmonella* were cultured in low (9mM) and medium (85mM) levels of NaCl (Median GMFI 51 and 42 respectively) despite Vi-expression (as inferred by anti-Vi binding) at these NaCl concentrations. Increasing the concentration of NaCl to 500mM increased anti-O:9 antibody binding (Median GMFI 161) (Figure 4.5 B).

Anti-Vi and anti-O:9 antibody binding to non-Vi-expressing *S.* Typhi and *S.* Enteritidis was also measured as a control. Anti-O:9 binding was higher for *S.* Typhi than *S.* Enteritidis (Median GMFI 205 and 118 respectively). Levels of anti-Vi binding to either Vi^- strain were negligible and similar to the PBS negative control most likely reflecting the absence of Vi capsule, and thus available anti-Vi antibody binding sites on these strains (Figure 4.5 C).

If anti-Vi binding is taken as an indicator of Vi-expression, these data show that increasing the concentration of NaCl downregulates Vi capsule expression in wild-type *S.* Typhi and this is in agreement with previous work (Arricau et al., 1998, Pickard et al., 1994). Growth in 500mM NaCl completely inhibited anti-Vi antibody binding to *S.* Typhi, suggesting capsular expression is switched off in this environment. In the same conditions, anti-O:9 binding to the cell was at its highest. We plotted anti-Vi antibody binding against anti-O:9 binding for each of the 5 strains grown in low, medium or high NaCl concentrations. There was a significant negative correlation between antibody binding to the two Ag (r_s=-0.62, p=0.016, 95% CI -0.86 - -0.14) (Figure 4.5 D).

Knowing that Vi expression is affected by media osmolarity, whereas O-Ag expression is not, we can infer that levels of anti-Vi binding to *S.* Typhi at different concentrations of NaCl are indicative of Vi expression levels. However, because O:9 expression is

unaffected by osmolarity, changes in anti-O:9 antibody binding to *S.* Typhi under these conditions do not represent changes in O:9 expression levels, but merely the level of O:9 Ag availability. Thus, to reduce confusion, herein anti-Vi binding will be referred to as "Vi-expression" while anti-O:9 binding will be referred to as "O:9 availability".

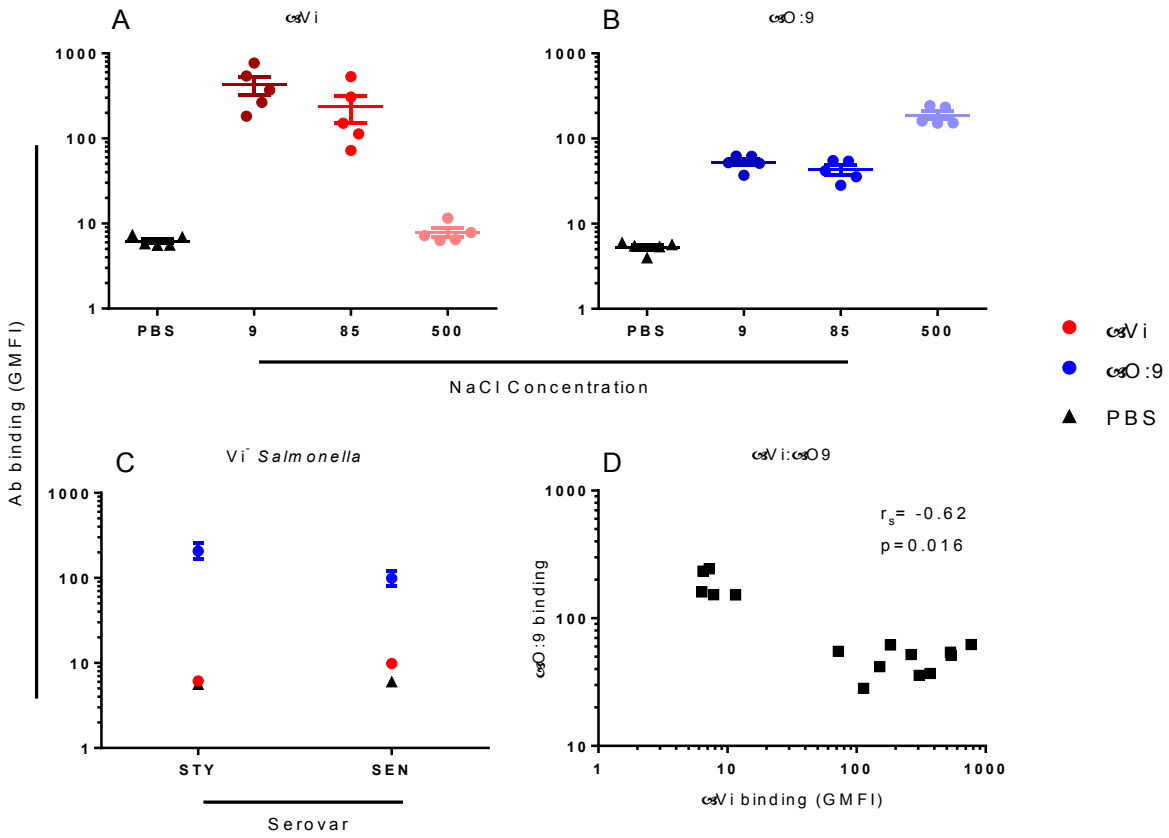


Figure 4.5. Increasing the osmolarity of growth media is associated with reduced Vi expression by *S.* Typhi but increased O:9 Ag availability.

The effect of media osmolarity on antibody binding as a proxy for Ag expression and/or availability was determined by flow cytometry. Vi expression levels decreased with increasing osmolarity and were no different to background levels at 500mM NaCl (A). No difference between anti-O:9 binding at 9mM or 85mM NaCl was observed. Anti-O:9 binding peaked when *Salmonella* were grown in 500mM NaCl (B). Anti-O:9 binding to both *S.* Enteritidis and Vi$^-$ *S.* Typhi was observed whilst anti-Vi binding was not (C). There was a significant negative correlation between Vi expression and anti-O:9 binding (r_s= -0.62, p=0.016). Colours in A and B represent five WT *S.* Typhi strains grown in different concentrations of NaCl, with the darkest colour representing the lowest concentration. Data are mean values from three separate experiments +/- SEM.

4.3.6.2 Human anti-Vi and anti-O:9 antibodies have bactericidal activity against wild-type *S.* Typhi

Having shown we were able to increase and also reduce levels of Vi-expression (as inferred by anti-Vi binding), we examined the effect that expression and/or absence of Vi capsule has on the bactericidal activity of anti-Vi and anti-O:9 antibody.

14 wild-type *S.* Typhi isolates were cultured in ordinary LB broth (85mM NaCl). Anti-*S.* Typhi antibody depleted serum was used as a complement source and combined with purified human anti-Vi or anti-O:9 antibody then tested against wild-type *S.* Typhi in the SBA. Purified antibody was tested in a four-fold dilution series ranging from a 1:4 – 1:1024 final dilution.

Anti-Vi antibody at a 1:4 dilution (corresponding to 188 IgG EU) effected between 2 – 3 $\log_{10}$ killing of wild-type *S.* Typhi isolates cultured in normal LB broth at 180 minutes. Similar levels of killing were observed after anti-Vi antibody was diluted to 1:16, while killing was reduced to between 0.5 – 1.5 $\log_{10}$ at a 1:64 final dilution. Killing was lost at dilutions thereafter (Figure 4.6 A, red lines). A 1:4 dilution of anti-O:9 antibody (corresponding to 1067 IgG EU) effected approximately 1 $\log_{10}$ killing of all but two *S.* Typhi strains, which underwent >2 $\log_{10}$ kill at this dilution. The bactericidal activity of anti-O:9 antibody for 12 of the 14 strains was lost after diluting to a 1:16 final dilution. Killing of the remaining two strains was lost at around a 1:64 dilution of antibody (Figure 4.6 A, blue lines).

The same 5 wild-type isolates as in 4.3.6.1 were cultured in low (9mM NaCl) (Figure 4.6 B) or high (500mM NaCl) (Figure 4.6 D) concentrations of NaCl and then tested in the

same SBA with purified anti-Vi and anti-O:9 antibody. When grown at 9mM NaCl, the killing of *S.* Typhi was similar to when *Salmonella* were cultured in normal LB broth (85mM NaCl) (Figure 4.6 A and C). Growth of *Salmonella* at 9mM NaCl did not reduce the activity of anti-O:9 antibody nor did they augment the bactericidal activity of anti-Vi antibody compared to growth in 85mM NaCl. Indeed, the bactericidal activity of anti-Vi antibody was slightly reduced when *S.* Typhi were grown in 9mM NaCl compared to 85mM NaCl.

Growth of *S.* Typhi at high NaCl concentrations (500mM) completely reversed the bactericidal action of the two antibodies against *S.* Typhi (Figure 4.6 D). Under these conditions, anti-O:9 antibody was highly bactericidal, effecting 3 $\log_{10}$ killing of *S.* Typhi between 1:4 and 1:64 final dilution of antibody. At the same NaCl concentration, the bactericidal activity of anti-Vi antibody was reduced effecting between 1 – 2 $\log_{10}$ killing at a 1:4 dilution after 180 minutes with killing being lost upon dilution of antibody to 1:256.

Killing of *S.* Typhi by anti-Vi antibody at 180 minutes was significantly greater than that of anti-O:9 antibody at NaCl concentrations of 9mM – 85mM (Figure 4.6 E, F and G, unpaired t-test $p<0.0001$, 0.018 and 0.032 respectively), whereas it was significantly reduced at 500mM NaCl (H, unpaired t-test $p<0.0001$).

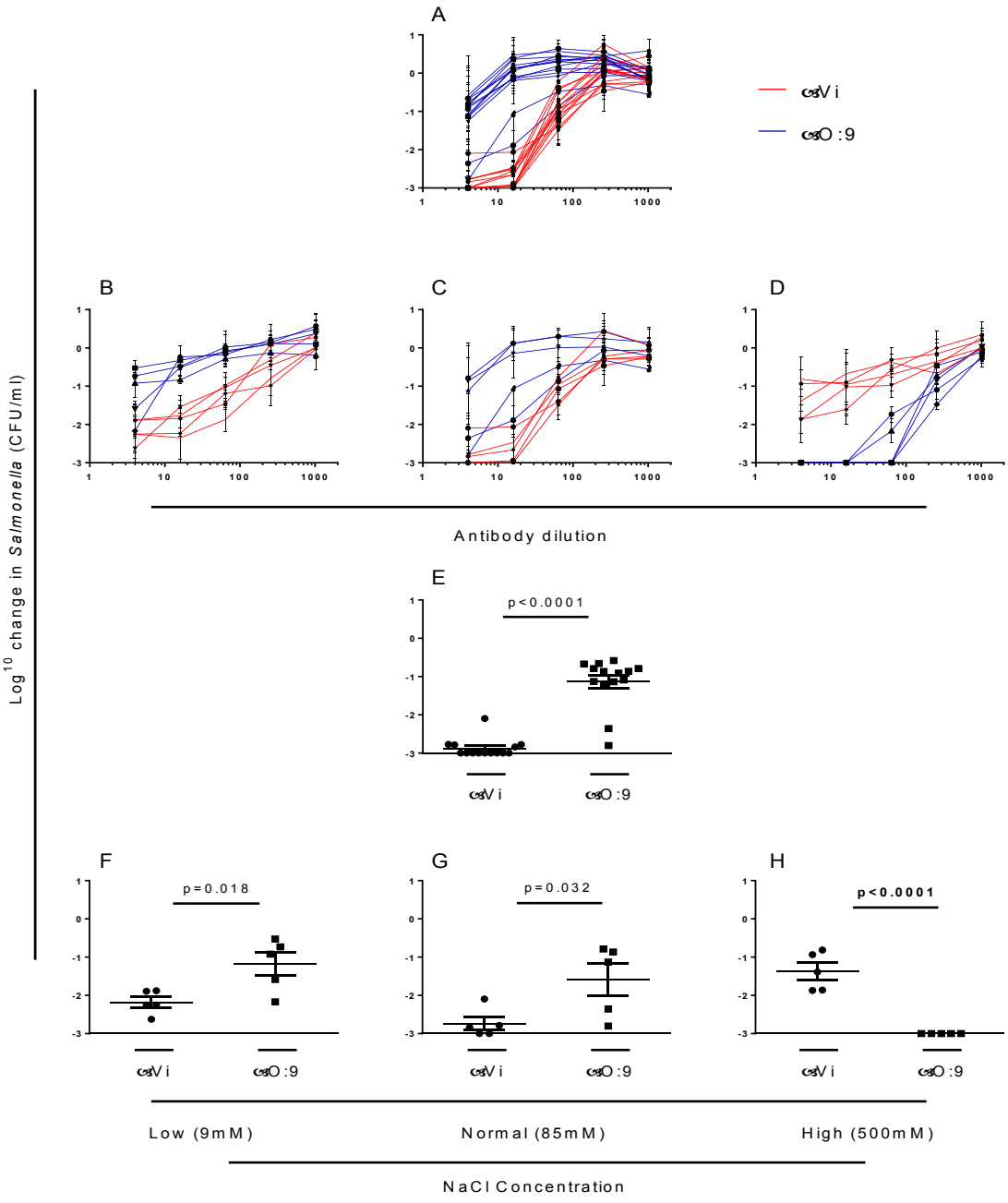


Figure 4.6. Growth at different osmolarity affects bactericidal activity of human anti-Vi and O:9 antibody against wild-type *S.* Typhi.

The bactericidal activity of anti-Vi antibody (red lines) and anti-O:9 antibody (blue lines) was tested in the SBA against wild-type *S.* Typhi grown in different concentrations of NaCl. Killing of 14 *S.* Typhi isolates by both antibodies are shown at 85mM NaCl (A and E). The killing of 5 of these isolates at 85mM NaCl is shown (C and G) with the killing of these same isolates at 9mM NaCl (B and F) or 500mM NaCl (D and H). Data are mean values from three separate experiments. Each line and point represents data for one strain. Statistical analysis was done using Student's t-test. Error bars represent SEM.

4.3.6.3 Activity of bactericidal antibody is associated with Vi expression and O:9 availability

We examined the relationship between Vi expression and O:9 availability levels and bactericidal activity of the respective antibody. SBA results for the five *S.* Typhi isolates grown under varying osmolarity conditions were plotted against Vi expression and O:9 availability levels, as determined by antibody binding by flow cytometry in section 4.3.6.1.

Vi expression by *S.* Typhi was significantly associated with bactericidal activity of anti-Vi antibody in the SBA (r_s= -0.55, p=0.034, 95% CI -0.8357 to -0.04154), with increased expression being associated with increased killing of *S.* Typhi by Vi-specific antibody (Figure 4.7 A). There was a significant negative correlation between Vi expression and anti-O:9-mediated killing in the SBA, in that greater Vi expression was associated with less anti-O:9 mediated killing (r_s= 0.72, p=0.025, 95% CI 0.3142 to 0.9034) (Figure 4.7 C). O:9 availability correlated with increased anti-O:9-mediated killing and decreased anti-Vi-mediated killing in the SBA (r_s=-0.72, p=0.003, 95% CI -0.9034 to -0.3142 and r_s=0.67, p=0.008, 95% CI 0.2215 to 0.8833 respectively) (Figure 4.7 B and D).

These data indicate that binding of anti-Vi antibody to Vi capsule is associated with serum killing. They also suggest that expression of Vi capsule decreases the bactericidal activity of anti-O:9 antibody. Increased levels of O:9 availability correlated with reduced anti-Vi bactericidal activity, most likely due to a reduction in Vi expression, and thus a reduction in the availability of the target Ag, under high NaCl conditions. Anti-O:9 binding was associated with anti-O:9-mediated bactericidal activity in a defined L-

shaped pattern. This most likely reflects a threshold effect suggesting that anti-O:9 becomes highly bactericidal once Vi expression is switched off.

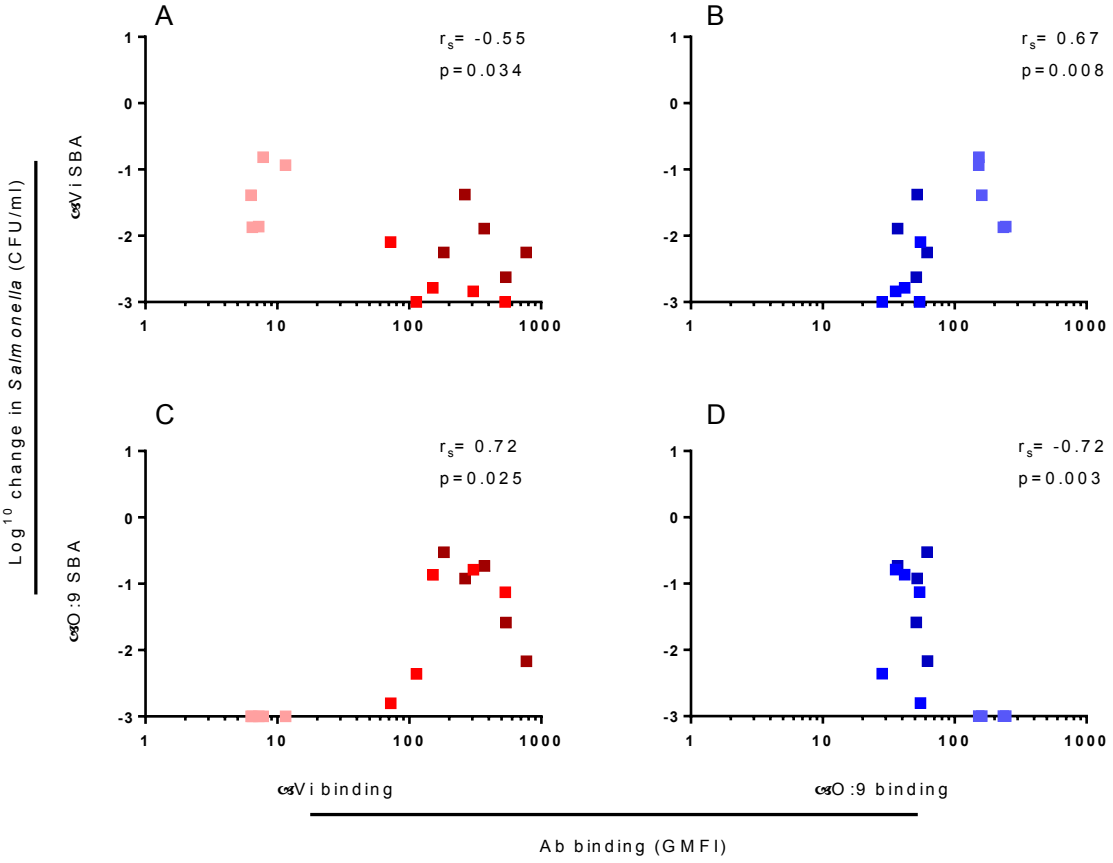


Figure 4.7. Vi expression and O:9 availability were associated with the bactericidal activity of their respective antibodies in the SBA

Killing of the five wild-type *S.* Typhi isolates grown at either 9mM (dark colours), 85mM (intermediate colours) or 500mM (light colours) was plotted against levels of anti-Vi or anti-O:9 antibody binding. Levels of anti-Vi binding and killing by anti-Vi in the SBA (A) or anti-O:9 in the SBA (C) are plotted. Anti-O:9 binding and the killing by anti-Vi (B) or anti-O:9 (D) are also shown. Points represent SBA data plotted against antibody binding data, both from three separate experiments. Each point of one colour represents one of five wild-type *S.* Typhi strains grown in either 9mM, 85mM or 500mM NaCl.

4.3.6.4 Anti-Vi, but not anti-O:9, mediated MAC deposition is associated with bactericidal activity in the presence of Vi capsule

MAC deposition onto the bacterial cell surface is a prerequisite for effective bactericidal activity of sera against *Salmonella.* We wanted to assess how effective anti-Vi and anti-O:9 antibodies were at facilitating MAC deposition onto *S.* Typhi and how this correlated with bactericidal activity of the antibody.

Again, we grew the same 5 wild-type *S.* Typhi as in figure 4.6 (B – D) at 9mM, 85mM and 500mM NaCl and measured MAC deposition, by flow cytometry, onto the surface of *S.* Typhi in the presence of purified hAb. We also assessed MAC deposition onto *S.* Enteritidis and Vi$^-$ *S.* Typhi.

Anti-Vi antibody was effective at facilitating MAC deposition onto the surface of *S.* Typhi. The highest levels of anti-Vi-mediated MAC deposition occurred on *Salmonella* grown at low and medium NaCl concentrations, which corresponds with higher levels of Vi capsule expression. Growth in 500mM NaCl significantly reduced anti-Vi-mediated MAC deposition. No increases in MAC deposition onto the surface of *S.* Enteritidis or Vi$^-$ *S.* Typhi were observed with anti-Vi antibody when compared with the PBS negative control. This indicates that anti-Vi antibody specifically facilitates MAC deposition through binding to Vi capsular polysaccharide, which is lacking in these strains (Figure 4.8 A).

Surprisingly, high levels of anti-O:9-mediated MAC deposition onto *S.* Typhi were observed irrespective of the osmolarity of the growth media, but this is consistent with anti-O:9 antibody binding presented in figure 4.5 (B). This indicates that anti-O:9

antibody can facilitate MAC deposition onto *S.* Typhi regardless of the level of Vi expression. Anti-O:9 mediated MAC deposition was also observed on Vi$^-$ *S.* Typhi and *S.* Enteritidis which confirms the ability of anti-O:9 to mediate MAC deposition onto O:9 expressing *Salmonella* irrespective of the strain and/or serovar (Figure 4.8 B).

To investigate the effect of MAC deposition on cell viability and how this is affected by Vi capsule expression, we plotted serum killing of the five *S.* Typhi isolates grown in varying NaCl concentrations against levels of MAC deposition on the *Salmonella* cell surface. We observed a significant negative correlation between anti-Vi mediated MAC deposition and the bactericidal activity of anti-Vi antibody whereby higher levels of MAC deposition were associated with increased killing of *S.* Typhi (r_s = -0.8 , p=0.0004) (C). In contrast, no correlation between anti-O:9 mediated MAC deposition and the killing of *S.* Typhi was observed (Figure 4.8 D).

These data indicate that the poor bactericidal activity of anti-O:9 antibody against *S.* Typhi is not due to a failure of anti-O:9 antibody to facilitate MAC deposition. Rather, they suggest that in the presence of Vi capsule, anti-O:9 mediated MAC deposition is ineffective at killing the bacteria. Once Vi-capsule expression is reduced or switched off, anti-O:9 mediated MAC deposition becomes productive. This could be due to binding of antibody to proximal or distal O-antigens (dependent on the presence of Vi capsule) which results in differences in killing, even though the binding of antibody and complement directly to Vi capsule results in killing.

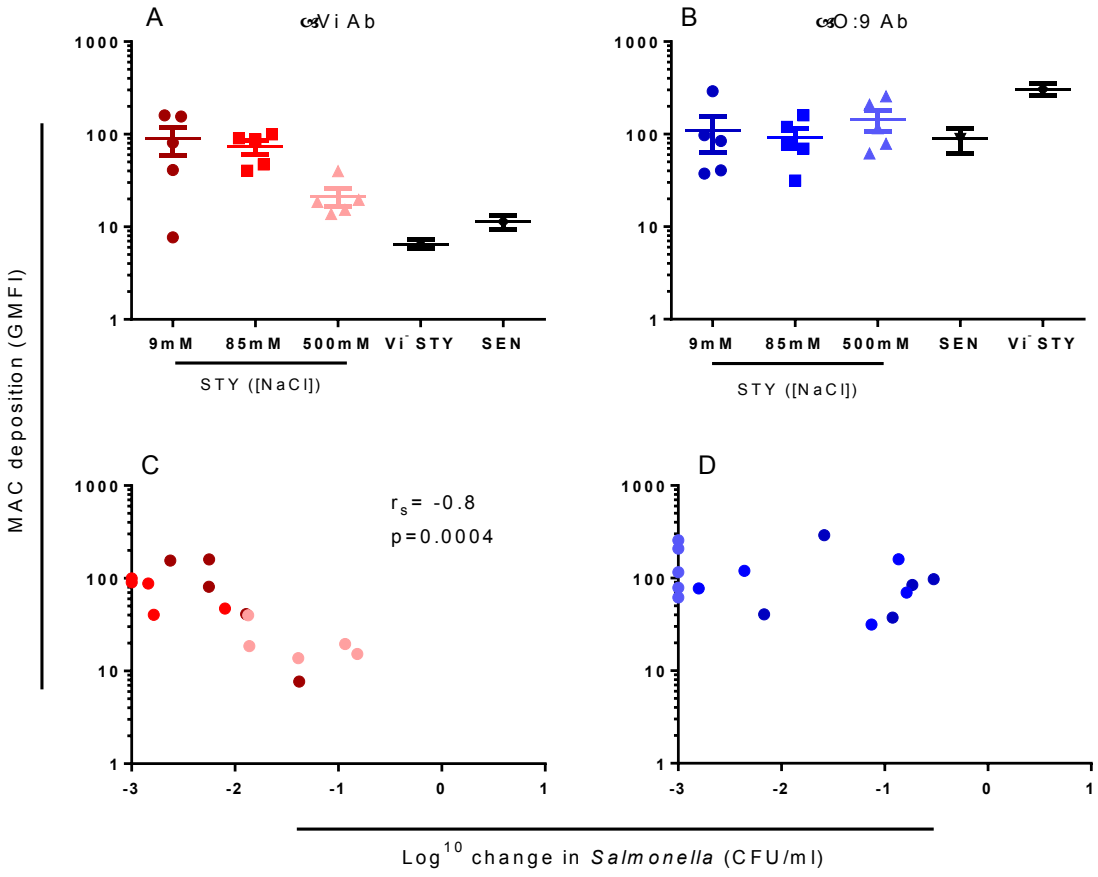


Figure 4.8. Anti-Vi mediated, but not anti-O:9-mediated, MAC deposition is associated with bactericidal activity.

Levels of anti-Vi-mediated MAC deposition onto five wild-type *S.* Typhi isolates grown at 9mM, 85mM or 500mM NaCl and Vi- *S.* Typhi and *S.* Enteritidis were measured (A). Levels of anti-O:9-mediated MAC deposition onto the same strains was also measured (B). We examined the relationship between MAC deposition and SBA activity by plotting the level of anti-Vi-mediated MAC deposition against killing by anti-Vi antibody in the SBA (C) or anti-O:9-mediated MAC deposition against killing by anti-O:9 antibody in the SBA (D). Red dots represent anti-Vi binding, and blue anti-O:9 binding, with the darker the shade corresponding to lower NaCl concentrations. Data in A and B represent mean of three separate experiments +/- SEM.

4.3.6.5 Levels of anti-Vi, but not anti-O:9 binding are associated with increased levels of MAC deposition

Both anti-Vi binding and anti-Vi-mediated MAC deposition were significantly associated with bactericidal activity against *S.* Typhi. To identify whether antibody binding was associated with MAC deposition and thus whether the bactericidal activity of the antibody is due to classical complement pathway activation, we plotted levels of the two variables against one another.

Levels of anti-Vi antibody binding to the surface of *S.* Typhi were significantly associated with levels of MAC deposition onto the cell surface, with higher levels of antibody binding correlating with increased MAC deposition ($r_s=0.71$, $p=0.004$) (Figure 4.9 A). There was no correlation between anti-O:9 binding to the surface of *S.* Typhi and MAC deposition onto the cell (Figure 4.9 B).

The association between anti-Vi binding and MAC deposition suggests that increasing levels of anti-Vi binding to the *Salmonella* surface correspond to greater levels of MAC deposition which could account for the bactericidal activity of anti-Vi antibody. While no correlation was observed between anti-O:9 binding and MAC deposition, no low levels of anti-O:9 binding were observed. This reflects what we have seen previously in that anti-O:9 antibody and MAC are able to bind to the *Salmonella* surface irrespective of the level of Vi-expression.

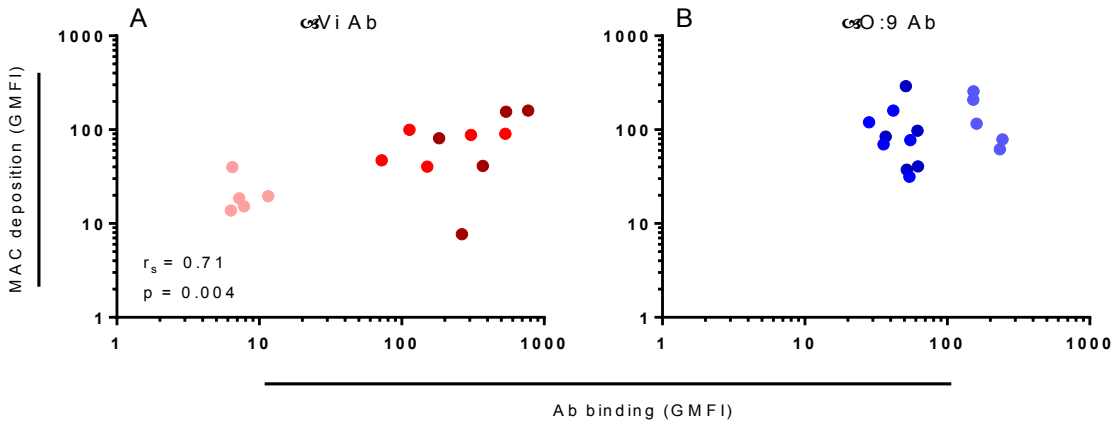


Figure 4.9. Anti-Vi, but not anti-O:9, mediated MAC deposition strongly correlated with antibody binding to *S.* Typhi

Antibody binding to *Salmonella* and levels of MAC deposition on the five wild-type *S.* Typhi grown at 9mM (Dark colours), 85mM (Intermediate) and 500mM (Lightest) NaCl were plotted. Anti-Vi binding to *S.* Typhi and Vi-associated MAC deposition (A) (r_s= 0.71, p=0.004) and anti-O:9 binding and anti-O:9 associated MAC deposition (B) are shown. Data points represent mean of three separate experiments.

4.3.6.6 Human anti-O:9 antibody is an ineffective opsonin of *S*. Typhi when Vi capsule is expressed

We assessed the opsonic potential of the purified hAb against wild-type *S.* Typhi strains. As in the SBAs, we tested the antibody against 14 wild-type isolates grown in ordinary LB broth (85mM NaCl) and 5 isolates (one from each geographical location) grown at 9mM and 500mM NaCl.

When grown in ordinary LB broth (85mM NaCl) all but one *S.* Typhi isolate underwent greater than 1 $\log_{10}$ killing by washed peripheral blood cells after opsonisation with anti-Vi antibody at a 1:4 final dilution after 180 minutes. Dilution of anti-Vi antibody to 1:16 decreased the killing of *S.* Typhi to between 0 and 1.5 $\log_{10}$ (Figure 4.10 A). The five strains chosen for growth in varying concentrations of NaCl also followed this pattern (Figure 4.10 C). Growth of *S.* Typhi in 9mM NaCl increased anti-Vi mediated opsonic killing by washed blood cells to 3 $\log_{10}$ for all but one isolate at a 1:4 dilution of antibody (Figure 4.10 B). Growth in 500mM NaCl reduced the opsonic activity of anti-Vi and isolates opsonised with a 1:4 final dilution of anti-Vi antibody were killed by approximately 1 $\log_{10}$ (Figure 4.10 D).

Anti-O:9 antibody exhibited poor opsonic activity against Vi-expressing *S.* Typhi. When isolates were cultured in 85mM NaCl and opsonised with anti-O:9 antibody, only two out of fourteen isolates underwent killing by peripheral blood cells (Figure 4.10 A). Similarly, when cultured at 9mM NaCl, only two of five isolates underwent killing by blood cells after opsonisation with anti-O:9 antibody (Figure 4.10 B). In contrast, anti-O:9 was a highly effective opsonin when *S*. Typhi was grown at 500mM NaCl. Washed peripheral blood cells effected 3 $\log_{10}$ killing of *S.* Typhi under these conditions when

Salmonella were opsonised with a 1:4 and 1:16 final dilution of anti-O:9. Killing by blood phagocytes was reduced to between -1 - 1 $\log_{10}$ at a 1:1024 final dilution (Figure 4.10 D).

When tested at the lowest dilution of antibody(1:4 final), killing of the fourteen *S.* Typhi strains grown at 85mM NaCl by washed blood cells was significantly lower after opsonisation with anti-Vi than with anti-O:9 antibody (Unpaired t-test, $p<0.0001$) (Figure 4.10 E). When the five wild-type *S.* Typhi strains were grown at 9 and 85mM, opsonisation with anti-Vi antibody resulted in significantly better killing than opsonisation with anti-O:9antibody(Unpaired t-test, $p=0.0036$ and 0.0049 respectively) (Figure 4.10 F and G). In contrast, opsonisation with anti-O:9 when *S.* Typhi was grown at 500mM NaCl resulted in significantly greater killing by blood cells than after opsonisation with anti-Vi antibody (Unpaired t-test, $p<0.0001$) (Figure 4.10 H).

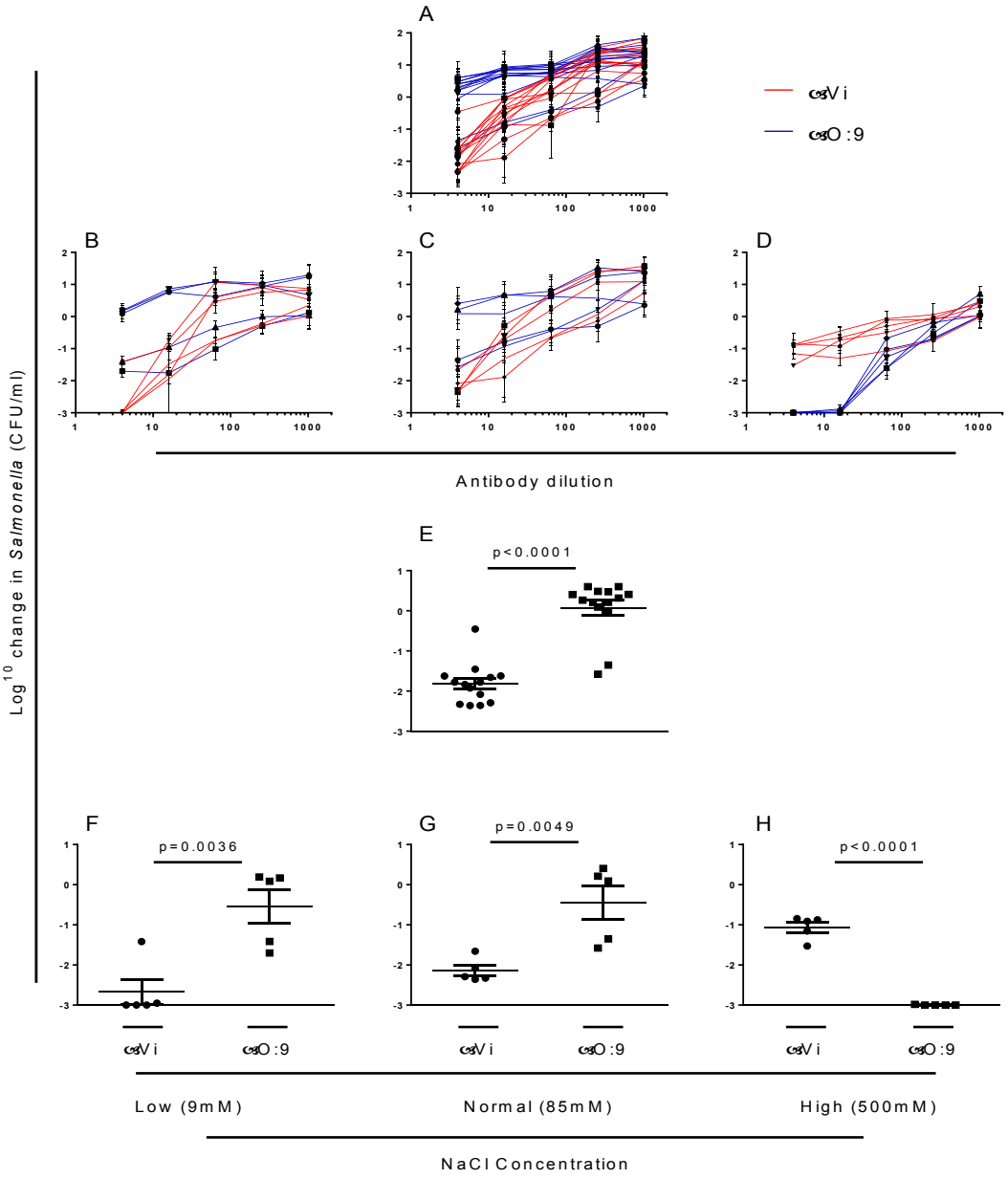


Figure 4.10. Opsonic activity of human anti-Vi and O:9 antibodies against *S.* Typhi expressing different levels of Vi capsule.

Washed peripheral blood cell killing of *S.* Typhi at 180 minutes when grown at different NaCl concentrations and opsonised with anti-Vi antibody or anti-O:9 antibody. Killing of 14 wild-type isolates (A) and 5 of those (C) by washed blood cells after opsonisation with either anti-Vi antibody (red lines) or anti-O:9 antibody (blue lines) in a 1:4 dilution series. The same 5 isolate as in (C) were cultured at 9mM (B) or 500mM (NaCl) and blood cell killing measured after opsonisation with anti-Vi or anti-O:9 antibody. Mean values for A, B, C and D are shown in E, F, G and H respectively. Statistical analysis was done using the Student's t-test. Data represent mean values from three separate experiments +/- SEM.

4.3.6.7 Anti-Vi antibody facilitates phagocytosis of Vi-expressing *Salmonella*

We next investigated how opsonisation with either anti-Vi or anti-O:9 antibody affected the phagocytosis of *Salmonella* by peripheral blood phagocytes and how this related to the data from the blood cell killing assay.

4.3.6.7.1. FITC-labelling of *Salmonella* is unaffected by media osmolarity

FITC-labelled *Salmonella* are used to assess levels of internalised bacteria after phagocytosis has occurred. To ensure FITC-labelling of *Salmonella* in the presence or absence of Vi capsule was equal, we screened FITC labelled *S.* Typhi by flow cytometry prior to use in the phagocytosis assay. No differences in levels of FITC-labelling were detected between *S.* Typhi cultured in 9mM, 85mM or 500mM NaCl when groups were compared by one way ANOVA ($p=0.97$) (Figure 4.11 A)

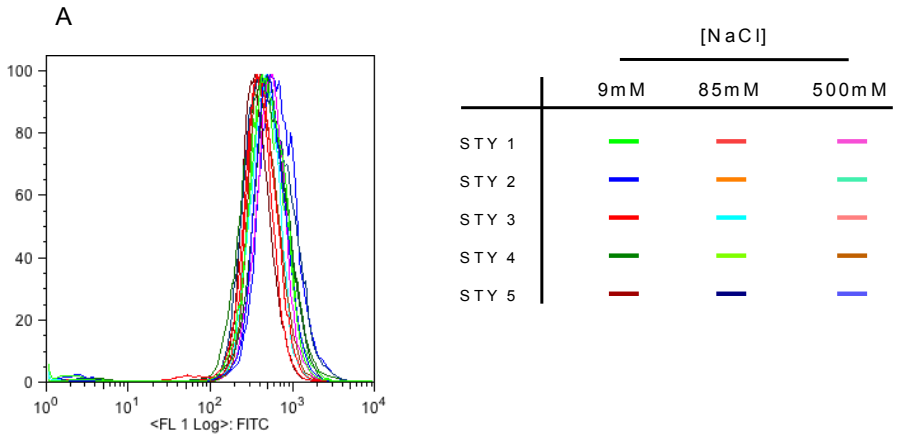


Figure 4.11. FITC-labelling is not different between *S.* Typhi cultured at different concentrations of NaCl.

Growth of *S.* Typhi in different concentrations of NaCl had no effect on resulting FITC-labelling of strains (one way ANOVA, p=0.97). Data are representative of one experiment. Each line represents one wild-type *S.* Typhi grown at 9mM, 85mM or 500mM NaCl.

4.3.6.8 Opsonisation of FITC-labelled *S.* Typhi with anti-Vi antibody increases the phagocyte GMFI and the percentage of phagocytosing cells compared to opsonisation with anti-O:9 Ab

Opsonisation with anti-Vi antibody increased the GMFI of human monocytes and neutrophils when *Salmonella* were grown in 9mM or 85mM NaCl suggesting anti-Vi opsonisation enhances *Salmonella* uptake and internalisation. When grown at 500mM NaCl, anti-Vi antibody opsonisation resulted in a small, but significant (unpaired t-test, $p<0.0001$) increase in the phagocyte GMFI compared to when *S.* Typhi was opsonised with complement alone. Opsonisation with anti-O:9 antibody only led to a small increase in the levels of internalised *Salmonella* within neutrophils or monocytes compared to opsonisation with complement alone but led to a marked difference in the percentage of phagocytosing cells. The concentration of sodium chloride in the growth media had no effect on levels of internalised *S.* Typhi after opsonisation with anti-O:9 antibody (Figure 4.12 A and B).

Opsonisation of *S.* Typhi with anti-Vi antibody increased the percentage of phagocytosing neutrophils and monocytes when *Salmonella* were grown at 9mM and 85mM NaCl in a concentration dependent manner. Opsonisation with anti-O:9 antibody did increase the percentage of phagocytosing cells, but this was not concentration dependent, and the lowest percentage of phagocytosing cells occurred when *Salmonella* were grown at medium NaCl concentrations. The percentage of phagocytosing monocytes and neutrophils after opsonisation with anti-O:9 antibody was highest when *Salmonella* were cultured in 500mM NaCl (Figure 4.12 C and D).

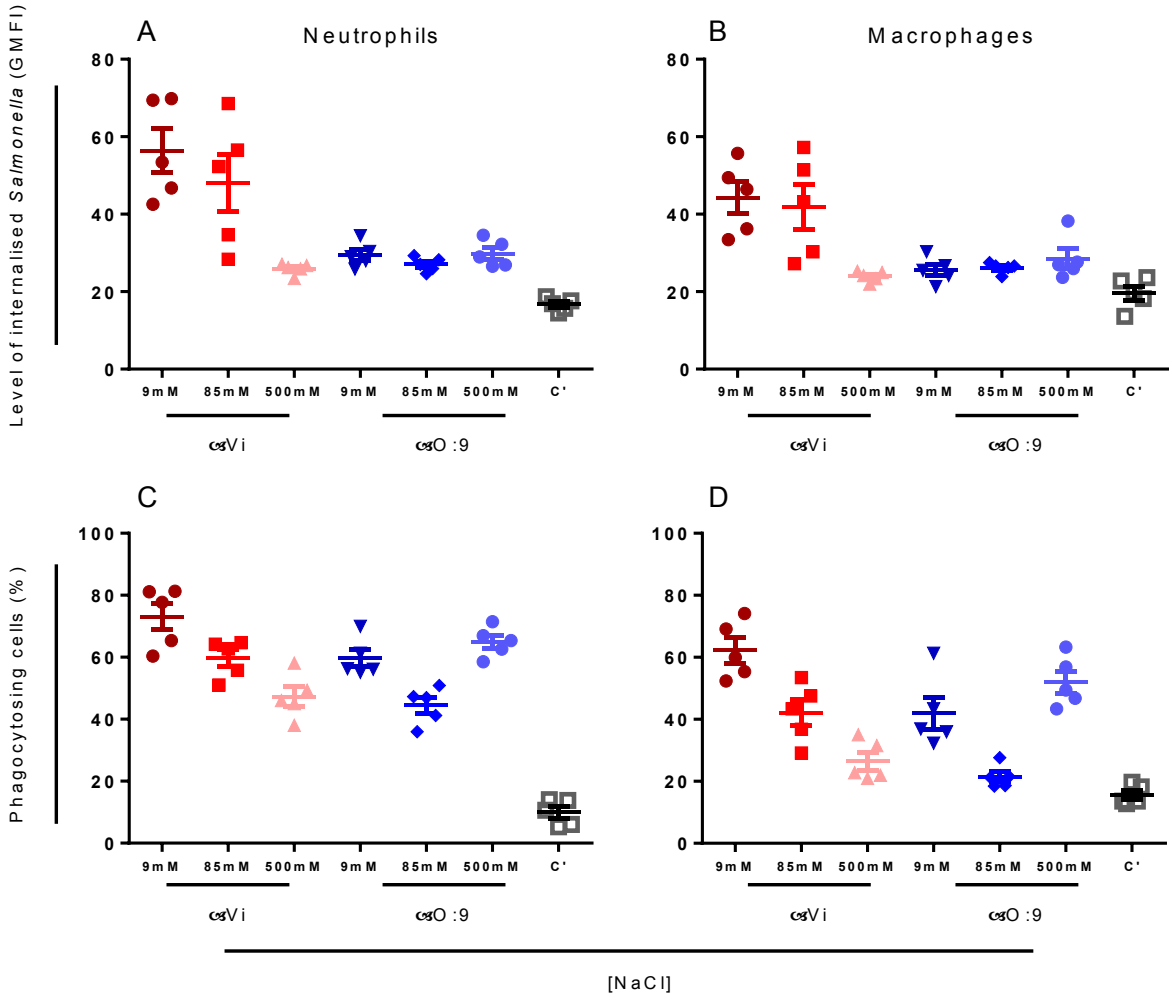


Figure 4.12. The effect of opsonisation with anti-Vi and anti-O:9 antibody on the internalisation of *Salmonella* and the percentage of phagocytosing monocytes and neutrophils.

The GMFI of monocytes and neutrophils was measured as an indicator of internalised FITC-labelled *Salmonella* and the percentage of phagocytosing cells recorded. Five wild-type *S.* Typhi isolates were cultured in 9mM (darkest colour), 85mM (intermediate colour) and 500mM (lightest colour) NaCl and opsonised with either anti-Vi (red dots) or anti-O:9 (blue dots) antibody. The internalisation of opsonised *Salmonella* by either neutrophils (A) or macrophages (B) was recorded. The percentage of neutrophils (C) and monocytes (D) phagocytosing opsonised *Salmonella* was also recorded. Levels of phagocytosis of *Salmonella* after opsonisation with Complement alone (C') are shown. Statistical analysis was done using a Student's t-test and data are mean of three separate experiments +/- SEM.

4.3.6.9 Levels of phagocytosis and internalisation of *Salmonella* by monocytes and neutrophils are associated with blood cell killing

To examine the extent that phagocytosis of *S.* Typhi accounts for the results seen in the blood cell killing assay we plotted killing in the assay against levels of internalised *Salmonella* and the percentage of phagocytosing cells.

There were significant correlations between the levels of neutrophil and monocyte (r_s= -0.83, p=0.0002 and r_s= -0.77, p=0.001 respectively) internalised *S.* Typhi after opsonisation with anti-Vi and anti-Vi antibody-mediated killing in the blood cell killing assay, with higher levels of internalised *S.* Typhi corresponding to greater killing by peripheral blood phagocytes (Figure 4.13 A and C). The percentage of neutrophils and monocytes which were phagocytosing *S.* Typhi after opsonisation with anti-Vi antibody was also significantly correlated with killing in the blood cell killing assay (r_s= -0.76, p=0.0017 and r_s= -0.81, p=0.0004 respectively) (Figure 4.13 E and G).

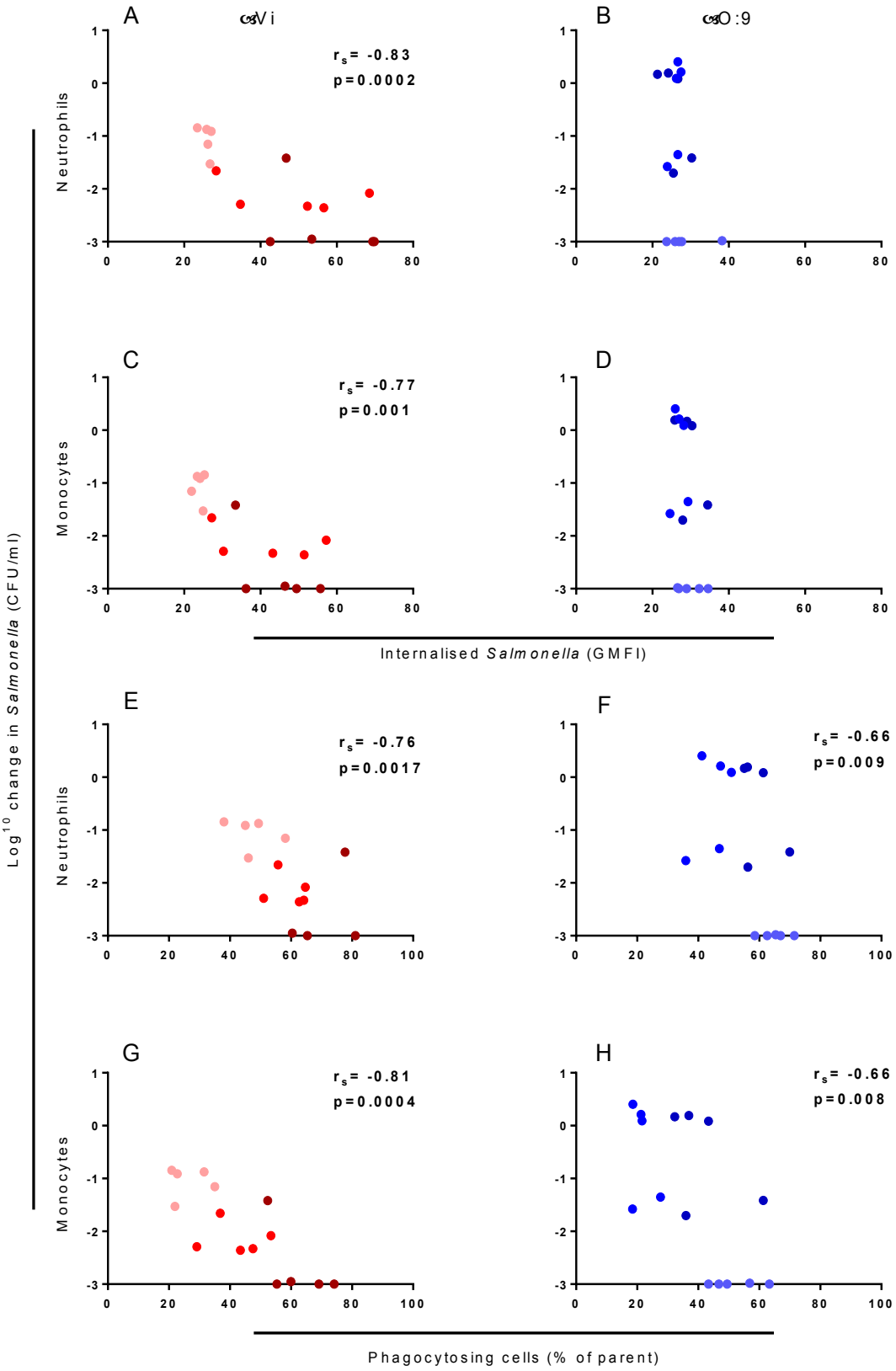
There were no correlations between the level of internalised anti-O:9-opsonsied *S.* Typhi and killing by blood cells (Figure 4.13 B and D). However, there was a significant correlation between the percentage of neutrophils and monocytes phagocytosing *S.* Typhi after opsonisation with anti-O:9 antibody and killing (r_s= -0.66, p=0.009 and r_s= -0.66, p=0.008 respectively) (Figure 4.13 F and H).

These data suggest that anti-Vi enhances killing by peripheral blood phagocytes by increasing both the internalisation of *S.* Typhi and the percentage of cells undertaking phagocytosis. In contrast, opsonisation with anti-O:9 antibody does not affect the levels

of internalised *S.* Typhi, at any concentration of NaCl, by neutrophils and monocytes. It is, however, associated with an increased percentage of phagocytosing monocytes and neutrophils compared to opsonisation of *S.* Typhi with complement alone.

Figure 4.13. Levels of internalised *Salmonella* and the percentage of phagocytosing cells correlates with killing activity by peripheral blood cells after opsonisation of *S.* Typhi with anti-Vi antibody.

There were significant associations between levels of internalised *S.* Typhi within monocytes and neutrophils and killing by peripheral blood cells after opsonisation of *Salmonella* with anti-Vi antibody (r_s= -0.83, p=0.0002 and r_s= -0.77, p=0.001 respectively). The percentage of phagocytosing monocytes and neutrophils was also associated with killing by blood cells for anti-Vi opsonised *S*. Typhi (r_s= -0.76, p=0.0017 and r_s= -0.81, p=0.0004 respectively). There was no association between internalised anti-O:9 opsonised *Salmonella* and killing, but there was between percentage of phagocytosing cells and killing (r_s= -0.66, p=0.009 and r_s= -0.66, p=0.008 respectively). Red and blue dots represent anti-Vi and anti-O:9 opsonised *S.* Typhi respectively. Dark colours represent lower NaCl concentrations.


A
αVi
r_s = -0.83
p=0.0002
B
αO:9
C
r_s = -0.77
p=0.001
D
E
r_s = -0.76
p=0.0017
F
r_s = -0.66
p=0.009
G
r_s = -0.81
p=0.0004
H
r_s = -0.66
p=0.008
Neutrophils
Monocytes
Log10 change in Salmonella (CFU/ml)
Internalised Salmonella (GMFI)
Phagocytosing cells (% of parent)

4.3.6.10 Depletion of anti-Vi antibody from whole human serum reduces killing of Vi-expressing *Salmonella*

Whilst studies have examined the effect of adsorption of anti-Vi antibody from rabbit and mouse sera on the killing of *S.* Typhi, the same has not been tested using human sera.

Having characterised the bactericidal activity of purified, human anti-Vi antibody against *S.* Typhi, we wanted to investigate the effect of removal of anti-Vi antibody on bactericidal activity of human sera against Vi-expressing *Salmonella.* To do this we used two isogenic $Vi^{+/-}$ pairs of *S.* Typhi and *S.* Typhimurium to remove specific antibody from human sera and examine the resulting effect on serum killing.

We used human serum from donor 1 (Table 3.1) since this serum contains IgG and IgM antibody to O:4,5, O:9 and Vi and is able to kill all four *Salmonella* strains. Prior to using the serum in SBAs, specific anti-*Salmonella* antibody in fresh serum from was adsorbed by incubation at 4°C with either Vi^{+} or Vi^{-} *S.* Typhimurium, or Vi^{+} or Vi^{-} *S.* Typhi.

In serum adsorbed with either Vi^{+} *Salmonella* strain there was a reduction in anti-Vi IgG and IgM to undetectable levels by ELISA (Figure 4.14 A and B). Similar reduction of anti-O:9 IgG and IgM levels occurred when serum was incubated with either *S*. Typhi strain (which express O:9), with a partial reduction in anti-O:9 IgG and IgM when incubated with both *S*. Typhimurium strains. Serum incubated with both *S*. Typhimurium strains underwent a large reduction in anti-LPS O:4,5 IgG levels, but some was still detectable, and a complete reduction in anti-LPS O:4,5 IgM levels. Anti-LPS O:4,5 IgG levels fell to a limited degree when serum was incubated with the *S*. Typhi strains

Fresh and antibody-depleted sera were then tested against the four *Salmonella* strains in the SBA. Sera adsorbed with each isolate were unable to kill the homologous isolate, with the exception of Vi$^-$ *S*. Typhi, where killing was significantly reduced at 45 and 90 min compared with unadsorbed serum (Unpaired t-test, $p<0.0001$ and 0.004 respectively) (Figure 4.14 C – F). This is consistent with a requirement for specific-Ab for C-mediated killing of each *Salmonella* strain, apart from Vi$^-$ *S*. Typhi which is sensitive to complement in the absence of antibody.

Adsorbed sera were able to kill heterologous strains, whether heterologous by serovar or Vi expression. Although sera depleted of non-Vi *Salmonella* antibody by adsorption with Vi$^-$ *Salmonellae* were unable to, or had reduced ability to kill their homologous strain, these sera could still effect significant killing of the Vi$^+$ *Salmonella* strains of the same serovar compared to adsorbed sera alone at 180 min (unpaired t-test, p=0.005 and <0.0001 for *S.* Typhimurium and *S.* Typhi respectively). This is consistent with antibody to Vi activating C-mediated killing. Hence, while expression of Vi increases general resistance to complement deposition and C-mediated killing, it renders *Salmonellae* susceptible to killing in the presence of Vi-specific antibody. Similarly, while sera adsorbed with the Vi$^+$ *Salmonella* strains, were unable to kill the homologous Vi$^+$ strain, they could still effect significant killing of the Vi$^+$ *Salmonella* strain of the heterologous serovar at 180 min (unpaired t-test, $p=0.018$ and 0.0003 for *S.* Typhimurium and *S.* Typhi respectively). This is consistent with killing of Vi$^+$ *Salmonellae* by non-Vi *Salmonella* antibody and suggests that the Vi capsule does not fully prevent access of non-Vi antibody to their targets.

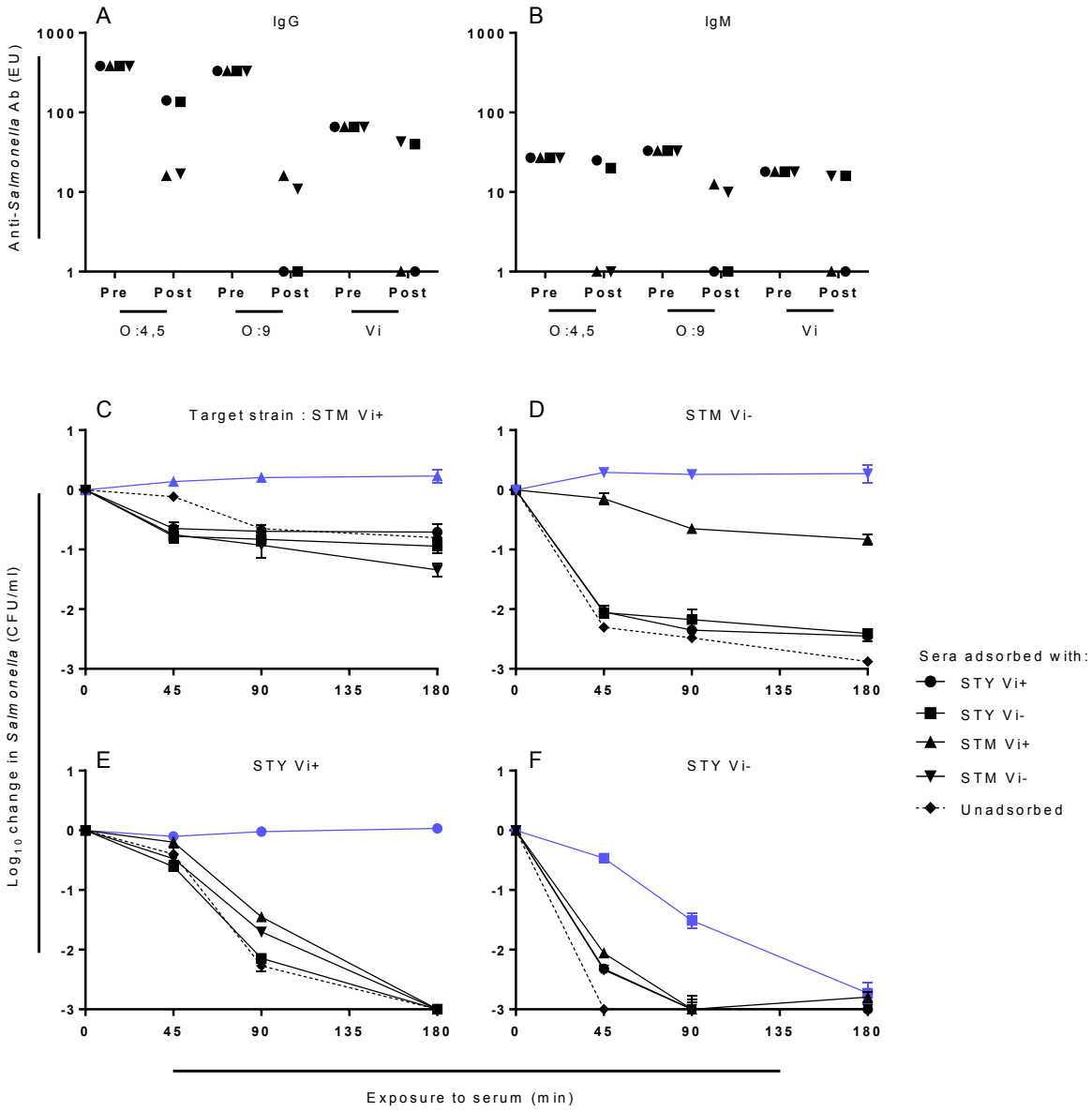


Figure 4.14. Depletion of anti-Vi antibody from human serum reduces killing of Vi-expressing *Salmonella*

Sera depleted of *Salmonella*-specific antibody lose their ability to kill the homologous *Salmonella* strain (Blue line). Levels of IgG (A), and IgM (B) against the *Salmonella* Ag LPS O:4,5, LPS O:9 and ViPS measured by ELISA in serum from donor 1 before and after adsorption with Vi^+ and Vi^- *S.* Typhi and *S.* Typhimurium. Killing of (C) Vi^+ and (D) Vi^- *S.* Typhimurium and (E) Vi^+ and (F) Vi^- *S.* Typhi by serum D1 adsorbed with Vi^+ and Vi^- *S.* Typhimurium and *S.* Typhi. Statistical analysis was done using a Student's t-test. Data are mean of two separate experiments +/- SEM. Initial concentration of bacteria in assay 10^6 CFU/ml.

4.3.6.11 Animal sera containing anti-Vi antibody kills Vi-expressing *Salmonella* in the presence of human complement

Multiple studies have investigated the bactericidal activity of immunized animal sera against *S.* Typhi (Osawa and Muschel, 1964, Ahmadi et al., 2013, Muschel and Treffers, 1956). However, none of these have compared anti-sera activity against isogenic Vi-*Salmonella* or the effect of anti-sera on a nontyphoidal Vi-expressing *Salmonella* strain. To further examine the role of anti-Vi Ab, we used sera from rabbits and mice immunized with a newly developed conjugate typhoid vaccine - Vi-CRM_{197}. Sera were tested for their bactericidal activity, using human serum depleted of *Salmonella*-specific antibody as a source of complement.

A 1:4 final concentration of mouse serum containing anti-Vi Ab, mixed with human sera depleted of antibody by adsorption with Vi^+ *S*. Typhimurium, exerted a bacteriostatic effect on the growth of Vi^+ *S*. Typhimurium (Figure 4.15 A). Rabbit serum containing anti-Vi antibody mixed at a 1:4 final concentration with human serum adsorbed with Vi^+ *S*. Typhimurium, killed Vi^+ *S.* Typhimurium by 0.5 $\log_{10}$ (Figure 4.15 B).

Mouse and rabbit immune sera, mixed with human C, were able to effect a 1 and 1.5 $\log_{10}$ kill respectively of Vi^+ *S*. Typhi at a 1:40 final dilution (Figure 4.15 C and D). A 1:4 final dilution of rabbit serum combined with human complement effected greater than a 2 $\log_{10}$ kill, similar to that seen with fresh serum (Figure 4.15 D). To ensure that the observed bactericidal activity of the animal sera was a consequence of anti-Vi Ab, the

experiments were tested using the isogenic Vi$^-$ *Salmonella* strains as target strains. Neither the mouse nor rabbit sera were able to kill Vi$^-$ *S*. Typhimurium in the presence of human complement (Figure 4.15 E and F). The addition of mouse or rabbit anti-Vi antibody did not increase the killing of Vi$^-$ *S*. Typhi by complement alone (Figure 4.15 G and H).

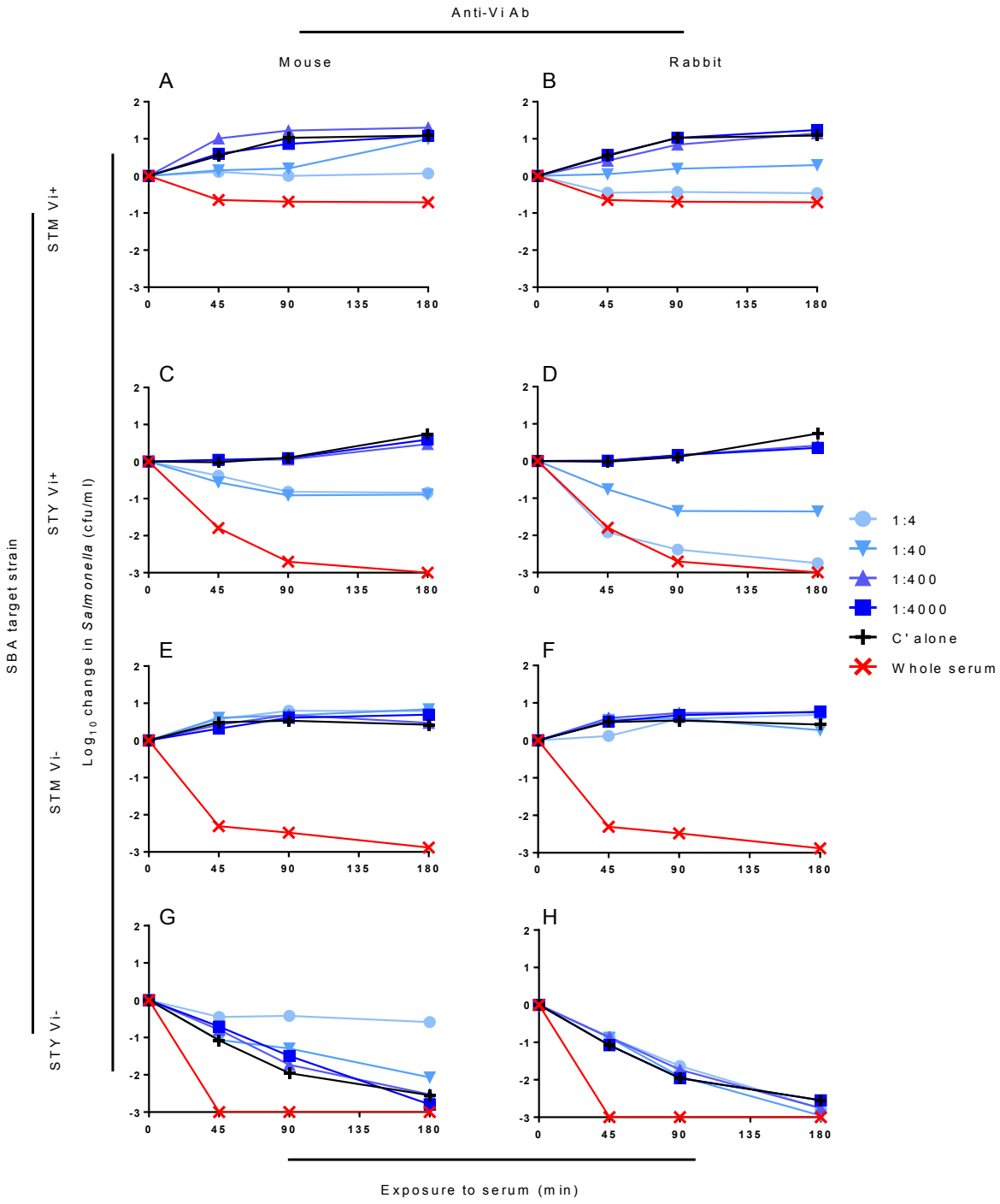


Figure 4.15. Sera from mice and rabbits immunised with a Vi conjugate vaccine, Vicrm197, effects killing of Vi-expressing *Salmonella*

Animal Anti-Vi antibody effects cell-free C-mediated killing of Vi^+ *Salmonella*. Killing of (A, B) Vi^+ *S*. Typhimurium, (C, D) Vi^+ *S*. Typhi, (E,F) Vi^- *S*. Typhimurium, (G, H) Vi^- *S*. Typhi by polyclonal antibody from mice (A, C, E, G) and rabbits (B, D, F, H) immunized with Vi-CRM_{197} supplemented with human complement. Initial concentration of bacteria in assay 10^6 CFU/ml.

4.4. Discussion

We have shown that human anti-Vi antibody effects bactericidal activity against Vi-expressing *Salmonella.* Although Vi capsule acts to reduce antibody and complement deposition (shown in section 3.3.3), Vi-specific antibody overcomes this immune evasion, and anti-Vi-mediated MAC deposition is strongly associated with C'-mediated cell-free killing of Vi^+ *S.* Typhi, even under conditions of high capsule expression.

In switching off capsular expression, we show that Vi^- *S.* Typhi is highly sensitive to the bactericidal action of anti-O:9 Ab, but less sensitive to anti-Vi antibody. This observation suggests that O:9 vaccines should protect against Vi^- strains of STY. The near universal presence of anti-*S.* Typhimurium O-Ag antibody has been documented in a relatively "clean" sample of a population in the USA (Trebicka et al., 2013). *S.* Enteritidis is one of the most prevalent serovars of NTS globally (Galanis et al., 2006), thus it is highly likely that circulating anti-O:9 are also present in a large proportion of the population, and indeed we see this in a collection of 500 sera from healthy African adults (MacLennan CA et al., unpublished data). Due to the sensitivity of Vi- *S.* Typhi to anti-O:9, it would seem unlikely that Vi^- *S.* Typhi strains will arise in nature, unless *S.* Typhi evolves adaptations to compensate for sensitivity to C'-mediated killing which accompanies the loss of capsular expression. Genetic changes in outer membrane proteins, *rck* genes, O-Ag chain length and other virulence factors associated with serum survival could be monitored to detect such a development.

There is a paucity of data examining the relationship between anti-O:9 titre and typhoid susceptibility. Here we show that anti-O:9 antibody is able to effect a degree of serum killing of *S.* Typhi even under conditions of capsule expression. This observation has

vaccinology implications, particularly given the development of O:9 O-antigen conjugate vaccines for use against O:9-expressing *Salmonella*. The relationship between anti-O:9 antibody titres and susceptibility to *S.* Typhi is something which warrants further study.

Interestingly, we observed moderate levels of anti-O:9 binding to *S.* Typhi, even at low NaCl concentrations which induce high levels of Vi expression. This was surprising as Vi^+ *S.* Typhi do not typically agglutinate or take longer to agglutinate with O:9-specific agglutinating antibody. It is possible a positive agglutination reaction requires a threshold level of binding/ or for the agglutinating antibody to bind in a certain way. Capsular expression at low NaCl concentrations may prevent this from happening, and when capsule expression is downregulated at 500mM NaCl, more anti-O:9 is able to bind which could lead to a positive agglutination result.

The observation that anti-O:9 antibody binds *S.* Typhi when capsule is expressed suggests some O:9 OAg epitopes are accessible under these conditions. O-antigen may extend beyond the Vi capsule, but binding of antibody to this distal O-antigen appears ineffective at eliciting both modes of killing. In accordance with this, we also observed anti-O:9 mediated MAC deposition onto *S.* Typhi, independent of media osmolarity. This indicates that anti-O:9 antibody is still able to facilitate MAC deposition onto Vi^+ *S.* Typhi. However, anti-O:9-mediated MAC deposition was not associated with serum killing or with anti-O:9antibody binding. *S.* Typhi was only killed by anti-O:9 antibody at 500mM NaCl when Vi capsule expression was low. These data suggest that Vi-expression may enhance bacterial survival by preventing productive anti-O:9-mediated MAC deposition.

The live attenuated vaccine strain, Ty21a, induces good immunity to *S.* Typhi despite lacking Vi capsule. This illustrates the protective potential of non-anti-Vi antibody in

immunity to *S.* Typhi. It is thought that Vi capsule is not expressed in the intestinal lumen due to high osmolarity (Tran et al., 2010). As such, any local antibody response, with antibacterial activity, is likely to be anti-O:9 IgA mediated (Wahid et al., 2012). The current project did not examine the role of anti-*S.* Typhi IgA due to its relatively paucity in serum. Extraction of mucosal anti-O:9 IgA from saliva, and subsequent testing of its antibacterial properties against *S.* Typhi would be a viable undertaking.

Newer live attenuated Vi^+ vaccines are being developed for oral delivery. As yet, these have been unable to stimulate adequate serum anti-Vi antibody responses but have succeeded at stimulating mucosal anti-Vi IgA responses (Tacket et al., 2004). Our data suggest production of mucosal anti-Vi responses in the absence of a serum response is unlikely to provide protection against typhoid infection, as we observed a reduction in the bactericidal activity of anti-Vi antibody at high osmolarity, such as that seen in the intestinal lumen. The most effective vaccine is likely to be one which stimulates solely mucosal anti-O:9 antibody responses in conjunction with serum anti-Vi responses. This would complement the tropism of Vi capsule expression within the host during typhoid infection.

Killing of *S.* Typhi by peripheral blood cells was similar to killing in the serum bactericidal assays, in that anti-Vi elicited better killing than anti-O:9 antibody when capsule was expressed, with this effect being reversed at 500mM NaCl. However, the O-9 mediated killing of *S.* Typhi at 500mM NaCl was not reflected in the phagocytosis, in terms of the levels of internalised *Salmonella,* but anti-O:9 opsonisation of *S.* Typhi grown at 500mM NaCl did increase and the percentage of phagocytosing cells compared with the negative control. The blood cell killing assay measures the killing of *S.* Typhi by

peripheral blood cells and results in this assay reflect multiple facets of cell-mediated immunity in particular phagocytosis and oxidative burst. Thus, the anti-O:9-mediated killing of *S.* Typhi at 500mM NaCl in the blood cell assay likely represents a combination of increased anti-O:9 binding but also may reflect the absence of Vi-mediated resistance to intracellular killing mechanisms such as the oxidative burst (Looney and Steigbigel, 1986).

Affinity chromatography was an effective way of purifying specific-human antibody. We found no evidence for impurities within the anti-Vi antibody preparation and only minor contamination of the anti-O:9 preparation with other immunodominant anti-*Salmonella* antibody. We did not measure the affinity of the eluted antibody compared to that of loaded antibody and it is possible that the elution buffers only remove low-affinity antibody from the columns. Even if this were the case, we still observed effective antibody-mediated bactericidal and opsonic activity with both purified antibody preparations, which suggests the affinity of eluted antibody binding is not an issue. An interesting observation was the efficacy of MgCl in the elution of anti-Vi antibody whereas glycine pH 2.4 was much more effective in the elution of anti-O:9 antibody. This may reflect the nature of bonds behind the respective antibody-antigen interactions, i.e. anti-Vi binding to Vi Ag may be based upon ionic forces or it could reflect differences in the affinities of the purified anti-Vi and anti-O:9antibodyto their antigens.

We noted that the isogenic Vi^- *S.* Typhi strain used in the antibody depletion and animal sera studies was susceptible to complement alone, and that antibody depleted sera was still able to kill this strain. This was not the case with the wild-type STY isolates when grown at 500mM NaCl, as these were not susceptible to complement alone. This likely

reflects inherent differences in susceptibility to complement between the lab strain and wild-type isolates. Moreover the lab strain was genetically engineered to lack the viaB locus and thus lacks the genetic machinery necessary to synthesize Vi capsule. In contrast, Vi-expression was merely suppressed in the wild-type strains. Also, due to the low osmolarity of human sera and/or blood, it is possible expression of Vi expression may be upregulated by the wild-type *S.* Typhi strains during assays using live *Salmonella.*

Adsorption of human serum with either Vi^+ *Salmonella* strain effectively removed all anti-Vi IgG and the ability to kill the homologous strain of *Salmonella* was lost. This is consistent with an important role for anti-Vi antibody in cell-free, C-mediated killing of *S*. Typhi. However, the killing of the heterologous Vi-expressing *Salmonella* was not lost. This indicates a role for antibody against other antigenic targets in C-mediated killing of *Salmonella*, even if access of these antibodies is reduced by the presence of the Vi capsule. Such antibodies are likely to include the O-Ag of LPS (Robbins et al., 1992) and outer membrane proteins (Secundino et al., 2006) and reflects what we have seen in our own work with regards to the bactericidal activity of anti-O:9antibodyagainst *S.* Typhi. It has previously been shown that the Vi capsule, when expressed by *S*. Typhimurium as described here, reduces, but does not fully remove, protection mediated by antibody to *Salmonella* porins (Marshall et al., 2012).

We used antibody induced in mice and rabbits immunized with a Vi-protein conjugate vaccine, Vi-CRM_{197}, to examine the bactericidal activity of anti-Vi antibody in further detail. The results from these experiments confirm that anti-Vi antibody have

bactericidal activity against Vi-expressing strains of *Salmonella*, irrespective of serovar. Addition of heat-inactivated sera from animals immunized with Vi-CRM_{197} to human sera adsorbed with either Vi^+ *S.* Typhimurium or Vi^+ *S.* Typhi is able to restore the killing of Vi-expressing *Salmonellae*. The bactericidal effect of anti-Vi antibody was more pronounced for *S.* Typhi than *S.* Typhimurium. This could be anticipated by the greater inherent sensitivity to C-mediated killing of *S*. Typhi compared with *S*. Typhimurium, together with the higher level of expression of Vi on our Vi^+ *S*. Typhi compared with the Vi^+ *S*. Typhimurium. As expected, animal serum containing anti-Vi antibody was unable to kill Vi^- *S.* Typhimurium or increase the killing of Vi^- *S.* Typhi by complement alone.

4.5. Conclusions

The findings from this study show that human both human anti-Vi antibody and anti-O:9 antibody have bactericidal activity against wild-type *S.* Typhi. Under conditions of capsule expression anti-Vi antibody effects greater C'-mediated killing of *S.* Typhi than anti-O:9 Ab, but these effects are reversed when *S.* Typhi are grown at high osmolarity and capsular expression is switched off. Anti-Vi antibody is also an effective opsonin of *S.* Typhi, leading to cell-mediated killing of *S.* Typhi by peripheral blood phagocytes. Anti-O:9 however, is a poor opsonin of *S.* Typhi when Vi capsule is expressed. We found that although Vi-capsule reduces anti-O:9 binding, it does not diminish it completely and anti-O:9 is still able to bind Vi^+ *S.* Typhi.

Anti-O:9-mediated MAC deposition also occurred onto *S.* Typhi despite capsule expression but did not kill *S.* Typhi unless capsule expression was downregulated. The

use of antibody depletion studies and immunised animal sera confirm the bactericidal role of both anti-Vi and other antibody in the killing of *S.* Typhi.

5. The effects of HIV infection on the immune response to *Salmonella*

5.1. Introduction

Nontyphoidal *Salmonella* are the leading cause of bloodstream infections (BSIs) in HIV-infected children and adults in Africa and Asia. HIV infection is associated with a significantly greater incidence of NTS BSIs (OR 6.42 – 8.2, p<0.0001, 95% CI, 3.51 – 11.73) (Huson et al., 2014, Reddy et al., 2010) and disease is typically severe and recurrent, with a high mortality rate (Gordon et al., 2002). In contrast, the incidence of typhoid in HIV-infected individuals in Africa is significantly lower than in healthy Africans (OR 0.07 – 0.12, p<0.0001, 95% CI, 0.03 – 0.49) (Reddy et al., 2010, Crump et al., 2011) and relatively few cases of concomitant infections have been reported (Manfredi et al., 1998).

The reasons behind this disparity in *Salmonella* infections in the context of HIV infection are unclear. Dysregulated cytokine and antibody responses and the disruption of the gut mucosa in HIV-infected individuals may be responsible for increased susceptibility of the host to NTS infection (MacLennan et al., 2010, Raffatellu et al., 2008, Gordon et al., 2007) but no evidence has been found to explain the apparent protective effect of HIV infection on typhoid.

Antibiotic prophylaxis in HIV-infected individuals has been suggested as a mechanism for the relative paucity of typhoid fever cases in the context of HIV infection (Crump et al., 2011). As discussed in section 1.6.2, in many of the study areas both *S.* Typhi and NTS exhibit similar levels of antibiotic resistance. If prophylaxis were the cause of the

reduction in HIV-associated typhoid, one would expect to see a similar effect for NTS, but this is not the case. Moreover, studies documenting this phenomenon report low adherence, of HIV-infected patients, to antibiotic prophylaxis (Crump et al., 2011), further undermining this hypothesis as a mechanism for the disparity in HIV associated *S.* Typhi and NTS infections.

It has also been suggested that a hospital selection bias, whereby patients with both HIV infection and typhoid succumb to illness prior to reaching hospital. However, when it does happen, concomitant typhoid-HIV infection does not appear to be more severe than typhoid in HIV-uninfected individuals. This suggests that a hospital selection bias is not responsible for the difference in *Salmonella* infections in the context of HIV infection (Crump et al., 2011, Manfredi et al., 1998).

Alternatively, a defective cell-mediated immune response in HIV infection has been proposed to be beneficial in *S.* Typhi infection by sequestering typhoid bacilli within the intracellular niche and preventing extracellular spread (Levine and Farag, 2011). While *S.* Typhi is able to survive within the intracellular niche inside the SCV (Waterman and Holden, 2003), it is also able to spread extracellularly, colonising secondary lymphoid organs. Defective lysis of *Salmonella* infected host cells by $CD8^+$ T cells in HIV infection may therefore abrogate infection by preventing extracellular spread of bacteria. However, even in the absence of intact cell-mediated immunity, phagocytes are able to kill intracellular bacteria via the oxidative burst and through the release of antimicrobial compounds such as defensins and lysozymes. Moreover, *Salmonella* replicate intracellularly and, upon reaching a threshold limit of numbers, induce caspase-1 and 3-mediated apoptosis of phagocytes (Chanana et al., 2007, Hersh et al., 1999), thereby

being liberated from the intracellular niche. Additionally, the lack of an increase in typhoid fever, upon successful ART treatment of HIV-infected patients, does not support this idea (Gordon, 2011, Feasey et al., 2014).

MacLennan et al observed that a subset of HIV-infected Africans produce high-titre anti-*S.* Typhimurium O-chain antibodies and exhibit HIV-associated hypergammaglobulinemia. It is thought that these antibody inhibit the killing of *S.* Typhimurium by blocking or diverting the access of complement and bactericidal antibody to *Salmonella* (MacLennan et al., 2010). *S.* Typhi expresses Vi capsular polysaccharide and we found that Vi-expression levels are negatively associated with O-chain antibody deposition (section 4.3.6.1). Masking of available O-chain epitopes by the Vi capsule or capsule induced steric hindrance of anti-O-antigen antibody binding may prevent such an effect occurring with *S.* Typhi. The effect, if any, of HIV infection on antibody titres to immunogenic *S.* Typhi antigens is unclear. If high titre anti-*S.* Typhi antibodies are produced in HIV infection, could these be protective? Alternatively, could the dysregulated cytokine milieu in macrophages from HIV-infected adults, noted by *Gordon et al*, be detrimental to the host in NTS infection but protective against typhoid fever? Whilst cytokines play an important role in the resolution of *Salmonella* infection, HIV infection results in extensive dysregulation of the host cytokine environment (Kedzierska and Crowe, 2001) and elevated and/or abrogated cytokine responses can have negative effects on disease outcome.

Evidence suggests the prevalence of *S.* Typhi is increasing in areas of Tanzania and Malawi, where it had previously been low or absent (Feasey et al., 2013, Biggs et al., 2014). This has been accompanied by a decrease in HIV infections. If there is a negative

association between the two infections and areas of Africa begin to bring HIV transmission under control, simultaneous typhoid vaccination in these areas may be warranted. In this chapter we investigate the role of antibody from HIV-infected and uninfected African adults in the bactericidal and opsonic killing of *S.* Typhi. We also characterised the cytokine response of whole blood from HIV-infected and healthy adults to different typhoidal and nontyphoidal *Salmonella* serovars.

5.2. *Aims*

The aim of this work was to compare the bactericidal and opsonic killing of *S.* Typhi by HIV-infected and control African sera to see if we could identify *in vitro,* humoral mechanisms accounting for the apparent protective effect of HIV infection on typhoid fever. The cytokine response of HIV-infected and healthy whole blood to *Salmonella* stimulation was investigated to establish whether the response differs according to HIV-status and *Salmonella* serovar.

5.3. Results

5.3.1. Wild-type Tanzania *Salmonella* isolates from both HIV-infected and healthy Tanzanian adults show a range of sensitivities to serum killing

First we wanted to establish whether *Salmonella* isolates from HIV-infected individuals were different, in terms of their serum sensitivity, to strains infecting healthy controls. To do this we tested a collection of wild-type *Salmonella* isolates, including 14 *S.* Typhi and 138 *S.* Typhimurium, from two sites in Tanzania in SBAs using a standardised human serum; pooled serum from healthy Malawian adults (PMS). Bacterial numbers were recorded at 45, 90 and 180 minutes after serum exposure. This enabled us to assess whether *Salmonella* isolates from HIV-infected patients are more susceptible to serum killing than isolates from healthy individuals but, due to defects in humoral immunity, thrive in the context of HIV infection.

All *Salmonella* isolates tested were killed to some extent after exposure to PMS, but nontyphoidal strains (Figure 5.1 A) showed greater variation and inherent resistance in their level of killing than *S.* Typhi (Figure 5.1 B) (unpaired t-test, p=0.001). 8% of NTS strains were classed as moderately serum resistant, undergoing between 0 to -1 $\log_{10}$ kill at 180 minutes (Figure 5.1 A). All *S.* Typhi strains were killed by 3 $\log_{10}$ at the same time point (Figure 5.1 B). We also examined the variability in serum killing of the two serovars by plotting the number of isolates and their corresponding $\log_{10}$ decrease in bacterial numbers at 180 or 90 minutes for NTS and *S.* Typhi respectively (Figure 5.1 C and D). Strains isolated from HIV-infected individuals are shown in red. These data show that *Salmonella* strains isolated from HIV-infected individuals are not similar to other strains of the same serovar isolated from HIV-infected individuals in terms of their

serum sensitivity. NTS strains infecting HIV-infected individuals displayed a broad range of sensitivities to serum killing. There were too few *S.* Typhi strains to arrive at the same conclusion as this isolate collection had only a limited number of *S.* Typhi isolates and only 2 of these were isolated from individuals with HIV infection. However, the few *S.* Typhi isolates we did have showed some variation in their susceptibility to serum killing.

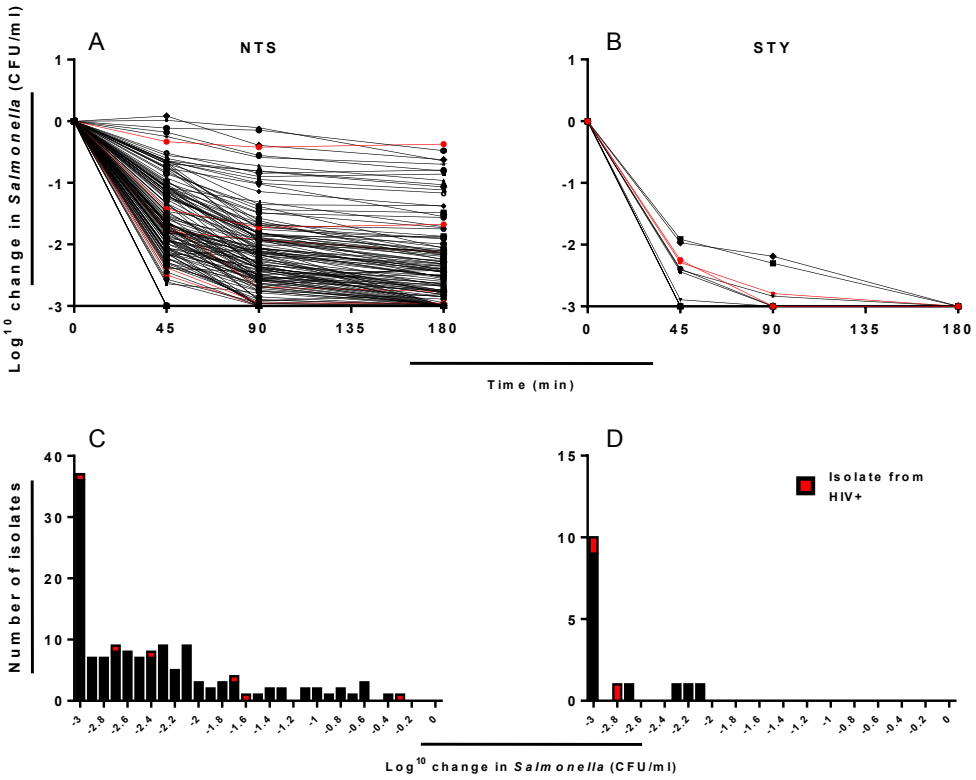


Figure 5.1. Different sensitivities of *S.* Typhi and *S.* Typhimurium Tanzanian isolates from HIV-infected, and HIV-uninfected, individuals to killing with pooled-Malawian serum (PMS)

The serum sensitivities of Tanzanian *Salmonella* isolates were measured in the SBA. The bactericidal activity of PMS against 6 NTS isolates from HIV-infected individuals (red lines) and 138 from HIV-uninfected individuals (black lines) was measured at 45, 90 and 180 minutes after serum exposure (A). The killing of 2 *S.* Typhi isolates from HIV-infected (red lines) and 12 isolates from HIV-uninfected individuals (black lines) in the same SBA was also measured at 45, 90 and 180 minutes (B). The serum sensitivity profiles of either NTS (C) or *S.* Typhi (D) were visualised by plotting the number of isolates against their corresponding kill in the SBA, with isolates from HIV-infected individuals represented as a red box (one per isolate). Initial *Salmonella* concentration was $1x10^6$ CFU/ml. Negative values show a decrease in viable *Salmonella* compared to the starting concentration. Each line represents one *Salmonella* isolate (A and B). Statistical analysis comparing HIV-infected and uninfected groups was done using a Student's t-test.

5.3.2. Serum and plasma from HIV-infected and uninfected Tanzanian Adults and children have a mixed ability to kill *S.* Typhimurium D23580

We did not observe a shared serum resistance phenotype in *Salmonella* isolates from individuals with HIV infection, suggesting that these isolates were similar to *Salmonella* isolates from HIV-uninfected individuals. However, low numbers of *S.* Typhi isolates limit this observation to NTS. In addition to a collection of Tanzanian wild-type *Salmonella* isolates, we had access to a collection of sera and plasma samples from HIV-infected and uninfected Tanzanians. This allowed us to investigate whether humoral activity against *Salmonella* and explore whether this was affected by HIV status of the donor. However, the status of the Tanzanian blood product samples, in terms of whether they were sera or plasma, was poorly defined.

The bactericidal activity of plasma is reduced or absent compared to that of serum due to the effect of the anti-coagulant on proteins of the complement system. Anticoagulants such as EDTA and Heparin abrogate complement activity either by binding directly to complement proteins such as factor H (heparin), or through the chelation of divalent metal cations required for functional complement activity (EDTA) (Baker et al., 1975, Almeda et al., 1983, Levine et al., 1953). The type of anticoagulant used determines the extent of the impairment of complement activity, with EDTA being the more potent inhibitor of the two (Mollnes et al., 1988).

Before testing the serum bactericidal activity of HIV-infected samples against *S.* Typhi, we needed to establish whether the Tanzanian samples were sera or plasma and whether they were able to kill *Salmonella* in the SBA. To do this we tested the undiluted, freshly thawed, Tanzanian samples against a representative African *S.* Typhimurium

strain – D23580. The aim being to identify which samples were plasma and which were sera by their ability to kill D23580. It is possible to identify the presence of EDTA in a sample by capillary electrophoresis. However, automated electrophoresis requires approximately 200µl of sample. For some of the Tanzanian samples we had less than 100µl of sample in total, thus capillary electrophoresis is not a viable method of determining sera or plasma status.

27 Tanzanian samples designated as serum samples effected between 1 – 3 $\log_{10}$ killing of *S.* Typhimurium D23580 at 180 minutes. 16 sera samples however, were unable to effect killing and in these sera D23580 experienced 0 – 1.5 $\log_{10}$ growth (Figure 5.2 A). The majority of plasma samples displayed relatively uniform killing of D23580, effecting between 0 – 2 $\log_{10}$ killing of D23580 by 180 minutes. 2 samples labelled as plasma killed D23580 by 3 $\log_{10}$ after 90 minutes (Figure 5.2 B). Fluid samples of unknown designation displayed a broad range of killing of D23580 (Figure 5.2 C). Some were able to kill D23580 by >2 $\log_{10}$ after 90 minutes and these might be sera. A number of samples gave a dense band of killing between -0.5 and -2 $\log_{10}$ similar to that seen with the plasma samples. A small number of samples were unable to kill D23580 resulting in 0 – 1 $\log_{10}$ growth by 180 minutes.

Serum and plasma samples from HIV-infected individuals displayed a range of killing or growth of D23580 (Figure 5.2 D). Only two HIV-infected sera were able to kill D23580 while the rest could not (yellow lines). HIV-infected plasma samples (blue lines) gave ~ 1 $\log_{10}$ kill of D23580 similar to that seen with the HIV-uninfected plasma samples. HIV-infected samples of unknown designation (green lines) displayed mixed ability to kill D23580.

The initial aim of this experiment was to identify sera or plasma samples based on their bactericidal activity against *S.* Typhimurium D23580 in the SBA. However, it is not possible to differentiate sera and plasma samples based on their bactericidal activity because the level of killing effected by sera samples overlapped that of plasma samples. The data also show that some serum samples were completely unable to kill D23580. A lack or excess of antibody and complement within these sera may underlie this absence in bactericidal activity and without assessing the complement function and/or antibody titres it would be difficult to determine the causes of the lack of killing in these samples.

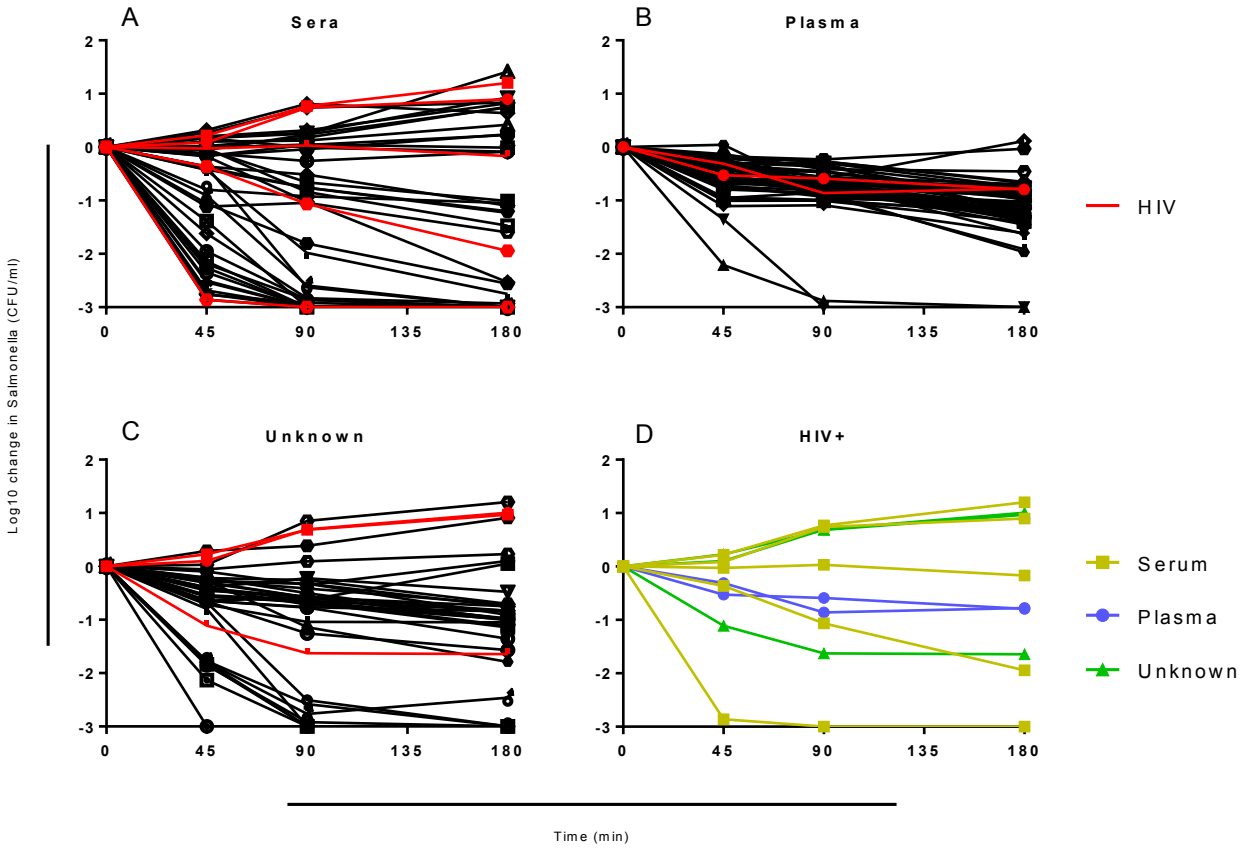


Figure 5.2 Bactericidal activity of sera and plasma from HIV-infected and uninfected Tanzanians against *S.* Typhimurium D23580.

Tanzanian samples labelled as sera (A), plasma (B) and samples of unknown sera/plasma status (C) from HIV-infected (red line, A – C) and uninfected Tanzanians were tested in the SBA against the invasive, wild-type *S.* Typhimurium isolate, D23580. Sera (yellow line), plasma (blue line) and samples of unknown sera/plasma status (green line) from HIV-infected individuals were also tested against D23580 (D). Numbers of viable *Salmonella* were calculated at 45, 90 and 180 minutes. Initial concentration of *Salmonella* was $1x10^6$ CFU/ml. Each line represents sera or plasma from one individual donor.

5.3.3. Addition of divalent cations restores complement activity of human plasma

It is possible to restore complement activity in EDTA plasma samples with the addition of exogenous divalent Calcium and Magnesium cations (Merchant et al., 2005). To use Tanzanian plasma samples in SBAs it was important to determine the correct concentration of divalent Ca^{2+} and Mg^{2+} cations required to restore complement and bactericidal activity to that of serum. To do this we venesected three healthy adults and took serum and plasma samples from each. Ca^{2+} and Mg^{2+} cations were titrated into the plasma and the bactericidal activity of supplemented plasma against *S*. Typhimurium D23580 was compared to that of the homologous serum in the SBA after 180 minutes.

For all donors, the addition of divalent cations was able to restore the bactericidal activity of plasma. However, the optimal concentration required to restore killing varied from donor to donor. For donor 1, 2.5-5 mM Mg^{2+} and 1-5 mM Ca^{2+} restored the bactericidal activity of plasma in excess of that of the homologous serum (Figure 5.3 A). Only 5mM Ca^{2+} and 2.5mM Mg^{2+} was able to restore killing activity of plasma from donor 2 (Figure 5.3 B) and only a combination of 5mM Ca^{2+}, 1mM Mg^{2+} or 1mM Ca^{2+}, 2.5mM Mg^{2+} restored bactericidal activity of plasma from donor 3 (Figure 5.3 C). These data show that it is possible to restore functional complement activity of human plasma with exogenous divalent Calcium and Magnesium yet they also highlight the issue that the optimal concentration of cations varies for different sera. Moreover, in the case of donor 1, addition of Calcium and Magnesium resulted in killing in excess of that of homologous serum. These data indicate that using divalent cations is not a viable method of restoring complement function to plasma. Whist effective, the optimal concentration of Ca^{2+} and Mg^{2+} required to restore killing for each plasma sample is

unknown and without having comparable donor sera samples, we do not know if the restored level of killing represents the physiological level of killing.

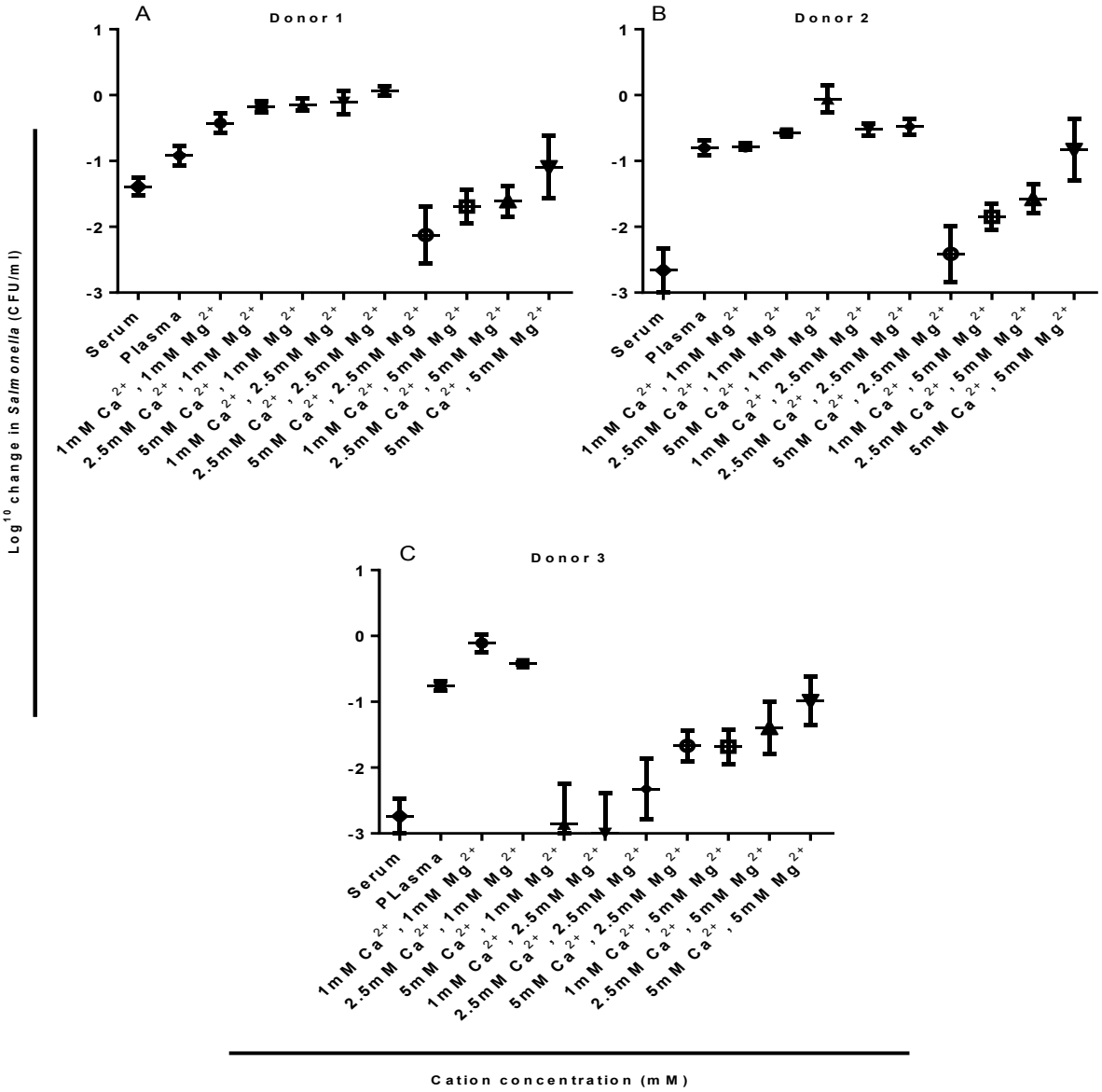


Figure 5.3 Restoration of the bactericidal activity of human plasma with the addition of exogenous divalent cations.

We assessed whether complement-mediated bactericidal activity of plasma could be restored with the addition of exogenous divalent Magnesium and Calcium cations. $MgCl_2$ and $CaCl_2$ were titrated into plasma from three donors; donor 1 (A), donor 2 (B) and donor 3 (C) and the killing of *S.* Typhimurium D23580 by supplemented plasma was measured and compared to killing effected by homologous donor serum or plasma. Data represent mean SBA values taken at 180 minutes +/- SEM from three separate experiments.

5.3.4. Sera from HIV-infected African adults has impaired bactericidal killing of *S.* Typhi compared to healthy controls

The problems associated with the Tanzanian sera and plasma collection, in that they were poorly labelled and a substantial number were plasma samples, meant that these samples were ill-suited for assessing whether humoral responses against *Salmonella* differ according to the HIV status of the donor. To test the involvement of antibody from HIV-infected and uninfected individuals in the bactericidal and opsonic killing of *S.* Typhi better, we used a serum collection from HIV-infected adults and healthy controls from Malawi. These sera were from a larger number of HIV-infected adults and controls and were known to have functional complement activity (MacLennan et al., 2010). Using a representative African *S.* Typhi strain, ISP-04-06979, we assessed the bactericidal activity of 149 HIV-infected and 19 HIV-uninfected sera in SBAs at 40, 90 and 180 minutes.

Sera from HIV-infected adults displayed a mixed ability to kill *S.* Typhi with 5 out of the 149 sera being unable to kill and the majority of sera effecting between 2-3 $\log_{10}$ kill after 180 minutes (Figure 5.4 A). Sera from HIV-uninfected Malawians showed some variability in killing however all but one serum effected greater than 2 $\log_{10}$ killing of *S.* Typhi with the majority effecting a 3 $\log_{10}$ kill after 180 minutes (Figure 5.4 B). When compared as groups, sera from healthy controls effected significantly greater killing than sera from HIV-infected adults after 45 (Figure 5.4 C), 90 (D) and 180 (E) minutes (unpaired t-test, p=0.005, 0.007 and 0.046 respectively) (Figure 5.4 C). Instead of suggesting any kind of protective effect of HIV infection against *S.* Typhi, these data

indicate that HIV infection significantly impairs the bactericidal activity of African sera against *S.* Typhi compared to that of healthy controls.

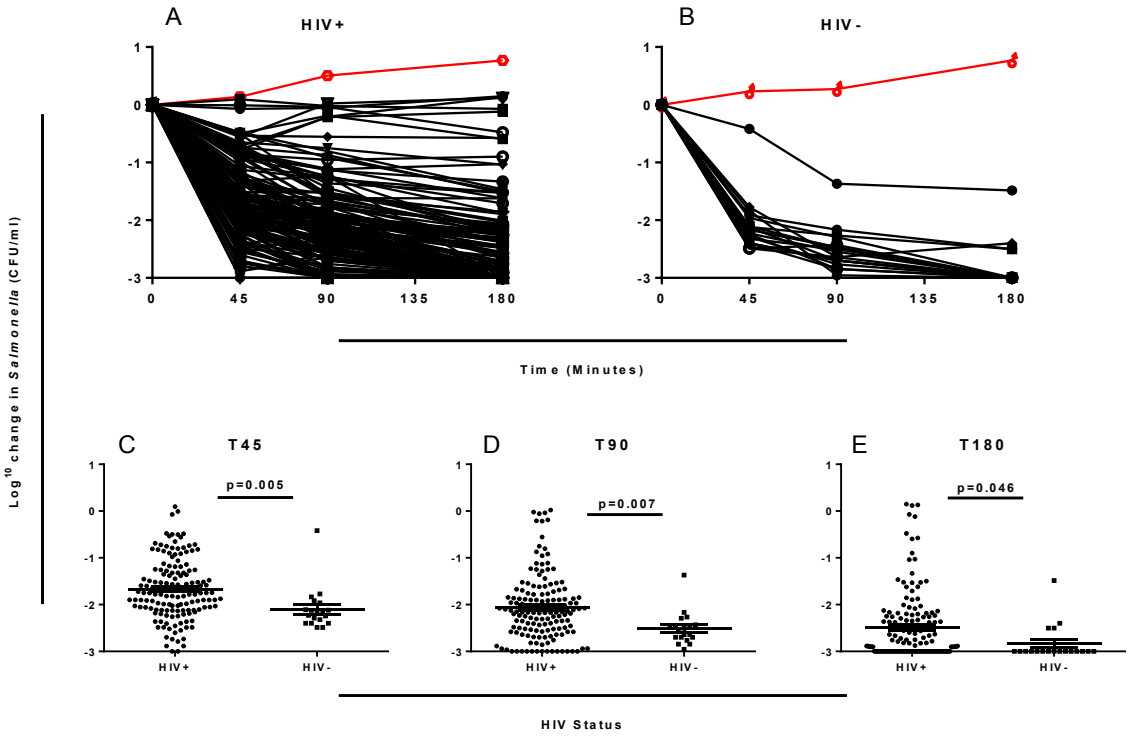


Figure 5.4 Killing of *S.* Typhi by HIV-infected sera is reduced compared to killing by sera from healthy African adults.

The ability of HIV-infected (A) and uninfected (B) Malawian sera and heat-inactivated sera (red lines) to kill wild-type *S.* Typhi ISP-04-06979 was tested in the SBA. The number of viable *Salmonella* was calculated, and killing of *S.* Typhi by HIV-infected and uninfected sera compared, at 45 (C), 90 (D) and 180 (E) minutes. Initial concentration of *Salmonella* was $1x10^6$ CFU/ml. Groups were compared statistically using a Student's t-test and each line represents data for serum from one individual. Bars in (C-E) represent mean +/- SEM.

5.3.5. Killing of *S.* Typhi by washed peripheral blood cells is reduced following opsonisation with HIV-infected sera compared to control sera

Anti-*Salmonella* antibody contributes to the killing of *Salmonella* either by effecting bactericidal activity or acting as an opsonin, facilitating phagocytosis. To assess whether the difference between *S.* Typhimurium and *S.* Typhi infections in the context of HIV infection were due to differences in the activity of opsonic antibody, we opsonised *S.* Typhi ISP-04-06979 with HIV-infected and healthy Malawian sera and observed killing of *Salmonella* by washed peripheral blood cells. We opsonised *S.* Typhi with 28 HIV-infected and 14 control sera with the highest and lowest anti-O:9, O:4,5 and Vi titres and measured the killing in the blood cell killing assay.

Similarly to the SBA results, opsonisation with HIV-infected sera resulted in a wide range of killing of *S.* Typhi by washed peripheral blood cells after 180 minutes (Figure 5.5 A). Two HIV-infected sera had absent opsonic activity and *S.* Typhi was not killed by peripheral blood cells following opsonisation with these sera. Opsonisation with the remainder of the HIV-infected sera did result in blood cell killing of *S.* Typhi and this ranged from 0.5 – 3 $\log_{10}$ after 180 minutes. All of the control sera had highly effective opsonic activity, effecting >2 $\log_{10}$ reduction in the number of *S.* Typhi by washed blood cells after 180 minutes (Figure 5.5 B). Opsonisation of *S.* Typhi with control sera resulted in significantly greater killing by blood cells at 45 (Figure 5.5 C) and 180 minutes (E) (unpaired t-test, p=0.003 for both) but not at 90 minutes (D) compared to opsonisation with HIV-infected sera. *S.* Typhi was not killed when opsonised with serum then added to RPMI (red line) or when opsonised with PBS and added to blood (blue

line). These data suggest that HIV infection has a negative impact on the opsonic activity of African sera.

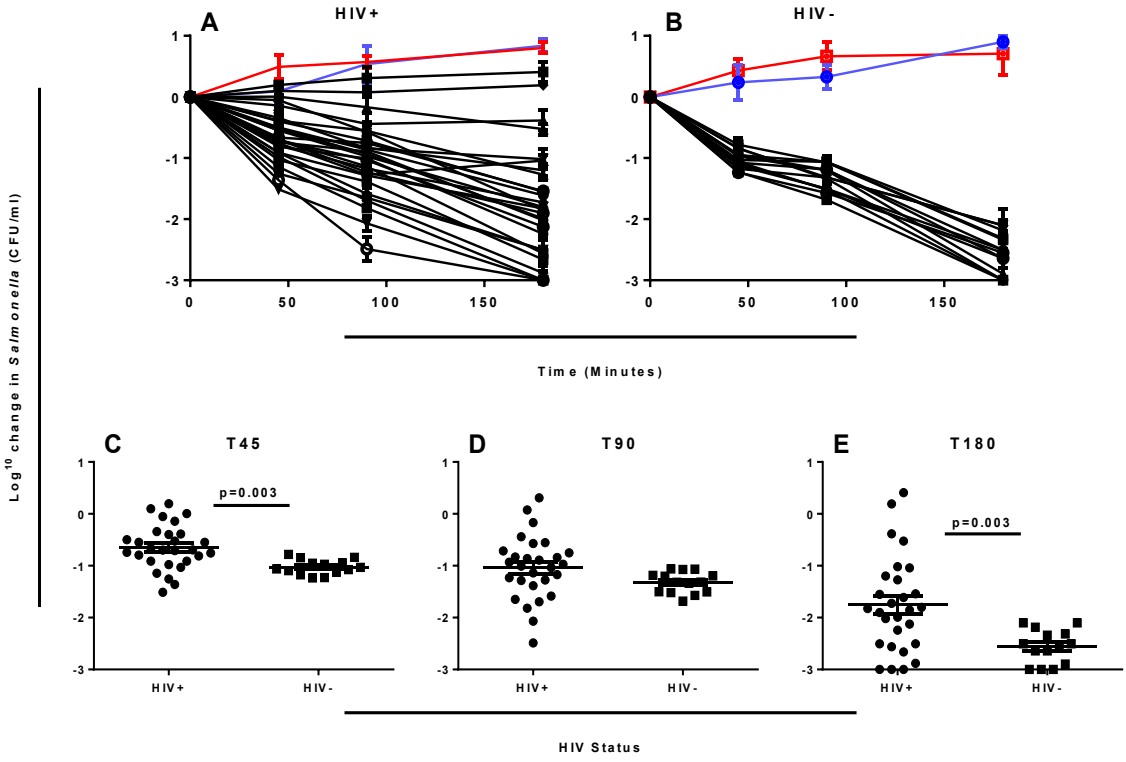


Figure 5.5 Blood cell killing of *S.* Typhi after opsonisation with HIV-infected sera is impaired compared to killing after opsonisation with healthy sera.

The killing of *S.* Typhi ISP-04-06979 after opsonisation with HIV-infected (A) and uninfected (B) Malawian sera was assessed in the blood cell killing assay. The number of viable *Salmonella* was calculated at 45 (C), 90 (D) and 180 (E) minutes. Serum opsonised *Salmonella* added to RPMI (red line) or PBS-opsonised *Salmonella* added to washed blood (blue line) were included as negative controls. Initial concentration of *Salmonella* was $1x10^6$ CFU/ml. Each line represents data for serum from one individual. Statistical analysis was done using a Student's t-test. Bars in (C-E) represent mean +/- SEM.

5.3.6. Sera from HIV-infected individuals had significantly higher anti-O:4,5 IgA titres, and lower anti-Vi, O:9 or O:4,5 IgM titres compared with HIV-uninfected sera

Both antibody and complement are required for bactericidal and opsonic activity of sera against *Salmonella* (Gondwe et al., 2010). The HIV-infected and control Malawian sera had already been tested and found to have functional complement activity (MacLennan et al., 2010). To examine whether a lack or excess of anti-*Salmonella* antibody were responsible for the differences in killing by HIV-infected sera in the SBA and BCK we tested all Malawian sera for anti-Vi, O:9 or O:4,5 IgG, A and M titres by ELISA.

When compared as a whole, no significant differences were found between anti-IgG titres against each of the three antigens; Vi (Figure 5.6 A), O:9 (B) and O:4,5 (C). There was no significant difference in the levels of anti-Vi or anti-O:9 IgA between HIV-infected and uninfected sera, but levels of anti-O:4,5 IgA were significantly higher in HIV-infected individuals (MW test, p=0.009). IgM titres against Vi (Figure 5.6 A), O:9 (B) and O:4,5 (C) were significantly lower in HIV-infected sera compared with HIV-uninfected sera (MW test, p=0.005, 0.026 and 0.004 respectively). Median IgA titres for both the HIV and control groups were lower than IgG and IgM for all three antigens, which is not surprising given the relative paucity of IgA in human serum. A small proportion of sera from HIV-infected individuals did have very high levels of anti-O:9 and O:4,5 IgG and A but not IgM compared to the control sera. These data suggest that the impaired bactericidal or opsonic activity of HIV-infected sera against *S.* Typhi is not due to a lack of IgG against Vi or O:9, but could be due to a lack of anti-Vi or anti-O:9 IgM, or the presence of elevated anti-O:4,5 IgA titres.

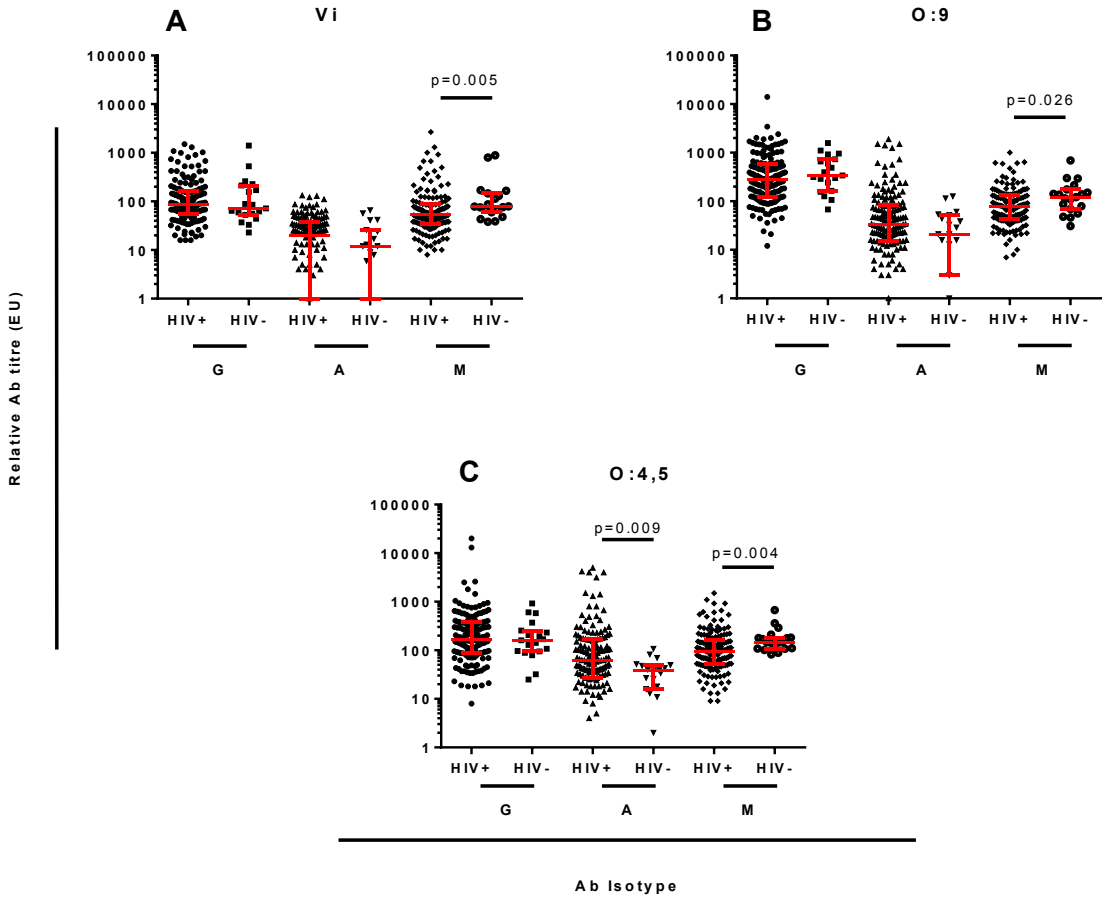


Figure 5.6 Levels of anti-Vi, O:4,5 and O:9 IgG, A and M in HIV-infected and uninfected African sera.

Levels of anti-*Salmonella* antibody in 149 HIV-infected and 19 HIV-uninfected Malawian sera were assayed by ELISA. Anti-Vi IgG, IgA and IgM (A), anti-O:9 IgG, IgA and IgM (B) and anti-O:4,5 IgG, IgA and IgM titres (C) are shown. Statistical analysis was done using the Mann-Whitney U-test. Data are from one experiment. Lines represent median and interquartile range.

5.3.7. Relationship between anti-*Salmonella* antibody titres and the killing of *S.* Typhi in the SBA

5.3.7.1 HIV Sera

We observed elevated anti-O-Ag antibodies in a subset of the HIV-infected Malawian sera and reduced IgM titres to all three antigens. To examine the relationship between anti-*Salmonella* antibody titres and the bactericidal activity of the HIV-infected and uninfected Malawian sera, we compared levels of anti-Vi, O:9 and O:4,5 IgG, IgA and IgM with the killing of *S.* Typhi in the SBA.

At 45 minutes, small but significant negative correlations were identified between anti-O:9 IgG and IgM titres and the killing of *S*. Typhi (r_s=-0.26, p=0.001, 95% CI -0.4 to -0.1 and r_p=-0.17 and p=0.038, 95% CI -0.32 to -0.01 respectively). After 90 minutes, there were small but significant correlations between anti-Vi IgM and O:9 IgG and the killing of *S.* Typhi (r_p= -0.18, p=0.033, 95% CI -0.3264 to -0.01427 and r_s= -0.21, p=0.009, 95% CI -0.3639 to -0.04727 respectively) (Data not shown). No significant correlation between anti-Vi IgG, A, or M and the bactericidal activity of sera against *S*. Typhi at 180 minutes was observed (Figure 5.7 A, B and C respectively). Small, but significant, negative correlations were observed for anti-O:9 IgG (Figure 5.7 D) and IgA (E) (r_s =-0.217, , p=0.0078, 95% CI -0.3694 to -0.05363 and -0.1996, p=0.014, 95% CI -0.3534 to -0.03525 respectively), but no association between anti-O:9 IgM titres (Figure 5.7 F) and the bactericidal activity of the sera was found at 180 minutes. No correlation between anti-O:4,5 IgG (Figure 5.7 G) or IgM titres (I) and killing was noted, however a small negative association for anti-O:4,5 IgA (H) and killing was observed (r_s =-0.1693, p=0.0390, 95% CI -0.3257 to -0.003883).

The small but significant negative correlation between anti-O:4,5 IgA titres and the killing of *S*. Typhi was surprising as *S*. Typhi does not express the O:4,5 o-chain antigens and thus there should be no available target sites for anti-O:4,5 IgA to bind. We thought this association may arise because individuals who produce high levels of anti-O:9 IgA also produce high level of anti-O:4,5 IgA and vice versa. We plotted titres of the two antibodies against each other and looked at the correlation. There was a highly significant positive correlation between the two (Figure 5.7 J) (r_s =0.67, p<0.0001, 95% CI 0.5631 to 0.7493)

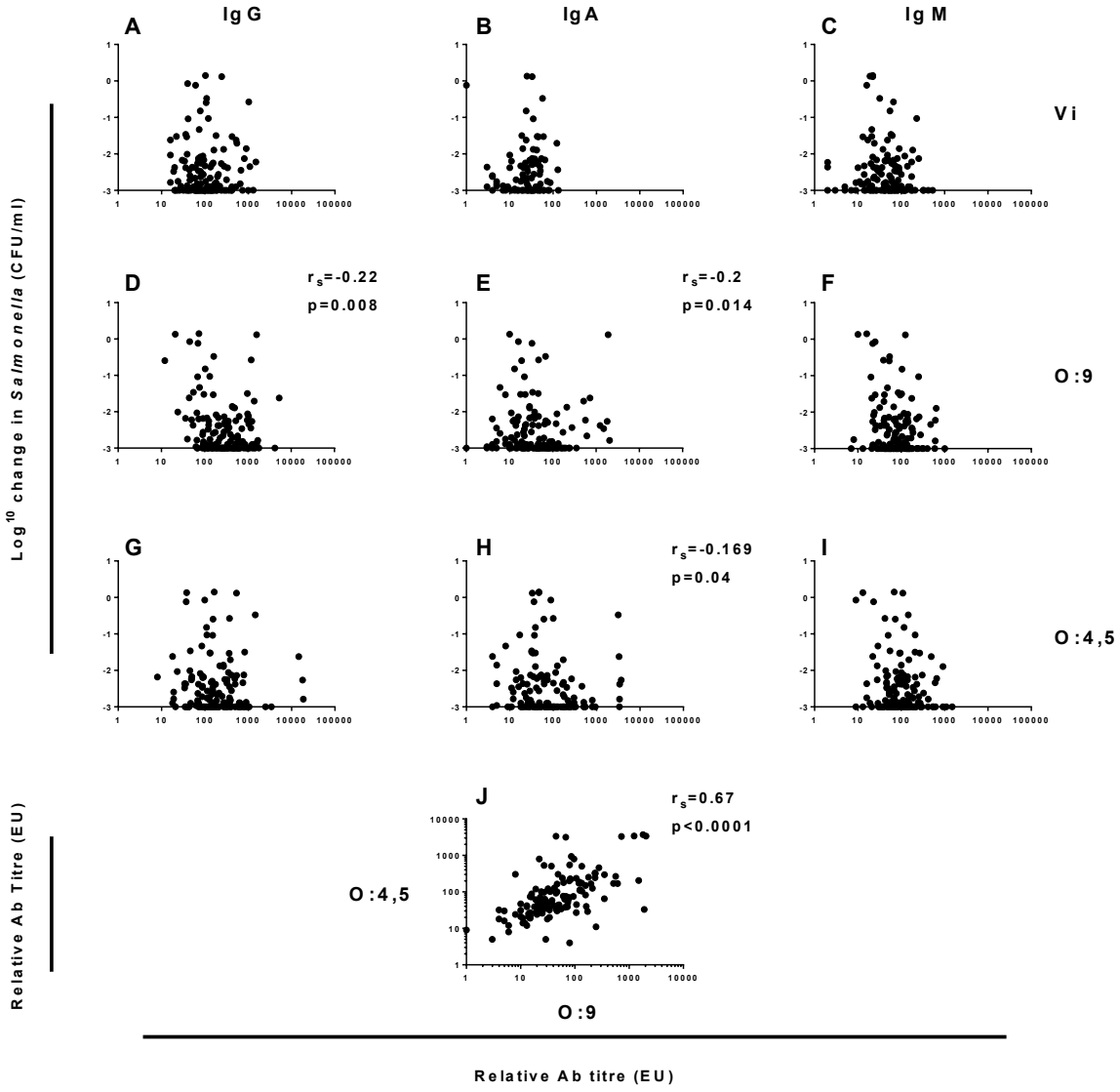


Figure 5.7 Relationship between anti-O:4,5 O:9 and Vi IgG, A and M antibody titres and bactericidal activity of HIV-infected sera at 180 minutes.

Levels of anti-Vi IgG (A), IgA (B) IgM (C), anti-O:9 IgG (D), IgA (E), IgM (F) and anti-O:4,5 IgG (G), IgA (H), IgM (I) compared with killing of *S.* Typhi in the SBA at 180 minutes. Anti-O:9 IgA plotted against anti-O:4,5 IgA is shown (J). Each circle represents values for serum from one individual.

5.3.7.2 Control sera

No correlations were observed between anti-Vi, O:9 or O:4,5 IgG, A or M titres and the bactericidal activity of the control sera against *S*. Typhi after 180 minutes exposure to serum (Figure 5.8 A - I). Because the control sera were so effective at killing *S*. Typhi, after 180 minutes a large number of data points fell on the X-axis making correlations difficult to establish. To address this, we examined the relationship between anti-Vi, O:9 and O:4,5 IgG, A and M titres and killing at the 45 and 90 minute time points. No relationship between titres and the bactericidal activity of the control sera against *S*. Typhi was observed at any time point (Data not shown).

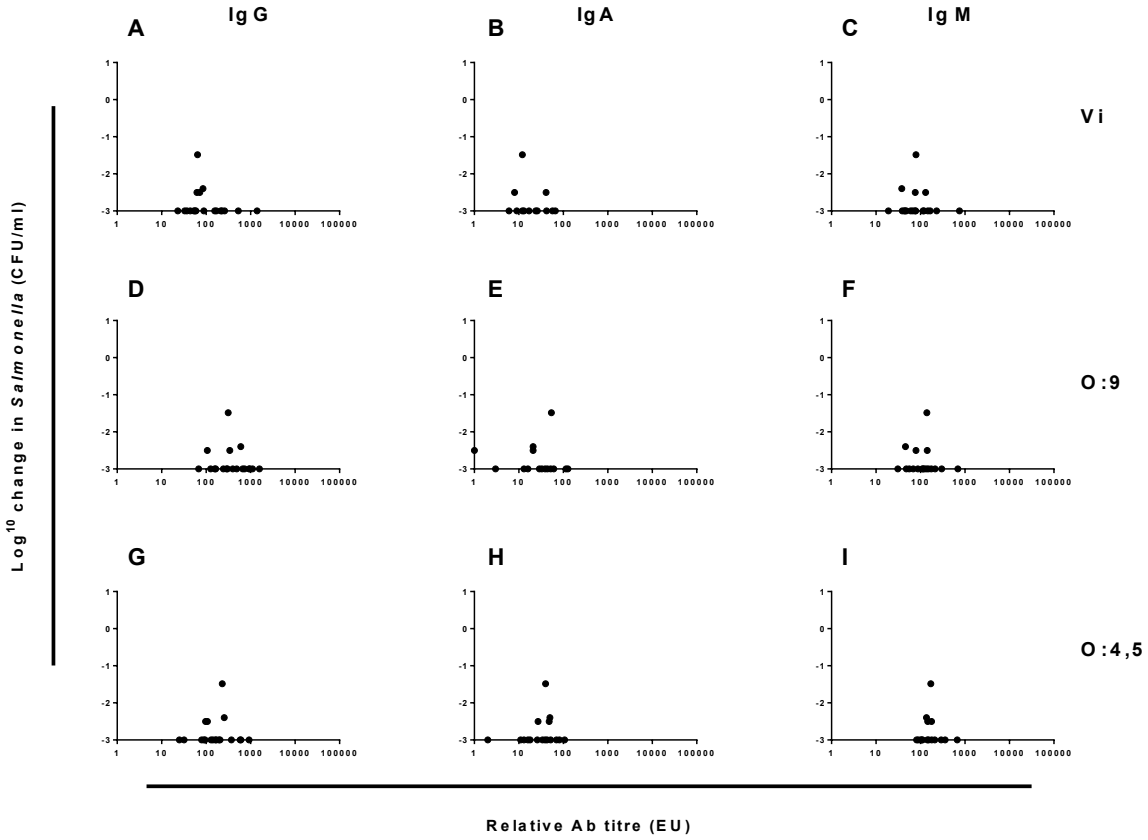


Figure 5.8. Lack of association between anti-Vi, O:9 or O:4,5 antibody titres and bactericidal activity of HIV-uninfected sera against *S.* Typhi.

No relationship between anti-*Salmonella* antibody titres and killing of *S.* Typhi was found for HIV-uninfected sera at 180 minutes. Anti-Vi IgG, A and M (A – C), O:9 IgG, A and M (D – F) and O:4,5 IgG, A and M (G – I) are shown. Each point represents data from one individual serum.

5.3.8. Anti-Vi IgM and O:9 IgG and IgM are associated with opsonic killing of *S.* Typhi by HIV-infected sera.

5.3.8.1 HIV Sera

To better understand the differences in killing of *Salmonella* by peripheral blood cells after opsonisation with control or HIV-infected sera, we examined the relationship between antibody titres and killing in the BCK assay.

No correlations between anti-Vi IgG or IgA, anti-O:9 IgA and all isotypes of anti-O:4,5 titres and killing of *S*. Typhi in the BCK assay were observed (Figure 5.9 A, B, E and G – I respectively). Anti-Vi IgM was significantly associated with killing of *S*. Typhi after 180 minutes (Figure 5.9 C) (r_p =-0.40, p=0.038) as were anti-O:9 IgG and IgM (r_s =-0.42 and -0.57, p=0.024 and 0.0017 respectively) (Figure 5.9 D and F). We previously observed significantly lower anti-O:9 and anti-Vi IgM titres in HIV-infected sera compared with uninfected sera. As both anti-Vi and anti-O:9 IgM were associated with opsonic activity and cell mediated killing of *S.* Typhi, it may be that lower anti-Vi and anti-O:9 IgM titres in HIV-infected sera are responsible for the reduced opsonic activity of these sera compared with that of HIV-uninfected sera.

5.3.8.2 Control Sera

Unlike HIV-infected sera, no correlations were observed between anti-Vi, O:9 and O:4,5 titres in control sera and the killing of *S*. Typhi in the blood cell killing assay at 45, 90 or 180 minutes (Data for 180 minutes shown in Figure 5.10).

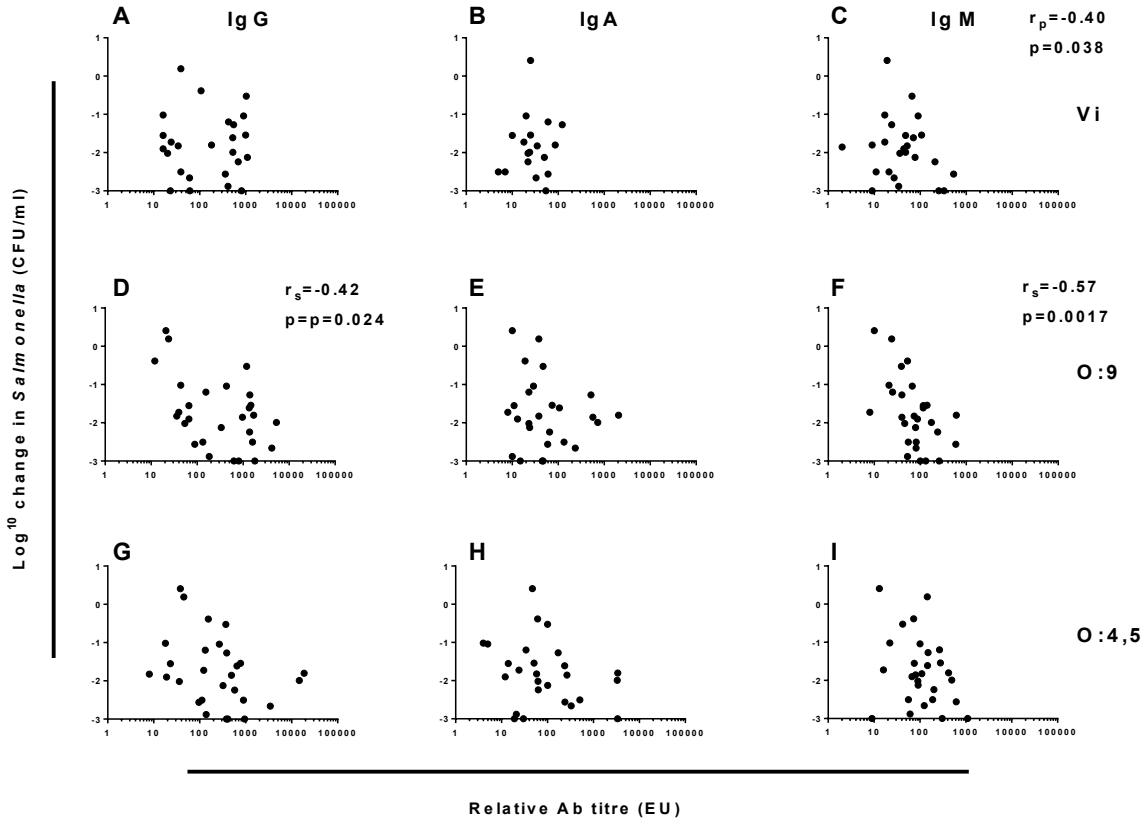


Figure 5.9. Anti-Vi IgM and anti-O:9 IgG and M from HIV-infected sera are associated with opsonic killing of *S.* Typhi.

We examined the relationship between anti-*Salmonella* antibody titres in the sera and the killing of *S.* Typhi in the blood cell killing assay at 180 minutes. Anti-Vi IgM, but not anti-Vi IgG or IgA, was significantly associated with killing (r_p=-0.40, p=0.038) (C). Anti-O:9 IgG and IgM were associated with killing (r_s=-0.42 and -0.57, p=0.024 and 0.0017 respectively) while IgA was not. No correlations were observed for any isotype of anti-O:4,5 antibody and killing. Each point represents data from one individual serum.

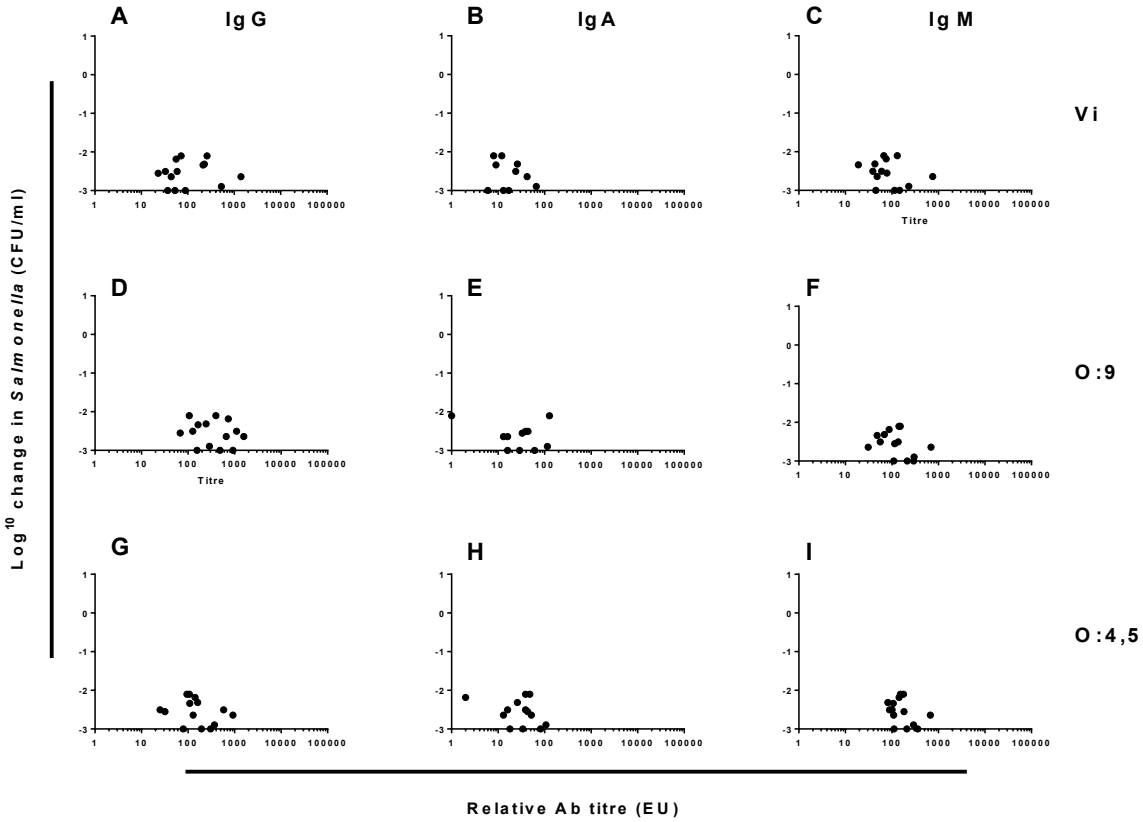


Figure 5.10. No association was found between anti-*Salmonella* antibody titres in healthy Malawian sera and the killing of *S.* Typhi.

No relationship was found between the titres of anti-Vi (A – C), O:9 (D – F) or O:4,5 (G – I) IgG, A or M at 180 minutes and the opsonic activity of the healthy Malawian sera against *S.* Typhi. Each data point represents titre and opsonic killing activity of one sera measured in the blood cell killing assay.

5.3.9. Antibody-depleted human serum, but not baby rabbit complement, is a suitable complement source for SBAs against *S.* Typhi.

Previous work from our group has shown that a subset of HIV-infected Africans produce high titre anti-O chain antibodies which interfere with the normal killing of *S*. Typhimurium possibly by blocking or diverting access of bactericidal antibody and complement. Dilution of these high-titre anti-O chain antibodies was able to restore the bactericidal activity of the antibody (MacLennan and Tennant, 2013). We wanted to examine whether a similar effect occurs with anti-*S.* Typhi antibody and whether this accounts for the reduction in the killing of *S*. Typhi when using HIV-infected sera in SBAs and BCKs.

To do this we needed to establish a complement source which can be combined with varying dilutions of antibody and tested in SBAs against *S*. Typhi. Baby rabbit complement (BRC) is produced in 3 – 4 week old rabbit kittens and has low heterophile antibody activity. It has been used successfully as a complement source against *S.* Typhi (Pulickal et al., 2009). However, our group have experienced problems with its use, such as high inherent toxicity to *Salmonella,* poor stability and variation between batches and/or manufacturers. An alternative source of complement is human serum depleted of specific anti-*Salmonella* antibody. From experience we have found this is not inherently toxic to *Salmonella,* is relatively stable and effects physiological levels of killing when combined with exogenous antibody and is tested against NTS. We assessed both sources of complement for their viability in SBAs against wild-type *S*. Typhi.

5.3.9.1 Baby rabbit complement alone is toxic to *S*. Typhi at low concentrations and effects poor killing of *S*. Typhi when combined with antibody

We established what concentration of BRC alone is non-toxic but still able to effect killing of *S.* Typhi when combined with heat inactivated human serum as the antibody source. We tested 5, 10, 20, 50, 75 and 100% BRC against three wild-type *S*. Typhi strains from Kenya, Nepal and Indonesia (Figure 5.11 A, B and C respectively). Concentrations of BRC at 20% or above were toxic to all three strains, however, the extent of toxicity varied between strains. 10% BRC alone was toxic to the Nepalese and Indonesian strains (B and C) effecting ~ 1 $\log_{10}$ kill at 180 minutes but was not toxic to the African strain (A). 5% BRC alone was not toxic to any of the three strains. Heat inactivation removed the toxicity of BRC to *S*. Typhi (red line).

To assess whether killing of *S*. Typhi could be induced by combining non-toxic concentrations of BRC with an exogenous antibody source, we tested 5, 7.5 and 10% BRC with heat-inactivated human serum, in a 1:10 dilution series, against the African *S*. Typhi strain (ISP-04-06979). Human serum used was from either a healthy Caucasian donor which contained anti-*Salmonella* antibody or a pool of sera from healthy Malawian adults (PMS). Addition of exogenous Ab, whether it be Caucasian or African, had very little effect on the ability of 5, 7.5 and 10% BRS to kill *S*. Typhi, with the maximum kill achieved by any combination of BRC and antibody being less than 1 $\log_{10}$ (Figure 5.11 5.11 D and E). Dilution of the antibody from the Caucasian donor had very little effect on the ability of BRC to kill *S*. Typhi suggesting exposure to BRC alone, even at low concentrations, is detrimental to *S.* Typhi survival (Figure 5.11 E).

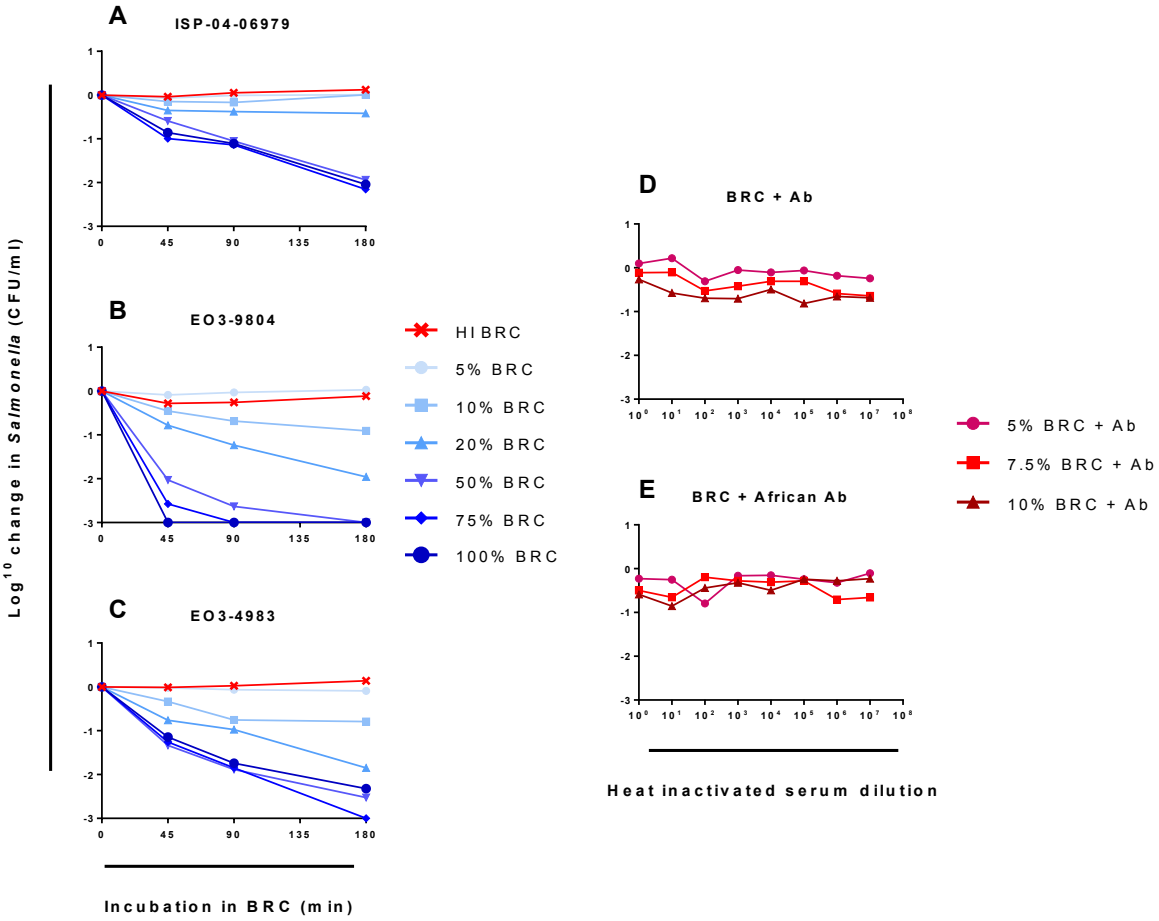


Figure 5.11 BRC alone is toxic to *S.* Typhi at relatively low concentrations but effects poor killing when combined with antibody at non-toxic concentrations.

5 and 10% BRC alone were not toxic to a representative African *S.* Typhi strain but concentrations of 20% and higher effected -2 $\log_{10}$ killing at 180 minutes (A). Only 5% BRC was non-toxic to two Asian *S.* Typhi strains. Concentrations of 10% or higher effected killing of *S.* Typhi in a concentration dependent manner (B and C). When combined with either Caucasian (D) or African (E) Ab, non-toxic percentages of BRC effected < 1 $\log_{10}$ killing of *S.* Typhi irrespective of the antibody dilution. Data are from one experiment.

5.3.9.2 Incubation of *S.* Typhi with human serum is effective at removing *Salmonella*-specific antibody and produces a functional complement source

We found that BRC is not viable as a source of complement for SBAs against *S*. Typhi due its inherent toxicity at relatively low concentrations and the lack of effective killing it induces when combined with exogenous antibody. To test the role of antibody from HIV-infected sera in SBAs, we assessed the suitability of antibody-depleted human serum as a complement source. In its entirety, human serum is not a suitable source of complement due to the presence of natural and specific anti-*Salmonella* antibody. Individuals lacking antibody are usually young or suffering from immune defects, making it unpractical and unethical to source sera from them. We tested the viability of depleting *S.* Typhi-specific antibody from healthy human serum as a method for producing C'.

We incubated fresh human serum with live, wild-type *S*. Typhi ISP-04-06979 to deplete it of *Salmonella*-specific antibody and assessed pre and post-incubation antibody titres and antibody binding by ELISA and FACs. Antibody depleted sera was then tested against *S*. Typhi in SBAs alone or combined with heat inactivated sera as a source of exogenous antibody.

Prior to incubation with *S*. Typhi, IgG, A and M binding to the surface of *S.* Typhi was detectable by flow cytometry. After antibody-depletion, antibody binding to *Salmonella* was reduced to background levels (Figure 5.12 A). High titres of anti-O:9 IgG and M, and anti-Vi IgG were detectable by ELISA in sera before depletion of antibody. Minimal amounts of anti-O:9 IgG but no IgA, M or anti-Vi IgG, A or M were detectable after incubation with *Salmonella* (Figure 5.12 B). We then assessed the killing activity of the

antibody-depleted sera in SBAs either alone or combined with exogenous antibody. Heat-inactivated sera from either a Caucasian donor (Figure 5.12 C) with known anti-*Salmonella* antibody or heat inactivated pooled Malawian sera (Figure 5.12 D) were used as an antibody source.

When combined with neat antibody from the Caucasian donor, 75 and 50% antibody-depleted sera effected 3 $\log_{10}$ killing of *S*. Typhi and approximately 2 $\log_{10}$ kill when antibody was diluted 1:10. 25% antibody depleted sera effected 2 $\log_{10}$ and 1 $\log_{10}$ killing when combined with neat exogenous antibody or a 1:10 dilution of antibody respectively. Killing was lost when antibody was diluted 1:100 or more (Figure 5.12 C). Neat African antibody effected 3 $\log_{10}$ killing of *Salmonella* when combined with either 75 or 50% antibody-depleted sera or 2 $\log_{10}$ kill with 25%. Killing was reduced to approximately -0.5 $\log_{10}$ at 1:10 dilution of antibody regardless of the percentage of antibody depleted serum used and killing was lost at antibody dilutions of 1:100 and greater (Figure 5.12 D). In all cases, neither antibody depleted sera nor heat inactivated sera were able to kill *S*. Typhi alone and gave between 0 – 1 $\log_{10}$ growth at 180 minutes (green and purple circles). When combined with Ab, 50 and 75% complement effected similar levels of killing to that of serum from the homologous antibody donor (black circle).

These data show that depletion of specific anti-*Salmonella* antibody from human serum creates a viable source of complement that is unable to kill *Salmonella* alone but effects physiological levels of killing when combined with human antibody.

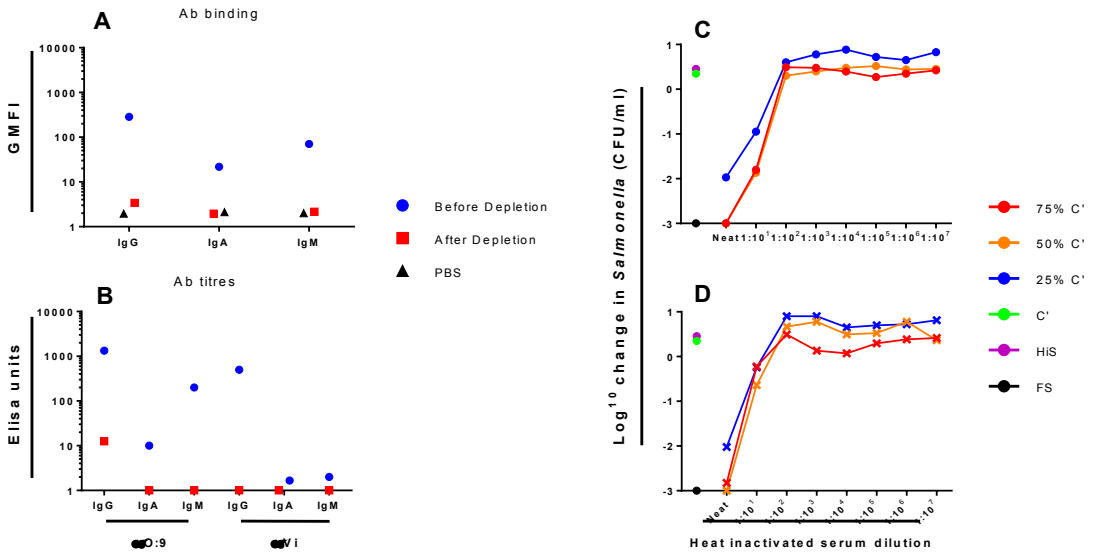


Figure 5.12. Incubation with *S.* Typhi depletes specific-Ab of sera whilst leaving complement intact.

We assessed the levels of anti-Vi and O:9 antibody before and after incubation of sera with *S.* Typhi by FACS and ELISA. Prior to depletion, sera contained IgG, A and M which was able to bind the surface of *S.* Typhi (A), and had high levels of anti-O:9 Ig and M and anti-Vi IgG (B). After incubation, total antibody binding to *S.* Typhi was no different to a PBS negative control and all but anti-O:9 IgG were undetectable by ELISA. When combined with exogenous antibody from either Caucasian (C) or African (D) donors both 50 and 75% human complement effected 3 $\log_{10}$ killing of *S.* Typhi. 25% human complement effect a 2 $\log_{10}$ kill for both antibody. Neither antibody-depleted sera alone (C', green circle) or heat-inactivated serum (HiS, purple circle) were able to kill *S.* Typhi at 180 minutes. Killing of *S.* Typhi with antibody and 50 or 75% complement was equivalent to physiological killing for serum from the antibody donor (Black circle). Data are representative of one experiment.

5.3.10. Killing of *S.* Typhi in a subset of HIV-infected and control sera can be restored by diluting Ab

Having established a viable complement source, we were able to examine the effect of diluting the antibody from HIV-infected and control sera, on the killing of *S*. Typhi and identify if a prozone effect occurs.

HIV-infected and control sera were heat-inactivated to remove endogenous complement and added to 50% antibody-depleted sera in a 2-fold dilution series ranging from neat (1:2 final dilution) to 1:256 (1:512 final). The killing of *S*. Typhi relative to the starting value was measured at 180 minutes.

Ab from 149 HIV-infected sera effected a broad range of killing of *S*. Typhi (Figure 5.13 A-C). Some antibody lost the ability to kill *S*. Typhi after being diluted 1:2 whereas other antibody retained the ability to kill *S.* Typhi at the highest dilution. 17 out of 149 antibody sources from HIV-infected individuals (11.4%) effected greater killing after dilution than at a neat concentration – a phenomenon known as the prozone effect (Figure 5.13 D). The mean results for non-prozone HIV-infected sera (blue line) and for those sera which exhibited a prozone effect (green line) are displayed in panel E. This illustrates how the majority of antibody from HIV-infected sera loses its ability to induce killing of *S*. Typhi in a linear fashion as the antibody source is diluted. For those sera which induce a prozone effect, at 1:2, 1:4 and 1:8 dilutions of antibody killing is increased compared to that of neat antibody to approximately -2.5 $\log_{10}$ compared to ~ -2 $\log_{10}$ for neat antibody on average. Of the 19 control sera, the killing of *S*. Typhi was more uniform as the antibody was diluted and all sera except 1 lost the ability to kill *S*. Typhi at the highest dilution (Figure 513 F). 2 out of 19 control sera (10.5%) also

exhibited a prozone effect with killing increasing from -2 $\log_{10}$ to -3 $\log_{10}$ at a 1:2 and 1:4 dilution of these sera. There were no significant differences for total anti-Vi, O:9 or O:4,5 or for the IgG, A or M isotypes of each antibody in sera exhibiting a prozone effect compared to those that did not (Data not shown).

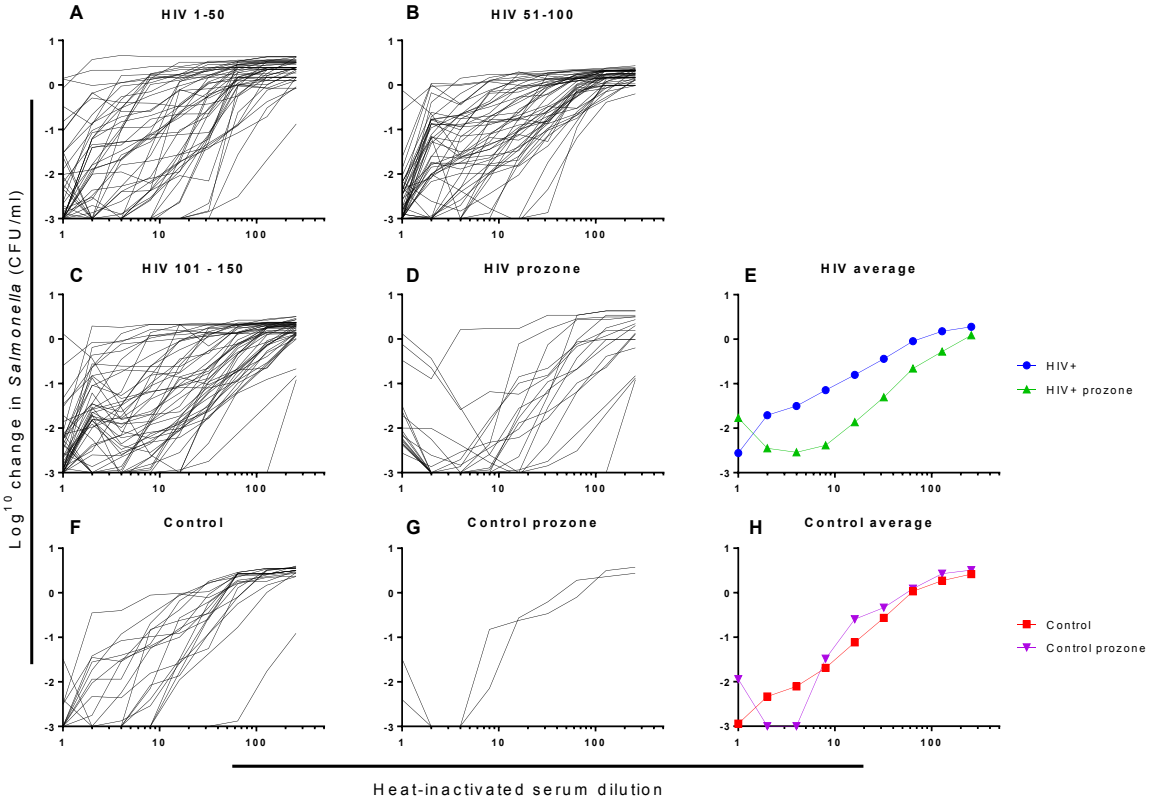


Figure 5.13 A subset of sera from HIV-infected and uninfected adults exhibits a prozone effect in the killing of *S.* Typhi.

Using antibody-depleted human serum as a complement source we measured the ability of antibody from HIV-infected and uninfected African sera, to kill *S.* Typhi. Antibody from 149 HIV-infected sera (A – C) was tested and effected mixed killing of *S.* Typhi, with killing generally being lost on dilution of the antibody. Antibody from 17 of 148 HIV-infected sera displayed greater killed upon dilution than at the lowest dilution, indicative of a prozone effect (D). Average values show a decrease in killing associated with dilution of antibody (blue line). Sera for which a prozone effect occurs, effected increased killing after dilution until further dilution (green line). All but 2 of the antibody from control sera lost killing with dilution (F) whilst two displayed a prozone effect at a 1:4 and 1:8 final dilution before killing was lost (G). Average values for antibody from control sera are shown in (H).

5.3.11. Interleukins 6 and 10, interferon-gamma and TNF-alpha but not interleukins 4 or 17 are produced by whole peripheral blood following stimulation with *S.* Typhimurium or *S.* Typhi.

We have investigated the humoral relationship between HIV infection and the effect of antibody against *S*. Typhi. These data suggest that, in contrast to observations in the literature, HIV infection leads to impaired *in vitro* killing of *S*. Typhi by both bactericidal and opsonic antibody. Primary macrophages from HIV-infected adults have a dysregulated cytokine response to *S*. Typhimurium challenge (Gordon et al., 2007). We wanted to investigate whether there was a dysregulated cytokine response in whole blood following stimulation with either *S*. Typhimurium or *S*. Typhi and whether this response differed according to HIV status. To do this, we first needed to establish the optimal concentration of *Salmonella* with which to stimulate the blood and the length of stimulation which gave a quantifiable response. We stimulated whole blood, venesected from two healthy adults, with 10^1 – 10^6 CFU/ml of *S*. Typhimurium D23580 or *S*. Typhi ISP-04-06979 for a total of 5 days at 37°C. Plasma from stimulated blood was prepared at 1, 2, 4, 8, 24, 48, 72, 96 and 120 hours and the levels of TNF-alpha, IFN-gamma, IL-10, IL-17, IL-4 and IL-6 quantified by cytokine ELISA.

IL-10 production began between 4-8 hours after stimulation with *S*. Typhi or *S*. Typhimurium (Figure 5.14 A-D). For *S*. Typhi, 10^4 CFU/ml and above was required to stimulate a response from both donors (Figure 5.14 A and B). However, for *S*. Typhimurium 10^3 CFU/ml was also sufficient to stimulate IL-10 production. Levels of IL-10 peaked at approximately 350 pg/ml at 24 hours followed by a plateau in levels until approximately 72 hours after which they decreased to baseline at 120 hours.

TNFα levels began to increase very early in the stimulation with 10^6 CFU/ml of either *Salmonella* serovar producing approximately 200-300 pg/ml after 1 hour. For both donors and serovars, the quantity of *Salmonella* used determined when TNFα levels began to rise and for how long production was sustained. With higher concentrations of *Salmonella* inducing earlier and longer production. 10^6 CFU/ml *Salmonella* sustained production at 500 – 600 pg/ml from 2 to between 64 and 96 hours. Low doses of *Salmonella* (10^1 and 10^2 CFU/ml) were unable to stimulate TNFα production until >72 hours after which levels climbed to approximately 600 pg/ml (Figure 5.14 E – H).

IFNγ production began to increase between 4 – 8 hours after stimulation irrespective of the serovar or the donor. For *S*. Typhi, only 10^6 CFU/ml was sufficient to stimulate a response and IFNγ levels rose in excess of 100 pg/ml (Figure 5.14 I and J). 10^5 and 10^6 S. Typhimurium were able to stimulate >100 pg/ml (Figure 5.14 K and L). Donor 2 produced approximately 3 times more IFNγ than donor 1 in response to stimulation with the same quantity of *Salmonella*. IFNγ levels began to decrease to baseline at 72 hours after stimulation.

IL-6 production was stimulated in a dose-dependent manner with levels increasing to ~250 pg/ml for either *S*. Typhi or *S*. Typhimurium at 10^4 CFU/ml and above. They remained elevated for the rest of the stimulation period (Figure 5.14 M – P) Low concentrations of *S*. Typhimurium (10^1 and 10^2) were able to stimulate some IL-6 production at 8 hours and this varied depending on the donor (Figure 5.14 L and P). The same concentrations of *S*. Typhi were unable to stimulate any IL-6 production until approximately 96 hours (Figure 5.14 M and N).

Neither *S*. Typhi nor *S*. Typhimurium at any concentration were able to stimulate the production of IL-4 (Figure 5.14 Q – T) or IL-4 (U – X) at any time point irrespective of the donor.

Figure 1.14 *Salmonella* stimulation induces production of IL-6, IL-10, TNFα and IFNγ but not IL-4 or 17 by peripheral whole blood.

The *ex vivo* cytokine response of peripheral whole blood to *Salmonella* stimulation was measured over 120 hours. IL-10 production was stimulated by *S.* Typhi (A and B) and *S.* Typhimurium (C and D) and peaked between 24 – 72 hours. TNFα production was stimulated before 1 hour had elapsed irrespective of serovar (E – H). The height and length of the response was dependent on the concentration of *Salmonella.* IFNγ production (I – L) varied according to donor but was stimulated by both serovars and peaked around 24 hours. IL-6 production began between 2 -4 hours and stayed constant when $>10^3$ CFU/ml *Salmonella* was used (M – P) *Salmonella* did not stimulate the production of IL-17 (Q – T) or IL-4 (U – X) for either donor at any concentration. Each line represents plasma from blood stimulated with a different concentration of *Salmonella.*

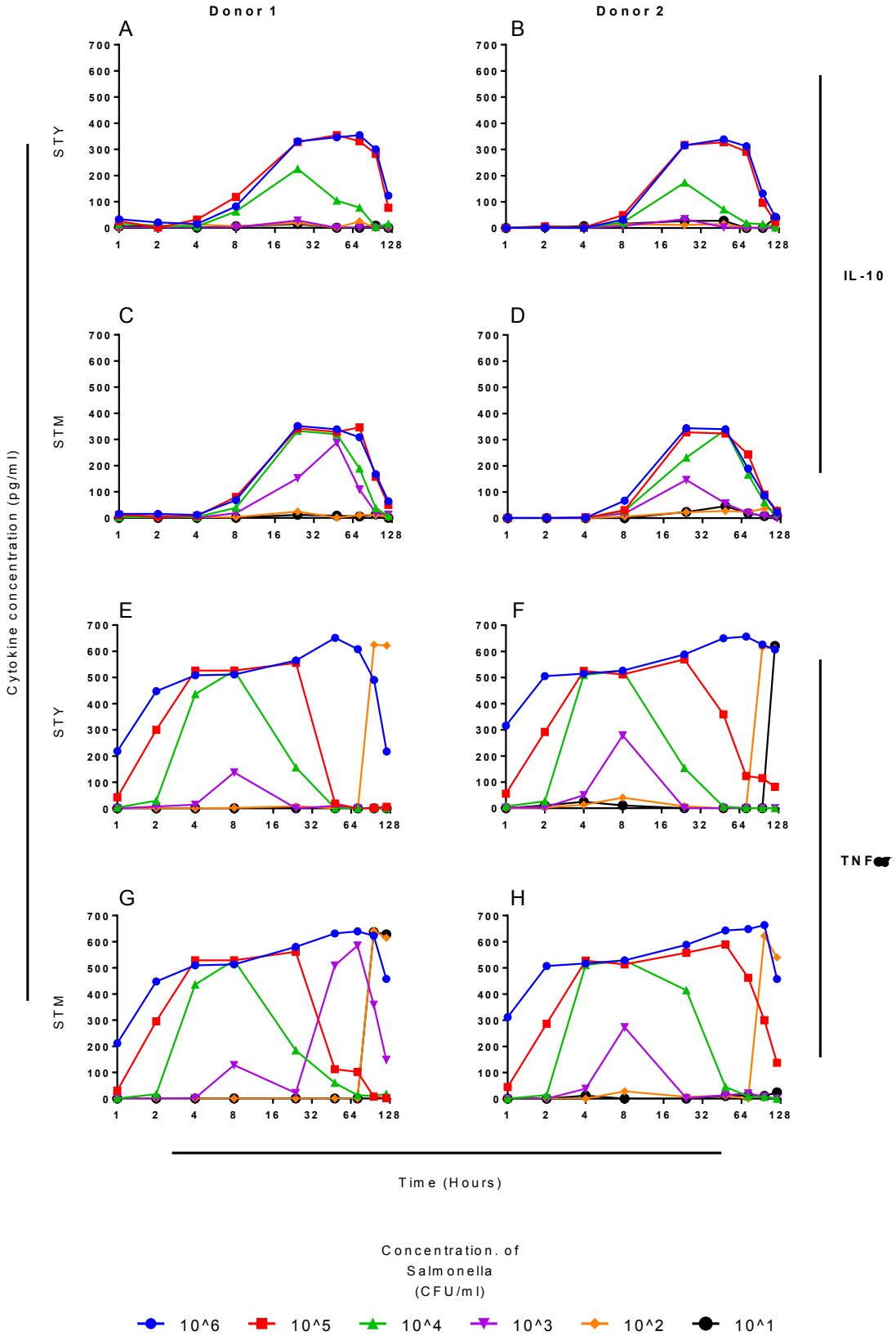
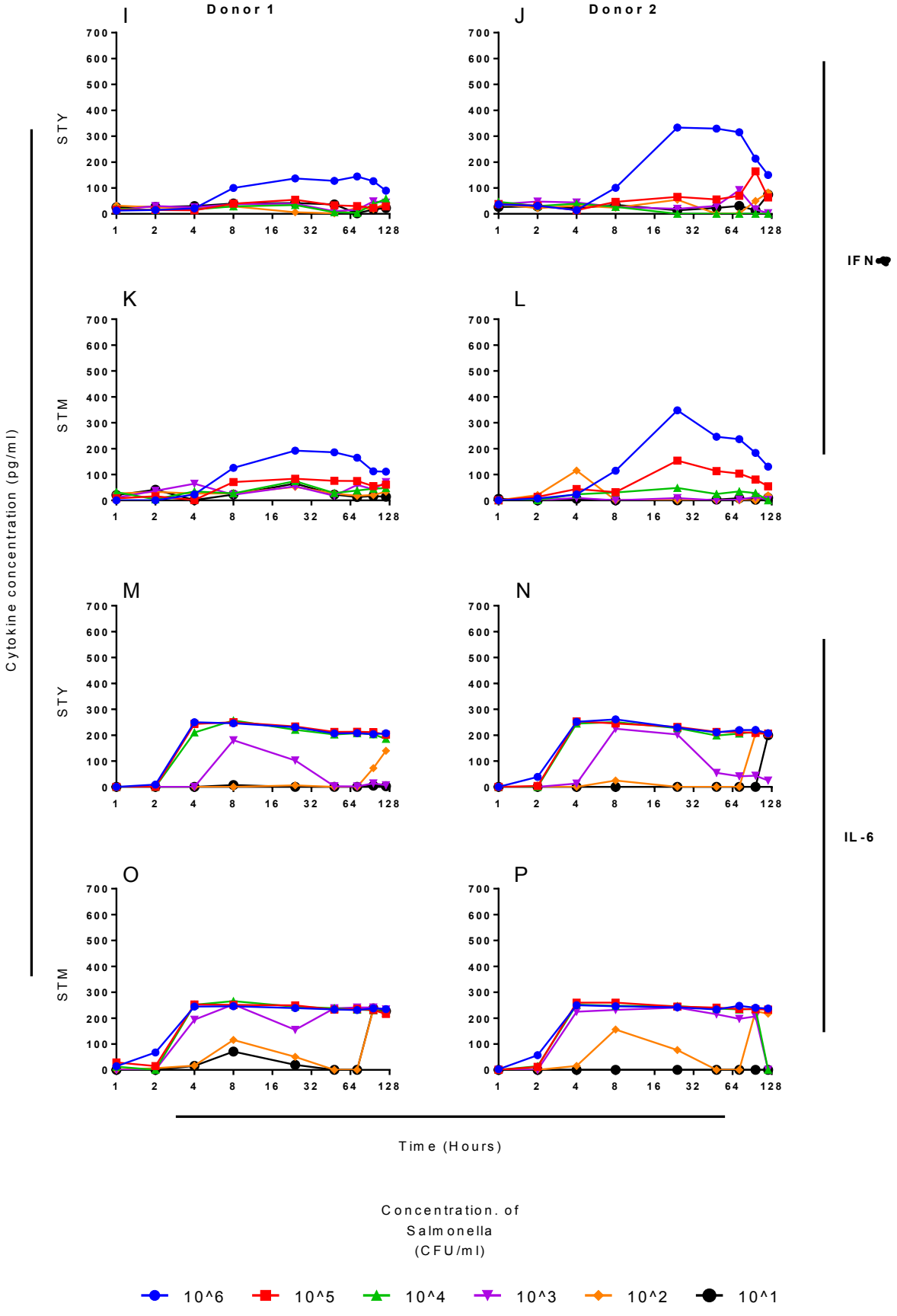
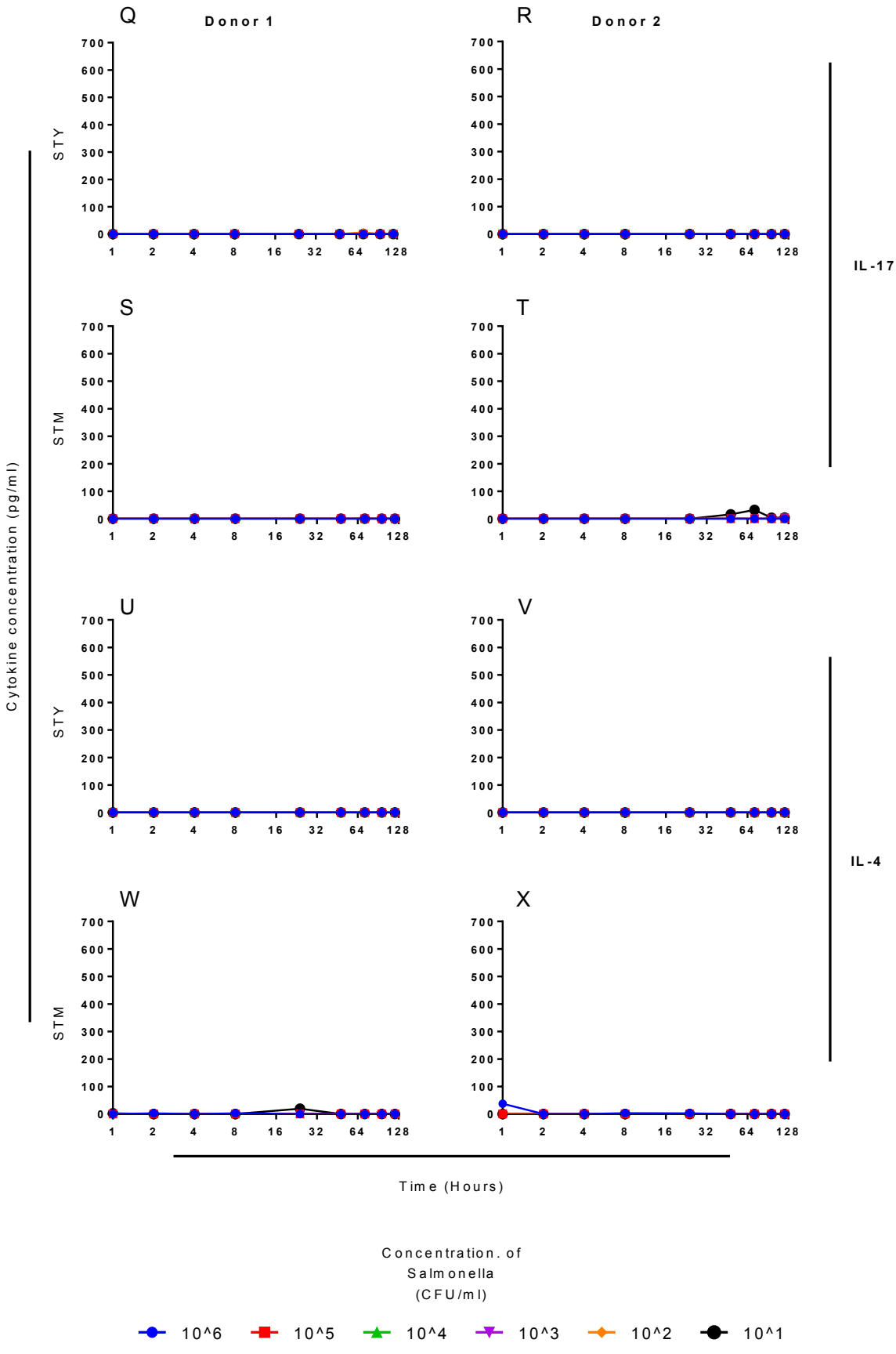

Donor 1
Donor 2
A
B
C
D
E
F
G
H
STY
STM
STY
STM
Cytokine concentration (pg/ml)
IL-10
TNF
Time (Hours)
Concentration. of Salmonella (CFU/ml)
10^6
10^5
10^4
10^3
10^2
10^1

Figure 5.14


I
Donor 1
J
Donor 2
K
L
M
N
O
P
STY
STM
STY
STM
700
600
500
400
300
200
100
0
1
2
4
8
16
32
64
128
Cytokine concentration (pg/ml)
IFN
IL-6
Time (Hours)
Concentration. of
Salmonella
(CFU/ml)
10^6
10^5
10^4
10^3
10^2
10^1


Q
Donor 1
R
Donor 2
S
T
U
V
W
X
STY
STM
700
600
500
400
300
200
100
0
1
2
4
8
16
32
64
128
IL-17
IL-4
Cytokine concentration (pg/ml)
Time (Hours)
Concentration. of Salmonella (CFU/ml)
10^6
10^5
10^4
10^3
10^2
10^1

5.3.12. Whole blood of HIV-infected African adults has altered cytokine responses to *Salmonella* stimulation for 22 different cytokines and chemokines compared to healthy African blood

From the pilot study we know that whole blood upregulates cytokine production in response to *Salmonella* stimulation and for those cytokines tested, production varied according to donor and responses were dose-specific. From these data we chose 10^6 CFU/ml *Salmonella* as a concentration which would give us a quantifiable response when stimulating blood with *Salmonella* to examine the response for a larger number of cytokines. Whilst not being clinically representative of *Salmonella* bacteraemia, when we tested 10^1 and 10^2 CFU/ml *Salmonella*, which are more representative, these levels often failed to stimulate an adequate cytokine response. We chose 24 hours as a practical time point to work with and one at which cytokine responses were at or near their peak levels in the pilot study.

We investigated whether the cytokine response of whole blood to *Salmonella* stimulation differs according to serovar and HIV status. We venesected 11 African adults, 8 of which were HIV positive. To maximise the data from the study we decided to select HIV-infected adults based on CD4 counts and ART status. We stimulated bloods from each adult with different strains and serovars of *Salmonella* to attempt to identify strain-and serovar-specific responses. Details of donor information such as CD4 count and viral load in addition to stimulation *Salmonella* information are listed in Table 5.1.

Table 5.1. Donor and strain information for *Salmonella* blood stimulations

Blood stimulation donor information		
HIV-Infected donors		
CD4 (cells/mm³)	**Viral Load (Sex/Age)** ART (VL <40)	No ART (VL >10,000)
<350	1 (M/49)	1 (M/34)
350 – 500	1 (M/32)	1 (F/44)
500 – 700	1 (F/47)	1 (M/45)
>700	1 (F/42)	1 (F/41)
Control Donors	3 (F/39, 35, 37)	

Stimulation *Salmonella* information		
Strain	**Serovar**	**Vi-expression**
D23580	Typhimurium	-
SGB1	Typhimurium	-
C5.507	Typhimurium	+
D24954	Enteritidis	-
ISP-04-06979	Typhi	+
BRD948	Typhi	+
BRD948 *tviB*-	Typhi	-

Blood was venesected from 11 African adults for stimulation with *Salmonella.* 3 donors were healthy African adults aged between 35- 39. Of 8 HIV-infected donors, 4 were on ART. 1 patient on ART and 1 not on treatment were chosen for each CD4 cohort. Donors were aged between 32 – 49 years and were recruited at the Queen Elizabeth Hospital, Birmingham, UK. *Salmonella* strains used for stimulation were serovars Typhi, Typhimurium and Enteritidis. D23580, ISP-04-06979 and D24954 are wild-type invasive isolates. C5.507 and SGB1 and BRD948 and BRD948 *tviB*- are isogenic pairs of Vi-expressing and non-expressing *S.* Typhimurium and *S.* Typhi respectively.

Heparinised bloods were stimulated with 10^6 CFU/ml *Salmonella* for 24 hours at 37°C. Stimulated plasma was then assayed for levels of 51 different cytokines and chemokines (listed in Table 5.1) by luminex immunoassay.

One donor had extremely high levels of all cytokines regardless of stimulation condition and was thus his/her data were excluded from analysis. We compared levels of analytes in whole blood stimulated with *Salmonella* to blood mock-stimulated with PBS. This enabled us to exclude analytes for which *Salmonella* stimulation was not responsible for their production.

Four analytes were not significantly different in PBS-stimulated bloods compared to those stimulated with *Salmonella* (Listed in red, Table 5.2). These were MCP-1, MIF, sICAM-1 and sE-selectin. These analytes were excluded from further study. We compared levels of analytes in blood stimulated with *S*. Typhi ISP-04-06979 to *S*. Typhimurium D23580 and also blood stimulated with typhoidal strains (ISP-04-06979 and BRD948) to blood stimulated with nontyphoidal strains (D23580, D24954 and SGB1). For all these comparisons *P*-values were greater than 0.05 indicating that the

cytokine and chemokine response did not differ according to strain or serovar (Data not shown).

We looked at whether HIV-status affected the cytokine and chemokine response to *Salmonella* stimulation as a whole (Table 5.3). 22 out of 47 different analytes were significantly different in HIV-infected and uninfected individuals and of these, 21 were significantly higher in those with HIV (Figure 5.15).

Figure 5.15. Relative expression of cytokines in peripheral whole blood from HIV-infected and uninfected individuals after *Salmonella* stimulation.

Blood from HIV-infected and uninfected Africans was stimulated with the *Salmonella* strains listed in table 5.1. The relative expression levels of 22 cytokines which were significantly different between HIV-infected (red boxplots) and uninfected blood (blue boxplots) after *Salmonella* stimulation are plotted.

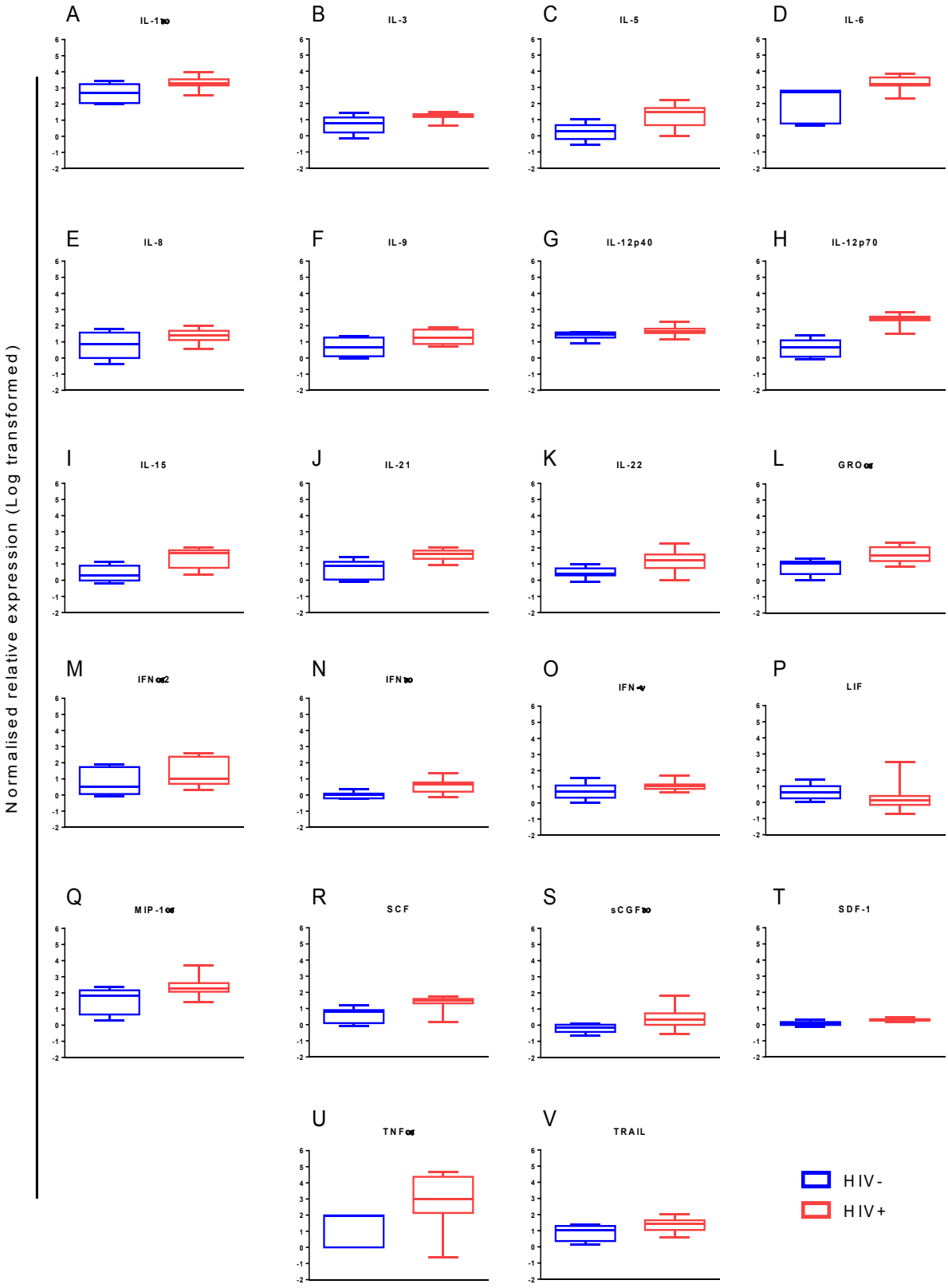

A
IL-1α
B
IL-3
C
IL-5
D
IL-6
E
IL-8
F
IL-9
G
IL-12p40
H
IL-12p70
I
IL-15
J
IL-21
K
IL-22
L
GROα
M
IFNα2
N
IFNα
O
IFN-γ
P
LIF
Q
MIP-1α
R
SCF
S
sCGFβ
T
SDF-1
U
TNFα
V
TRAIL
Normalised relative expression (Log transformed)
HIV-
HIV+

Table 5.2. Difference in cytokine levels between *Salmonella* stimulation and PBS mock-stimulation.

Four analytes were not significantly different between blood stimulated with *Salmonella* or blood mock-stimulated with PBS (Listed in red). The remainder were significantly different (Holm's corrected Wilcoxon *U*-test, $p<0.05$).

Wilcoxon-U test					
Cytokine/ Chemokine	**p-value**	**Adjusted p-value**	**Cytokine/ Chemokine**	**p-value**	**Adjusted p-value**
IL.6	4.86E-13	2.48E-11	**IL.8**	3.75E-12	9.37E-11
IFNgamma	5.32E-13	2.66E-11	**IL.1RA**	8.26E-12	1.98E-10
MIP.1alpha	5.33E-13	2.66E-11	**IL.4**	2.03E-11	4.67E-10
G.CSF	5.33E-13	2.66E-11	**TNFalpha**	3.42E-11	7.52E-10
IL.1alpha	5.33E-13	2.66E-11	**IL.7**	3.69E-11	7.76E-10
IL.23p19	5.33E-13	2.66E-11	**IL.5**	3.86E-11	7.76E-10
GROalpha	5.33E-13	2.66E-11	**GM.CSF**	1.17E-10	2.21E-09
IL.10	5.33E-13	2.66E-11	**IP.10**	3.30E-10	5.94E-09
IL.12.p40.	5.33E-13	2.66E-11	**M.CSF**	5.64E-10	9.59E-09
IL.1Beta	5.33E-13	2.66E-11	**MIG**	4.32E-09	6.91E-08
MIP.3alpha	5.33E-13	2.66E-11	**MCP.3**	7.97E-09	1.20E-07
TRAIL	5.33E-13	2.66E-11	**IL.2**	2.78E-08	3.90E-07
MIP1.Beta	5.57E-13	2.66E-11	**IFN.beta**	6.93E-08	9.01E-07
IL.3	6.35E-13	2.66E-11	**CTACK**	2.47E-07	2.97E-06
IL.9	8.17E-13	3.02E-11	**IL.13**	2.79E-07	3.07E-06
IL.22	9.33E-13	3.36E-11	**IL.27**	3.20E-07	3.20E-06
IL.21	9.82E-13	3.44E-11	**NGF**	1.49E-06	1.34E-05
IL.12.p70.	1.02E-12	3.45E-11	**IL.16**	1.89E-06	1.51E-05
SCF	1.06E-12	3.50E-11	**IFNomega**	1.79E-05	0.00012548
Eotaxin	1.11E-12	3.57E-11	**LIF**	0.0011437	0.00686237
IL.17A	1.16E-12	3.61E-11	**sCGF.b**	0.0020758	0.01037911
IL.15	1.27E-12	3.81E-11	**sICAM.1**	0.1099262	0.43970504
TNF.beta	1.33E-12	3.86E-11	**sE.Selectin**	0.3509646	1
IFNalpha2	1.50E-12	4.20E-11	**MCP.1**	0.6324255	1
IL.31	1.51E-12	4.20E-11	**MIF**	0.8202682	1
SDF.1	3.41E-12	8.88E-11			

Table 5.3. 22 analytes were significantly different in HIV-infected bloods compared to healthy bloods after *Salmonella* stimulation.

Levels of 22 out of 51 analytes were significantly different in HIV-infected blood compared to HIV-uninfected blood after *Salmonella* stimulation (listed in black) (Holm's corrected Wilcoxon *U*-test, p<0.05). 29 analytes were not significantly different between HIV-infected and uninfected blood after *Salmonella* stimulation (Listed in red) (p>0.05)

Wilcoxon-U test					
Cytokine/ Chemokine	*P* value	Adjusted *P* value	Cytokine/ Chemokine	*P* value	Adjusted *P* value
IL.12.p70.	9.18E-11	4.68E-09	IL.31	0.015764	0.3941166
SCF	2.73E-09	1.36E-07	IL.23p19	0.020276	0.4866453
SDF.1	6.14E-09	3.01E-07	MIF	0.024114	0.55464296
IL.21	7.77E-09	3.73E-07	CTACK	0.027770	0.61094777
GROalpha	5.11E-08	2.40E-06	NGF	0.036191	0.76002719
IFN.beta	1.26E-07	5.82E-06	IL.16	0.042335	0.84670443
IL.6	1.84E-07	8.28E-06	G.CSF	0.129919	1
IL.15	3.40E-07	1.50E-05	IL.7	0.134060	1
TNFalpha	2.23E-06	9.61E-05	IFNomega	0.160520	1
sCGF.b	2.58E-06	0.000108263	IL.1alpha	0.160924	1
IL.5	2.84E-06	0.000116633	MCP.3	0.196995	1
IL.22	1.67E-05	0.000667277	IL.4	0.208458	1
IL.12.p40.	2.73E-05	0.001065877	sICAM.1	0.220940	1
IL.3	3.57E-05	0.00135787	sE.Selectin	0.231316	1
MIP.1alpha	4.25E-05	0.001571256	MIG	0.236491	1
IL.1Beta	4.38E-05	0.001578386	Eotaxin	0.351090	1
IL.9	5.04E-05	0.001763403	IL.27	0.363619	1
TRAIL	9.51E-05	0.003232708	M.CSF	0.538545	1
IL.8	0.00046	0.015285191	MCP.1	0.737987	1
IFNalpha2	0.00054	0.017363518	IP.10	0.796476	1
IFNgamma	0.00076	0.023612218	IL.2	0.846159	1
LIF	0.00122	0.036648173	IL.10	0.859462	1
GM.CSF	0.00192	0.055693796	TNF.beta	0.889548	1
MIP1.Beta	0.00362	0.101411178	MIP.3alpha	0.911239	1
IL.1RA	0.00494	0.133533236	IL.13	1	1
IL.17A	0.00769	0.200044853			

5.3.12.1 The response of HIV-infected blood to *Salmonella* stimulation is characterised by elevated TNFα, IL-22 and IL12p40 and IFNα/β

Using principal component analysis (PCA) and the data for cytokines which were significantly different in HIV-infected bloods compared to healthy blood after *Salmonella* stimulation, we were able to identify four principal components which explained 85% of the variance within the data set. PCA works by finding gradients, known as principal components (PC), within a dataset which account for the most variance in the dataset. It then assigns "loadings" onto factors (known as principal determinants) which influence those gradients. The greater the loading, the greater the contribution of that factor to the PC.

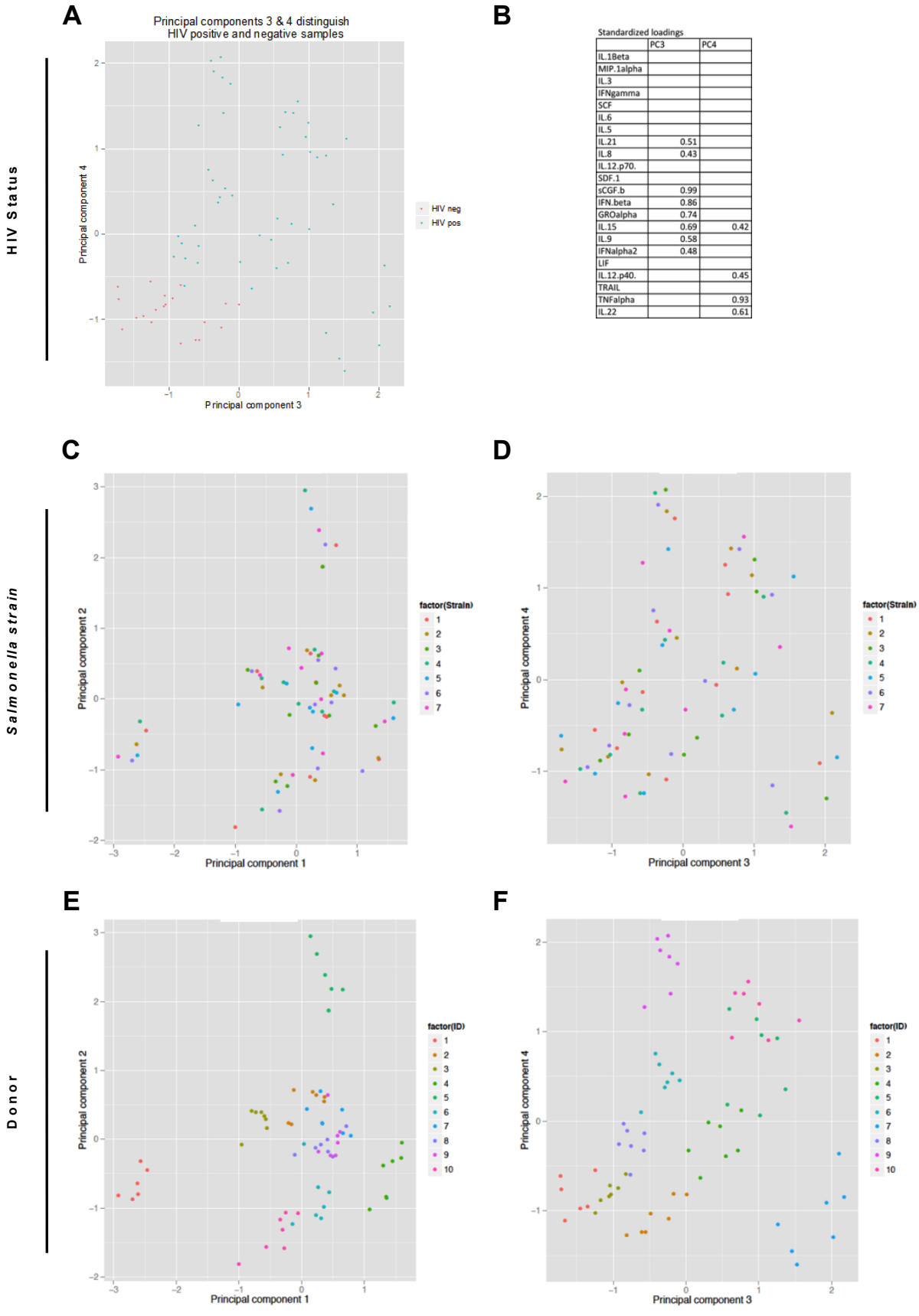
Two out of the four PCs we identified were highly discriminating for the HIV-status of the donor – PC3 and PC4 (Figure 5.16 A). The significant loadings for each PC can be seen in (Figure 5.16 B). These data show that in response to *Salmonella* stimulation, HIV-infected individuals score highly for PC3 and PC4, whereas HIV-uninfected have low scores for both. Looking at the loadings for each PC, this indicates that TNFα is elevated in HIV-infected individuals along with IL-12p40 and IL-22, whereas PC3 suggests a more Type-1 Interferon profile.

Superimposing the strains onto the PCs, PCA is unable to distinguish any of the strains (Figure 5.16 C and D) but is able to discriminate according to donor (E and F). This indicates that the variance in the data is caused by inter-individual variation and not inter-strain variation i.e. cytokine responses differ according to the individual donor and not the *Salmonella* strain used to stimulate the blood.

Whilst providing valuable insights into differential cytokine responses to *Salmonella* stimulation, PCA suffers from several limitations. Stimulating peripheral blood with multiple serovars and strains of *Salmonella,* and the small number of participants, left the study poorly powered to identify differences in the cytokine response which were dependent on the *Salmonella* serovar. Moreover, whilst being a useful tool for examining larger datasets, PCA is only a qualitative form of analysis, using loadings to identify which analytes contribute to the variance within a dataset. However, interpretation of these loadings and their meanings, relies on applying a biological hypothesis to the data which needs to be validated by further experiments.

Figure 5.16 Principal component analysis of the blood cytokine response to *Salmonella* stimulation.

PCA was able to discriminate between cytokine responses of HIV-infected (blue dots) and healthy (red dots) individuals (A). The cytokine response of HIV-infected blood to *Salmonella* was associated with increased TNFα, IL-22 and IL-12p40 along with sCGFβ and IFNα and IFNβ (B). PCA was unable to discriminate according to strain or serovar (C and D) suggesting the variance is due to inter-individual differences. All PCs were able to discriminate according to the individual donor (E and F). Each circle is representative of blood from one donor stimulated with one strain.



	PC3	PC4
IL.1Beta		
MIP.1alpha		
IL.3		
IFNgamma		
SCF		
IL.6		
IL.5		
IL.21	0.51	
IL.8	0.43	
IL.12.p70.		
SDF.1		
sCGF.b	0.99	
IFN.beta	0.86	
GROalpha	0.74	
IL.15	0.69	0.42
IL.9	0.58	
IFNalpha2	0.48	
LIF		
IL.12.p40.		0.45
TRAIL		
TNFalpha		0.93
IL.22		0.61

5.3.13. Differential blood cytokine responses to Salmonella stimulation are modified by HIV infection

Data from the first multiplex assay showed that HIV-infected blood produce a significantly altered cytokine response upon stimulation with *Salmonella* compared to healthy blood. However, having only 8 HIV-infected samples (one of which was later excluded) and 3 controls, meant the study was poorly powered to identify differences in the cytokine response due to stimulation with different strains or serovars of *Salmonella.*

We repeated the assay but this time to increase the power of the study, particularly in terms of identifying serovar-specific responses, we venesected 58 HIV-infected adults and 59 healthy controls, again stimulating their blood for 24 hours at 37C. This time only *S.* Typhi ISP-04-06979 and *S.* Typhimurium D23580 were used as wild-type, serovar representative strains.

Again, heparinised bloods were stimulated with 10^6 CFU/ml *Salmonella* for 24 hours at 37°C. Stimulated plasma was then assayed for levels of 51 different cytokines and chemokines (listed in table 5.2) in addition to IL-18 and RANTES by luminex immunoassay.

In contrast to our previous results we only observed 3 cytokines which were significantly different after *Salmonella* stimulation in HIV-infected blood compared to control blood. These were IL-6, GROα and MIG (P_{ADJ}=0.001, 0.005 and 0.03 respectively). Initially this was surprising but when the CD4 counts and VLs of the new HIV cohort

were examined it became clear that their HIV infection was well managed (as opposed to 50% of the HIV patients in the previous study which had viral loads >10,000 viral copies per ml of blood. The inclusion of these well managed HIV-infected patients in the comparison of cytokine responses likely accounts for the lower number of cytokines which were significantly different between HIV-infected and uninfected in response to *Salmonella* stimulation in this second study.

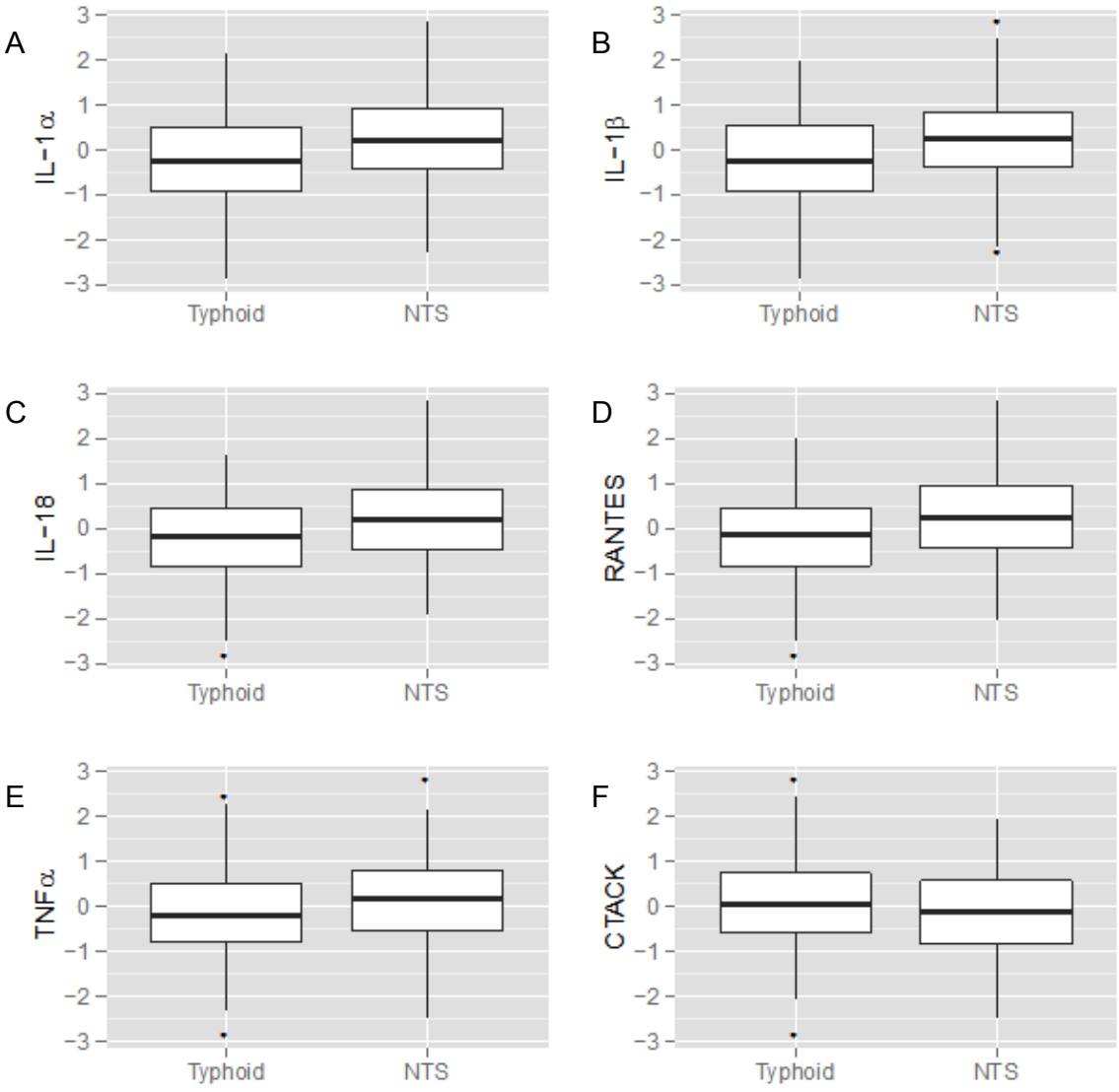


Figure 5.17. Significantly different Serovar specific blood cytokine responses after *Salmonella* stimulation.

Relative expression of blood cytokine responses after *Salmonella* stimulation were normally transformed and plotted according to serovar. Significantly different cytokines are plotted; (A) IL-1α, (B) IL-1β, (C) IL-18, (D) RANTES, (E) TNFα and (F) CTACK.

Importantly however, the power of the study was sufficient to allow us to identify serovar specific differences in cytokine responses in stimulated blood overall (in both control and HIV-infected bloods combined). We found that 6 cytokines were significantly different between stimulation of bloods with *S.* Typhi and *S.* Typhimurium. These were; IL-1α, IL-1β, IL-18, RANTES, TNFα and CTACK (P_{ADJ}<0.0001, 0.0001, 0.002, 0.005, 0.02 and 0.04 respectively) (Figure 5.17).

We then investigated whether any of the responses of these cytokines were modulated by HIV-infection. To do this we divided the HIV cohort into two groups; good control and poor control of HIV infection. Patients classed as having good control of HIV-infection had CD4 counts >350 and a VL <50, with poorly controlled patients having CD4 counts <350 and VLs >50. The relative expression data of the 6 significantly different cytokines between *S.* Typhimurium and *S.* Typhi stimulation were then normally transformed and fitted to a nested regression model. A likelihood ratio test (LRT) was used to calculate the effect of HIV infection on a particular cytokine. In Figure 5.18 we can see the effect of HIV infection and HIV infection control on each of the six cytokines. Out of the 6, only RANTES was significantly altered by HIV infection between NTS and STY according to the LRT (p= 0.03). IL-18 showed a similar, albeit non-significant, pattern as RANTES which tended towards significance (LRT p=0.06). The RANTEs observation suggests that there is a significant interaction between HIV status and *Salmonella* serovar stimulation in determining the RANTES response. This was a particularly interesting observation given the involvement of RANTES in HIV infection and the fact the direction of the RANTES response in HIV infection was lower after *S.* Typhi stimulation but higher after *S.* Typhimurium infection.

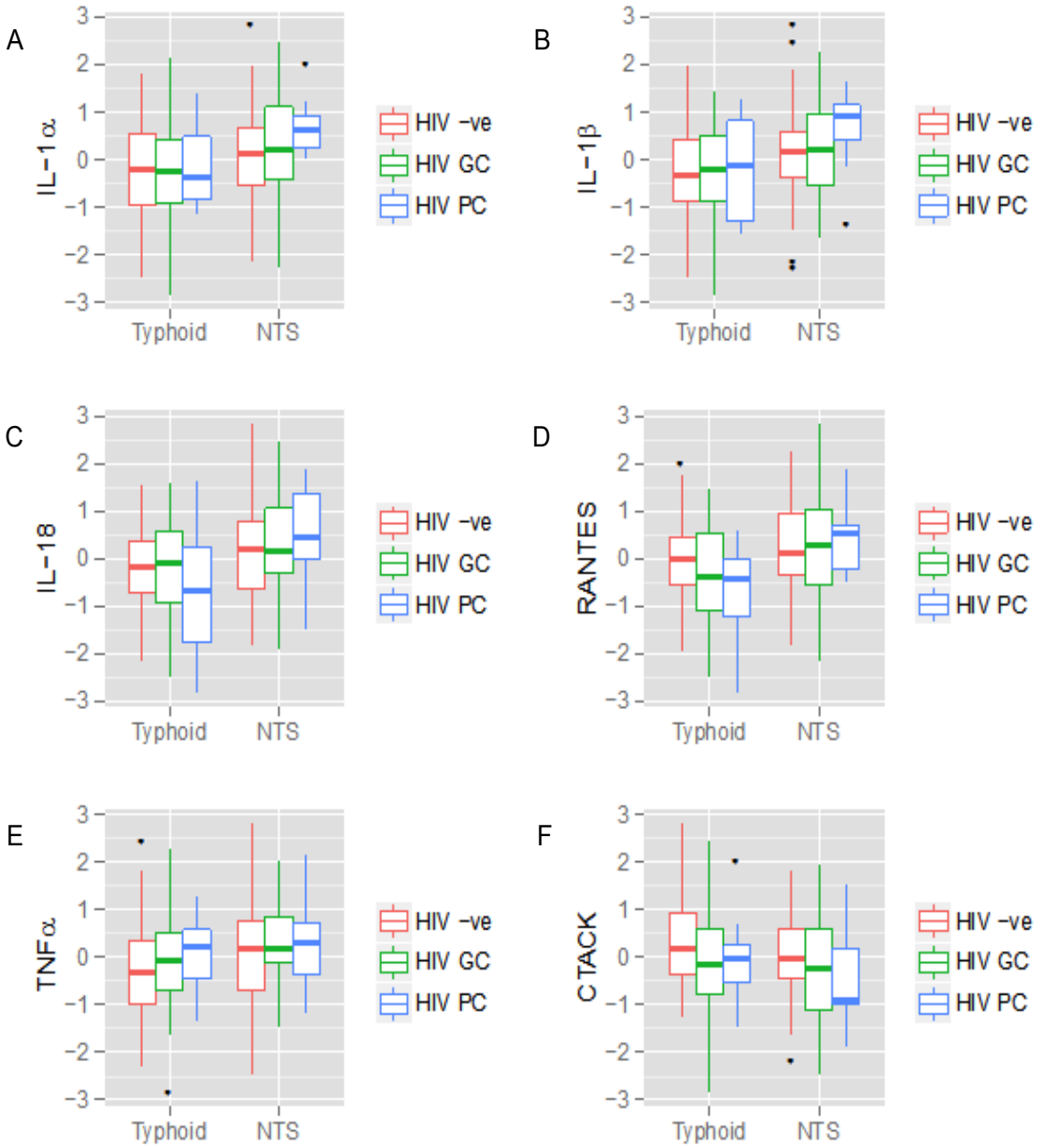


Figure 5.18. Serovar specific cytokine responses in HIV-infected and uninfected blood after *Salmonella* stimulation.

Normally transformed relative expression levels of six cytokines which were significantly different between *S.* Typhi and *S.* Typhimurium after stimulation of peripheral blood. Normally transformed relative expression levels of IL-1α (A), IL-1β (B), IL-18 (C), RANTES (D), TNFα (E) and CTACK (F) are shown and were stratified according to HIV status and control of HIV-infection (HIV -., red boxplot, well controlled HIV (GC, green boxplot) and poorly controlled HIV (PC, blue boxplot).

5.4. Discussion

Little is known about the genotypic or phenotypic differences between *Salmonella* isolates from HIV-infected individuals compared to isolates from uninfected individuals. Genotypic data suggests there is no difference between *S.* Typhimurium isolates from HIV-infected individuals compared to isolates from HIV-uninfected individuals (Msefula et al., 2012). Isolates from HIV-infected adults are significantly more resistant to antibiotics in certain locations, but whether they differ in terms of other virulence factors is unknown (Wolday and Erge, 1998). Recent genotypic data suggests *S.* Typhimurium isolates do not differ according to the HIV-status of the host (Msefula et al., 2012). We used wild-type, Tanzanian, *Salmonella* strains to demonstrate that isolates from HIV-infected individuals do not differ phenotypically in terms of their serum resistance. This was to examine whether HIV infection was selecting for infection with less virulent *Salmonella* strains which exploits HIV-associated immune defects in the host. Isolates from HIV-infected individuals varied considerably in their resistance to serum killing when tested against a standard serum. This was more obvious for *S.* Typhimurium, due to the greater number of strains we had available, but the phenotype of *S.* Typhi isolates did not appear to differ depending on whether the strain was isolated from HIV-infected or uninfected Africans and this suggests there is nothing unusual in terms of the serum resistance of these strains. The *S.* Typhi isolates from Tanzania have been collated into a sequencing project thus genotypic data for these isolates (including those from HIV-infected individuals) will be available in due course.

We initially wanted to test HIV-infected Tanzanian sera for its bactericidal activity against *S.* Typhi and measure how this compared to healthy sera to ascertain whether a protective humoral effect exists. Almost half of the Tanzanian blood products, however, had been prepared as plasma samples, thereby potentially impairing both their bactericidal and opsonic activity. We attempted to rectify this and salvage those samples by reconstituting the complement activity of plasma with the addition of exogenous divalent cations. Whilst effective, this method could not be used for the Tanzanian samples because the optimal concentration of cations required varied between donors. Moreover, addition of excess cations was able to enhance the bactericidal activity over that of homologous sera and thus we could not be sure that any killing observed after reconstitution was a natural or synthetic effect.

We used a different African serum collection from HIV-infected and uninfected Malawian adults to demonstrate that the bactericidal and opsonic activity of HIV-infected sera against wild-type *S.* Typhi is significantly impaired compared to sera from healthy adults.

Additionally we observed that anti-Vi and antiO:9 IgM titres correlated with opsonic activity and blood cell killing of *S.* Typhi in HIV-infected sera. Moreover we observed significantly lower IgM titres against Vi and O:9 in HIV-infected sera compared to uninfected. This raises the possibility that the reduced blood cell killing of *S.* Typhi after opsonised with HIV-infected sera is due to a lack of anti-Vi and anti-O:9 IgM within this sera.

Dysregulated humoral immunity to *S.* Typhimurium has been documented in HIV-infected Africans and this is due to high titres of anti-*S.* Typhimurium O-chain antibodies

which impair effective killing. We quantified titres of anti-*Salmonella* O-chain and capsular antibody but found no evidence of a relationship between titres and inhibition of killing of *S.* Typhi such as for *S.* Typhimurium. It is possible that there is an inhibitory factor present in HIV-infected sera, such as anti-OMP or flagellar antibody. However, as we only tested for levels of anti-O chain and capsular antibody we cannot say if this is the case and this is something that requires further study. The HIV-infected and control sera used in this study have previously been assessed for functional complement activity, stored at -80C and thawed in the same conditions, suggesting that Ab, and not C', is responsible for the differences in killing observed between sera from HIV-infected and healthy individuals.

Whilst these data do not identify an effect which may explain the clinical and field observations in Africa regarding HIV-typhoid co-infection, immunity to typhoid is complex and extends beyond humoral factors. Cell mediated immunity and cytokines are both important in host defence against *S.* Typhi so it may be that these are able to compensate for a defective antibody response (Kaur and Jain, 2012, Sztein, 2007). Following challenge with *S.* Typhimurium, primary macrophages from HIV-infected individuals exhibit dysregulated pro-inflammatory cytokine responses which may contribute to the increased severity of infection in the context of HIV (Gordon et al., 2007). Dysregulation of the NFκB pathway and the downstream effects of IFNγ have also been shown in HIV-infected blood after stimulation with *S.* Typhimurium LPS or flagellin (Schreiber et al., 2011). We reasoned that, while dysregulated cytokine responses in the HIV infection may be detrimental in NTS disease, they may be protective for *S.* Typhi.

We initially stimulated HIV-infected and uninfected blood from 11 Africans with 7 different *Salmonella* strains of three different serovars. However, in trying to maximise the value from the study we lacked statistical power and so whilst we observed dysregulated cytokine production in HIV-infected blood compared to control blood, we were unable to ascribe the causes of this response to any particular strain or serovar.

To increase the power of these findings, and to ascertain whether serovar dependent differences in the cytokine response occur, we stimulated a further 58 HIV-infected bloods and 59 control bloods with two wild-type, representative *Salmonella* strains of the Typhimurium and Typhi serovars; *S.* Typhimurium D23580 and *S.* Typhi. We observed significantly different cytokine responses for six cytokines after stimulation with *S.* Typhimurium compared to *S.* Typhi. Levels were significantly higher after stimulation with *S.* Typhimurium than with *S.* Typhi for 5 of the 6 cytokines, with levels of CTACK being significantly lower after *S.* Typhimurium stimulation than after *S.Typhi stimulation.*

We observed a classical pro-inflammatory response in *Salmonella* stimulated peripheral blood, which was characterised by significantly elevated levels of IL-1α/β, TNFa and Il-18 after stimulation with *S.* Typhimurium compared with *S.* Typhi. This observation is unsurprising as Vi-expression by *S.* Typhi has been shown to reduce inflammatory responses and TLR recognition of *Salmonella* epitopes (Raffatellu et al., 2005, Wilson et al., 2008).

The observation that RANTES levels were significantly different between *S.* Typhi and *S.* Typhimurium after *Salmonella* stimulation was an interesting finding given the role of RANTES as a T Cell chemokine and HIV suppressive factor (Cocchi et al., 1995,

Maghazachi et al., 1996). Moreover, RANTES was the only cytokine where HIV infection modulated the direction of the response, i.e. levels were lower after *S.* Typhi stimulation in the context of HIV infection, but higher in *S.* Typhimurium stimulation. RANTES is a relatively newly discovered cytokine and thus its role in non-HIV infectious diseases is poorly characterised. Interestingly, polymorphisms which reduce levels of RANTES expression have been associated with a reduced risk of recurrent bacterial infections in individuals with sickle cell anaemia (Dossou-Yovo et al., 2009). Fayoumi chickens which express RANTES at lower levels than other strains such as Broilers, are more resistant to *S.* Enteritidis infection (Coble et al., 2011). However, the mechanism by which differential RANTES expression might mediate *Salmonella* infection remains unknown.

It is clear that *Salmonella* stimulation of peripheral blood induces differential cytokine responses dependent on both the *Salmonella* serovar and the HIV status of the host. The 22 cytokines for which we found a significant difference in section X, in addition to RANTES and Il-18 should be further investigated to characterise their role in *Salmonella* infection, possibly through cytokine priming or murine studies. This would provide valuable insights into how these cytokines modulate *Salmonella* infection, particularly in the context of HIV infection.

5.5. *Conclusions*

The aim of this study was to identify whether a protective bactericidal or opsonic effect exists in sera from HIV-infected individuals against *S.* Typhi. Not only did we not find a protective effect, we observed that bactericidal or opsonic killing of *S.* Typhi by HIV-

infected sera was significantly impaired. This has been observed for a different serovar of *Salmonella* – Typhimurium, but not for *S.* Typhi. Killing of *S.* Typhimurium by HIV-infected sera is impaired due to the presence of inhibitory antibody. We found no evidence of a relationship between anti-*S.* Typhi LPS or capsular antibody and the inhibition of killing of *S.* Typhi and while HIV-infected sera displayed a prozone effect in the killing of *S.* Typhi so too did a similar percentage of control sera. Stimulation of HIV-infected blood with *Salmonella* resulted in a dysregulated pro-inflammatory and type-1 IFN response. Moreover we observed differential peripheral blood cytokine responses dependent on the *Salmonella* serovar. RANTES in particular was identified as a cytokine of interest, expression of which was lower in poorly controlled HIV-infected bloods after *S.* Typhi stimulation and higher in the same bloods after stimulation with *S.* Typhi. We found no evidence that antibody activity is responsible for the protective effect of HIV infection against *S.* Typhi. Instead dysregulated cytokine responses in HIV-infected individuals may account for the altered incidence of typhoid in individuals with HIV infection.

6. General Discussion

Despite advances in vaccine development and public health measures, both typhoidal *Salmonella* and NTS remain major causes of disease in endemic areas. iNTS disease is a particular problem in Africa where mortality rates are as high as 47% (Gordon et al., 2002) and the incidence of iNTS in HIV-infected individuals is approximately eight times that in HIV-uninfected (Reddy et al., 2010). Similarly, despite the existence of licenced vaccines, typhoid fever remains an endemic problem in parts of South East Asia, with the incidence as high as >500 cases/ 100,000 population in some locales (Bhan et al., 2005). No vaccines for NTS exist but several are in development (Maclennan et al., 2014), and many currently licensed typhoid vaccines are unsuitable for use in infants under two years of age or require multiple doses (Maclennan et al., 2014). To improve existing typhoid vaccines and develop effective vaccines against NTS, a better understanding of the immune response to *Salmonella,* and the cross protective nature of different anti-*Salmonella* antibodies, is required. In this PhD we explored the role of the *Salmonella* Vi capsule in resistance to the immune response and investigated the activity of two key types of anti-*Salmonella* antibodies; anti-Vi and anti-O:9 antibody, on wild-type *S.* Typhi.

In recent years an intriguing clinical phenomenon has been reported in Africa and Asia where the incidence of typhoid fever in HIV-infected individuals is, on average, approximately ten times lower than that in individuals without HIV infection (Huson et al., 2014, Reddy et al., 2010, Crump et al., 2011). The scientific basis for this phenomenon remains unclear, but understanding it may lead to improvements in our knowledge of immunity to these infections and contribute to the clinical management of invasive bacterial diseases in HIV-infected individuals. In Africa, temporal associations

between the incidence of HIV, Malaria, NTS and typhoid have been observed (Biggs et al., 2014, Feasey et al., 2013). Identifying the etiological mechanisms underlying these associations might also help in the prediction and prevention of outbreaks of these diseases.

Previous reports using both non-isogenic and isogenic *S.* Typhi have indicated that the Vi capsule increases the resistance of *S.* Typhi to the bactericidal activity of serum (Looney and Steigbigel, 1986), possibly by reducing C3 deposition (Wilson et al., 2011). In contrast *Bravo et al., 2008* reported that expression of Vi capsule does not contribute to serum resistance and suggested it acts in a similar fashion to the K5 capsule of *E. coli,* which contributes little to serum resistance but does protect against phagocytosis (Bravo et al., 2008, Burns and Hull, 1998, Burns and Hull, 1999). We found that, in comparison to their Vi^- partner strains, Vi capsule expression by either *S.* Typhimurium or *S.* Typhi was associated with significantly increased resistance to serum killing and significantly lower IgM, C3 and MAC binding and deposition. Vi-expression was also associated with significantly lower total IgG deposition in the absence of Vi-specific IgG.

Similarly, expression of Vi capsule by either serovar was associated with a significant increase in resistance to killing by washed peripheral blood cells at certain time points. This is in agreement with earlier work using non-isogenic strains (Looney and Steigbigel, 1986) which found expression of Vi capsule was associated with reduced phagocytosis and oxidative burst. In light of the reported literature and our findings, Vi capsule should be considered a *Salmonella* virulence factor which contributes both to serum resistance and resistance to blood cell killing, possibly by reducing antibody binding and complement deposition. When observations suggest Vi capsule does not

enhance resistance to serum and/or blood cell killing, methodological factors, such as the short length of the SBA used in *Bravo et al., 2008*, may be responsible and should be examined carefully.

In contrast to *Looney et al., 1986, w*e were unable to find a specific effect of Vi-expression on phagocytosis of *Salmonella* after exposure to blood for 20 minutes. It may be that this is methodological issue with our assay, or that Vi-expression does not affect the phagocytosis of *Salmonella* but instead protects against the intracellular, oxidative burst. To further understand the role of Vi capsule as a *Salmonella* virulence factor, it would be advantageous to examine the effect of Vi expression on both phagocytosis and intracellular oxidative burst production, using isogenic $Vi^{+/-}$ *Salmonella*, at different time points after *Salmonella* exposure to blood. Measuring responses over three hours would allow a comparison of data to the blood cell killing assay data and enable the contribution of phagocytosis and the oxidative burst to the blood cell killing of *Salmonella* to be examined.

We observed reductions in antibody and complement deposition onto Vi-expressing strains compared to their isogenic Vi^{-} counterparts. Previous work has suggested that the location of complement deposition is a significant factor in determining bactericidal activity, as opposed to the quantity of complement deposited (Grossman et al., 1987, Joiner et al., 1989b). To investigate this we have commenced an electron microscopy imaging project, assessing the quantity and location of IgG, IgM, C3, MAC and factor H binding to $Vi^{+/-}$ *Salmonella.* The results will help understand whether Vi capsule directs complement deposition to distal sites from the cell membrane. This would inform

vaccine development, particularly when choosing vaccine candidates other than the Vi antigen for use against Vi-expressing *Salmonella*.

The purification of human anti-O:9 and anti-Vi antibody allowed us to assess the bactericidal and opsonic potential of these antibodies against wild-type *S.* Typhi, something which to our knowledge has not been previously reported. We were able to compare the activity of anti-Vi antibody with that of anti-O:9 antibody against *S.* Typhi. Under conditions of capsule expression, anti-Vi antibody effected greater bactericidal and opsonic activity than anti-O:9 antibody. When cultured in high levels of NaCl (500mM) the activity of anti-Vi antibody was reversed with respect to that of anti-O:9 antibody, with anti-O:9 antibody effecting greater bactericidal and opsonic killing of *S.* Typhi cultured under these conditions.

These data demonstrate that both anti-Vi and anti-O:9 have bactericidal activity against *S.* Typhi, even when Vi capsule is expressed. This has implications for the development of O:9 O-chain antigen vaccines, in that an O:9 vaccine developed to protect against *S.* Enteritidis may induce antibody responses which kill *S.* Typhi. A combined Vi-O:9 vaccine should induce antibody responses which effect killing of Vi^+ *S.* Typhi and might also protect against *S.* Dublin, but further work would be required to assess this. Anti-O:9 antibody is however, an ineffective opsonin for *S.* Typhi when Vi capsule is expressed. As such, depending on the respective contribution of bactericidal and opsonic antibody in the killing of *S.* Typhi in the bloodstream, an O:9 vaccine inducing opsonic antibody may be ineffective against *S.* Typhi.

It would be prudent to test both anti-Vi and anti-O:9 combined against *S.* Typhi to assess the synergistic potential of the two antibodies as this would inform the development of a

bivalent or multivalent vaccines containing both the Vi and O:9 antigens. In light of recent observations that high titre anti-LPS antibodies are associated with the inhibition of killing of certain NTS serovars (MacLennan et al., 2010), the bactericidal and opsonic activity of very high titre anti-Vi and anti-O:9 antibodies against *S.* Typhi should be characterised to see whether a similar phenomenon occurs for *S.* Typhi and whether excess anti-O:9 antibody binding may inhibit killing of *S.* Typhi by preventing anti-Vi binding. We were unable to examine the effect of high titre antibody within our study. However, it is feasible to do so and should be tested in future as this could have implications for vaccinations of individuals prone to hypergammaglobulinemia, such as those infected with HIV.

Our data showed that Vi$^-$ *S.* Typhi is highly susceptible to both the bactericidal and opsonic action of anti-O:9 antibodies. In a separate project to this PhD, our group analysed 500 sera samples from healthy, HIV-uninfected and Malaria slide negative, Africans, all 500 sera had detectable levels of circulating anti-O:9 IgG and 70.2% had ≥ 100 O:9 IgG EU (MacLennan CA et al, unpublished data). Despite this, virulent Vi$^-$ *S.* Typhi isolates have been reported (Baker et al., 2005), raising fears of Vi$^-$ *S.* Typhi strain replacement as a result of widespread Vi vaccination. While the sera we analysed were African, and the Vi$^-$ *S.* Typhi isolates were recorded in Pakistan, these ELISA data still suggest that in areas of *Salmonella* transmission, the general population has circulating anti-O:9 antibody which might effect killing of Vi$^-$ *S.* Typhi. As such, Vi$^-$ *S* Typhi clinical isolates should be screened for possible mutations in genes affecting the chain length of the LPS O-antigen such as the *fepE* gene (Bravo et al., 2008, Crawford et al., 2013), or for changes affecting the structure of outer membrane proteins involved in resistance to complement-and cell-mediated killing such as *Rck* and *PgtE* (Ramu et al., 2007, Ho et al.,

2010). The presence of these changes could represent the evolution of new *S.* Typhi virulence mechanisms as a result of vaccine-related selection pressure.

A surprising finding was the observation that anti-O:9 antibody was able to bind to *S.* Typhi and facilitate MAC deposition onto Vi-expressing *Salmonella.* However the level of anti-O:9 mediated MAC deposition was not associated with bactericidal activity. We also observed approximately 1 $\log_{10}$ of killing of Vi^+ *S.* Typhi using anti-O:9 antibody in the SBAs. These findings were unexpected as it is thought that Vi capsule blocks access to LPS, explaining why Vi-expressing strains do not, or only weakly, agglutinate with anti-O:9 antibody. Moreover, Vi capsule inhibits TLR4 recognition of LPS (Wilson et al., 2008)(Figure 6.1, "shield" arrangement).

Our data indicate that the O-antigen of LPS is accessible to anti-O:9 antibody binding even when Vi capsule is expressed. This suggests that the O-antigen component of LPS extends beyond the Vi capsule (Figure 6.1 C, "net" arrangement). As the agonist of TLR4 is the lipid A portion of LPS (Kawai and Akira, 2010), it may be that the capsule extends beyond the lipid A portion of LPS but not beyond the O-antigen. With respect to agglutination, a lack of agglutination is not a definitive indicator of a lack of antigen, and Vi-expression might shift the agglutination reaction into the post-zone stage by limiting the amount of anti-O:9 antibody that can bind to the O:9 O-antigen. Thus agglutination results may be affected by capsular expression whilst still allowing anti-O:9 binding which is detectable by more sensitive assays such as flow cytometry.

Currently these are speculative ideas, but if the capsule is arranged in a way that O-antigen, but not lipid A, was exposed, this arrangement would agree with both our data

and the literature. We explore these potential LPS and Vi capsule arrangements on *Salmonella* in Figure 6.1.

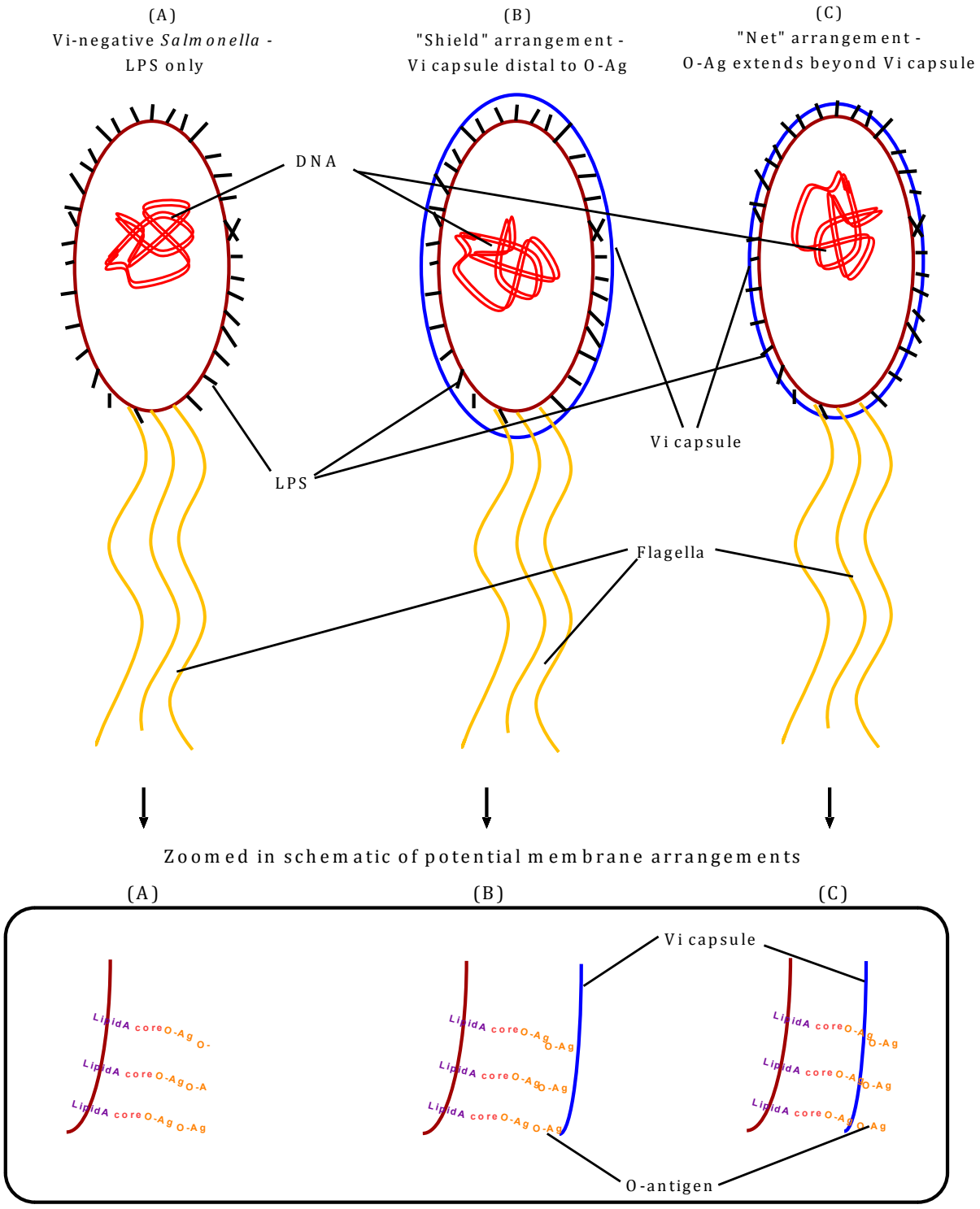

(A)
Vi-negative *Salmonella* -
LPS only
(B)
"Shield" arrangement -
Vi capsule distal to O-Ag
(C)
"Net" arrangement -
O-Ag extends beyond Vi capsule
DNA
LPS
Vi capsule
Flagella
Zoomed in schematic of potential membrane arrangements
(A)
(B)
(C)
Vi capsule
O-antigen

Figure 6.1. Schematic of possible Vi capsule and LPS arrangements on *S.* Typhi.

Vi$^-$ *S.* Typhi does not express capsule, agglutinates with anti-O:9 antibody and activates TLR4 (A). If Vi capsule is arranged as depicted in the "shield" variation (B), with capsule distal to the LPS O-antigen, it should not agglutinate with anti-O:9 antibody, activate TLR4 or experience detectable anti-O:9 antibody binding by flow cytometry. Vi$^+$ *S.* Typhi, with O-antigen extending distally to the Vi capsule and lipid A proximal to the cell surface such as in the "net" arrangement (C), should not activate TLR4, but could still experience anti-O:9 antibody binding such as we observe in our data, whilst having reduced or absent anti-O:9 agglutination. Vi capsule is represented by the blue line, O-antigen in orange and lipid A in purple.

Once optimised, affinity chromatography purification of antibodies from human sera is a viable way of isolating relatively high and pure amounts of specific antibody which can be used in *in vitro* assays to assess the killing potential of the specific-antibody. This method should be used to examine the potential of other *Salmonella* vaccine candidates, for instance *SseB*, *OmpC*, *OmpF* and *OmpD*. Conjugation of protein antigens to affinity chromatography columns is possible, provided the elution buffer is carefully selected so as to not disrupt the column protein-linker molecule bond, for example, if the protein in question precipitates at low pH, a high pH or ionic elution buffer should be used. Purification of antibodies to proteinaceous antigens is something which has been applied to *H. influenzae* specific antibodies (Hetherington, 1989) and should be considered for a greater variety of anti-*Salmonella* antibodies. This technique should contribute to the understanding of the bactericidal and opsonic activity of antibodies induced by potential vaccine candidates.

The aim of the HIV-*S.* Typhi/ NTS project was also to identify *in vitro* mechanisms which may contribute to the apparent protective effect of HIV-infection on *S.* Typhi infection. Dysregulated humoral immunity in HIV-infected Africans to NTS has been reported (MacLennan et al., 2010). We wanted to examine whether a similar phenomenon existed

in the case of *S.* Typhi as opposed to NTS, and whether in this scenario, the HIV-associated dysregulation conferred a protective effect against typhoid.

We found no evidence for such an effect, and using sera from HIV-infected and uninfected Africans, we observed that HIV-infected sera had significantly reduced bactericidal and opsonic activity against *S.* Typhi compared to control sera. We measured antibody titres to the immunodominant *Salmonella* antigens, Vi, O:4,5 and O:9 in both the HIV-infected and uninfected sera and observed reduced levels of IgM to all three antigens and elevated levels of anti-O:4,5 IgA in HIV-infected sera compared with uninfected sera. Given the association between anti-Vi and anti-O:9 IgM titres and the killing of *S.* Typhi in the blood cell killing assay, a lack of IgM against O:9 and Vi in the HIV-infected sera may be responsible for the reduced opsonic activity of these sera, but further work is required to confirm whether this is the case.

All the Malawian sera had also been tested for, and found to have functional complement activity, suggesting an absence of complement was not responsible for the reduced killing of *S.* Typhi observed in the HIV-infected sera. The HIV-infected and control sera should be retested and screened for antibody titres against a wider array of immunogenic *Salmonella* antigens to identify whether a deficit or abundance of antibody in the HIV-infected and/or control sera is responsible for the differences in killing activity.

Gordon et al .,2007 reported dysregulated cytokine responses in primary macrophages from HIV-infected individuals to *S.* Typhimurium which may increase the susceptibility to *Salmonella* infection (Gordon et al., 2007). We stimulated peripheral blood from HIV-infected and uninfected individuals with representative strains of *S.* Typhi and *S.*

Typhimurium. Significantly different cytokine responses were observed in HIV-infected individuals compared to controls, suggesting cytokine dysregulation in response to *Salmonella* stimulation does occur in peripheral blood. However, in the preliminary study we were unable to differentiate serovar-specific responses. In a second study using solely *S.* Typhi and *S.* Typhimurium and a greater number of donors, we observed dysregulation, and were able to differentiate serovar-specific responses. Intriguingly for one cytokine, RANTES, the direction of the response was significantly altered by HIV-infection, with levels being reduced in HIV-infected blood after *s.* Typhi stimulation but higher in the same bloods after *S.* Typhimurium stimulation. A similar, albeit non-significant, effect was observed for IL-18 which tended towards significance (p=0.06). The role of RANTES in bacterial infections is poorly understood, while IL-18 has a well described pro-inflammatory role, inducing IFNγ (Eckmann and Kagnoff, 2001, Dinarello and Fantuzzi, 2003). Why and/or how changes in levels of these chemokines may alter *Salmonella* infection in HIV-infected individuals is unknown. Further work is needed to characterise the effects of changes in levels of these cytokines and investigate the mechanisms responsible and potential impacts in a clinical setting. The stimulation assay should be repeated, focusing on only the 24 cytokines (22 from the preliminary study plus RANTES and IL-18) which were found to be significantly different in HIV-infected individuals compared to controls, after *Salmonella* stimulation. It would also be of value to stimulate blood for different time periods such as 4, 24 and 48 hours and also to be more stringent with donor selection to only select HIV-infected individuals classed as having "poor control" of the virus i.e. CD4 <350, VL >50. This should confirm whether the serovar specific effects on the cytokine responses between HIV-infected and uninfected observed exist and identify additional serovar specific responses which may

have been hidden in the second study due to the large number of well controlled HIV-infected patients.

Investigation into the role of RANTES and IL-18 in *Salmonella* infection should be done to examine the effect of these cytokine on different serovars of *Salmonella,* and how this might relate to HIV-infection. One could do this via *in vitro* cytokine priming and antibody neutralisation experiments to examine whether increasing or decreasing RANTES or IL-18 levels affects peripheral blood killing of both *S.* Typhimurium and *S.* Typhi. The effect of changing these cytokines on the production of other cytokines important to *Salmonella* infection such as IFNγ and TNFα, should also be measured. Murine infection studies using RANTES or IL-18 deficient mice, or supplying exogenous RANTES or IL-18 during the course of *Salmonella* infection may also provide insights into the role of these cytokines in *Salmonella* infection. As *S.* Typhi is host restricted, murine models are not suited to investigating immune responses to this pathogen. Tissue culture and/or humanised mice may be beneficial in this respect.

6.1. Future work

Currently, serological correlates of protection to either typhoid or iNTS remain elusive and poorly defined and the identification of such a correlate would have profound vaccinology implications (Maclennan et al., 2014). Given the vast number of *Salmonella* serovars and the likelihood that a serological correlate of protection will differ between serovars, identifying a correlate of protection will not be simple and should be prioritised for the most clinically important serovars such as *S.* Typhimurium, *S.* Enteritidis, *S.* Typhi and *S.* Paratyphi.

Establishing a correlate of protection could be done in one of two ways. Firstly, a serological sampling study could be run either alone, or attached to an existing longitudinal study, in a S*almonella* endemic area. The aim of this would be to collect frequent blood samples from a sizeable population whilst monitoring for reported or confirmed *Salmonella* infection. Familial groups, or other closely related groups such as school children or co-workers, with differential infection would be of particular interest, and should allow protective titres to be established given the likelihood of shared exposure within such groups. A study designed in this way has the potential for antibody titres to be correlated with protection and should identify serological factors important to the resistance to infection.

One of the major findings from this PhD is that different antibodies can effect bactericidal activity against *S.* Typhi, but that efficacy varies between antibodies. Given this finding, in a correlate of protection study, the widest possible range of antibodies to *Salmonella* antigens within the sample sera should be screened, including antibody isotypes. In theory, and partly based on observations herein, low titres of highly bactericidal and/or opsonic antibody have the potential to offer greater protection than high titre antibody of low efficacy (the differential killing of *S.* Typhi by anti-Vi and anti-O:9 antibodies for example). So, a wider antibody screen may detect antibody against other, less immunogenic, *Salmonella* antigens which may play a role in protection. Moreover, it is possible that different antibodies interfere with one another or with the action of complement (MacLennan et al., 2010) so the ratios of different antibody populations should be examined, as the effect of one antibody may be influenced by the action of a different antibody.

An alternative way of establishing a correlate of protection would be a human challenge study. A longitudinal study would not allow the infectious dose of *Salmonella* to be established, given the environmental source of infection. A human challenge study would allow the infectious dose to be controlled and the infection delivered in a monitored environment. Provided the sample group has sufficient variation within their antibody titres and populations, a correlate of protection could be established this way. A study designed in this way would allow dose-specific correlates of protection to be identified. Such an investigation is better suited to identifying correlates of protection against the enteric fevers as opposed to iNTS, given the immunocompromised status of the host and the severity of infection of the latter. However, infecting the required number of people to ensure the study is sufficiently powered poses both ethical and safety issues.

Within this thesis, we show that Vi expression is associated with increased resistance to serum and blood cell killing. As discussed previously, blood cell killing is a culmination of both the phagocytosis of *Salmonella* and their subsequent killing by the oxidative burst. We were unable to identify whether Vi-associated reductions in blood cell killing were a result of reduced phagocytosis, oxidative burst or both mechanisms. We observed no difference in phagocytosis of Vi-expressing and non-expressing *Salmonella*, however methodological issues may be responsible for this. To fully elucidate the role of Vi capsule in the phagocytic and oxidative burst processes, both should be measured by flow cytometry over a longer time period; 3 hours for example. Oxidative burst can be measured by Phagoburst text kits (BD) using flow cytometry. Using the isogenic $Vi^{+/-}$ *Salmonella* strains in such an experiment should allow the effect of Vi capsule on these processes to be characterised effectively.

Moreover, we observed that in the blood cell killing assay, under conditions of low osmolarity, opsonisation by purified human anti-Vi antibody lead to greater killing than with anti-O:9 antibody. The effects of opsonisation with either purified antibody on the oxidative burst could and should also be measured, and may enable greater understanding of the different effects of the purified antibodies on facilitating cell-mediated killing of *Salmonella*.

MacLennan et al., 2010 reported that high titre anti-LPS antibodies in a subset of HIV-infected individuals interfere with the killing of *S.* Typhimurium. Whether a similar effect occurs for other *Salmonella* serovars and for other antibodies is unknown. With O:9 conjugate vaccines, and newer, more immunogenic Vi vaccines in development, it would be prudent to examine the effect of very high titre anti-Vi and anti-O;9 antibodies on the bactericidal and opsonic killing of wild-type *S.* Typhi. Using the affinity columns it should be possible to purify excessively high titre, concentrated antibody against these two antigens which can then be tested in in vitro killing assays to detect whether a prozone effect occurs. Synergy of the two purified antibodies should also be assessed to ascertain whether they complement, or compete against, each other – the findings from this could influence vaccine design in terms of continuing with, or adopting either a monovalent or bivalent vaccine approach respectively.

Finally, we found no evidence of a beneficial effect of HIV infection on the bactericidal or opsonic activity of sera against wild-type *S.* Typhi. If a dichotomy of *Salmonella* infection in the context of HIV infection does exist, these preliminary data suggest that the underlying cause is not a humoral one. However, we did find evidence of differential cytokine expression in HIV-infected and uninfected blood after stimulation with either *S.*

Typhimurium or *S.* Typhi. RANTES in particular, but also IL-18, were identified as being of interest. Further work is required to consolidate this observation and investigate the mechanisms underlying it, ideally using a large cohort of HIV-infected individuals with low CD4 counts.

RANTES is a relatively nascent cytokine, but it is known to be a T cell and monocyte chemokine and a potent suppressor of HIV replication (Cocchi et al., 1995, Kedzierska and Crowe, 2001). Very little is known about the role of RANTES in bacterial infections, thus further investigation should help to clarify this in the context of *Salmonella.* Using the isogenic pairs of $Vi^{+/-}$ *Salmonella* it would be possible to investigate whether it is the Vi capsule which is responsible for the altered RANTES response that we observe after *Salmonella* stimulation. Moreover, priming of blood lymphocytes followed by in vitro killing assays, or murine in vivo RANTES depletion studies followed by infection, possibly with Vi^{+} *S.* Typhimurium should shed further light on the role of this chemokine in *Salmonella* infection.

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