UNIVERSITY OF
LIVERPOOL

NATURAL IMMUNITY TO *SALMONELLA* IN HUMANS

Thesis submitted in accordance with the requirements of the

University of Malawi and University of Liverpool for the degree of

Doctor in Philosophy

by

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University of Malawi and University of Liverpool

DECLARATION

THIS WORK HAS NOT PREVIOUSLY BEEN ACCEPTED IN SUBSTANCE FOR ANY DEGREE AND IS NOT BEING CURRENTLY SUBMITTED IN CANDIDATURE FOR ANY DEGREE

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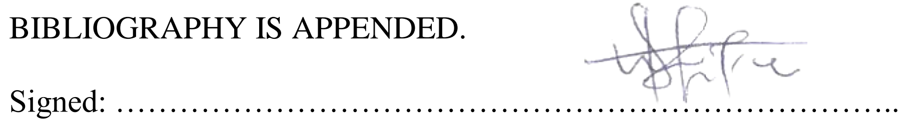
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I HEREBY GIVE CONSENT FOR MY THESIS. IF ACCEPTED, TO BE AVAILABLE FOR PHOTOCOPYING AND FOR INTER-LIBRARY LOAN AND FOR THE TITLE AND ABSTRACT TO BE MADE AVAILABLE TO OUTSIDE ORGANISATIONS.

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DECLARATION OF WORK DONE

This work was part of four studies titled "Development of adaptive immunity to nontyphoidal *Salmonella* in Malawian children", "*Salmonella* exposure and development of specific immunity in Malawian children", "Development of T cell and antibody mediated immunity in response to invasive *Salmonella* infection" and "Ty21a oral typhoid vaccine induced immunity in the peripheral blood and gut mucosa of healthy adults". All studies were under the supervision of Dr Melita Gordon, Dr Wilson Mandala, Prof Robert Heyderman and Prof Stephen Gordon. Some of the work in my study was shared among a number of individuals. My contributions for the reported work were as follows:

Activity	Responsibility
Study designs and protocols	shared
Ethical applications	shared
Study community and health care workers sensitizations	shared
Participant's recruitment and consent	others
Study participants follow up	others
Clinical assessment	others
Sample collection (stool, oropharynx swabs, blood, milk, biopsy)	others
HIV testing	others
Malaria testing	others

Blood stream infection surveillance	others
Immuno-phenotyping (IPT)	sole
Intracellular cytokine staining (ICS)	sole
Enzyme-linked immunosorbent assay (ELISA)	sole
Serum Bactericidal Assay (SBA)	sole
Preparation of *S.* Typhimurium homogenate	sole
Development of real time PCR primers	shared
DNA extraction	sole
Real time PCR testing	sole
Development of Milk Bactericidal Assay	sole
Isolation of *Salmonella* in stool and oropharynx	sole
ELISpot	shared

ABSTRACT

Background: *Salmonella* bacteraemia is an important public health problem in children from sub Saharan Africa (SSA). Understanding what constitutes natural acquired immunity to *Salmonella* is crucial for the development of *Salmonella* vaccine. It was hypothesized that natural *Salmonella* exposure within the GIT and peripheral blood induces the generation of specific-antibodies and T cells and these might provide protection to subsequent *Salmonella* infection.

Methods: Natural acquisition of antibody and T cell immunity to *Salmonella* was investigated in healthy and *Salmonella* infected Malawian children. Acquisition of typhoid vaccine induced T cell immunity in healthy adults from the United Kingdom (UK) was investigated to model natural immunizing events occurring within the gut associated lymphoid tissues (GALTs) following *Salmonella* infection. Acquisition of immunity was examined using immunological tools including the intra-cellular cytokine staining assay (ICS), serum bactericidal activity (SBA) assay, ELISA and ELISpot. Exposure to *Salmonella* was examined using microbiological tools including standard culture and real-time PCR.

Principal findings: CD4+ T cells and IgG antibodies to *Salmonella* develops sequentially in under-five children. Acquisition of *Salmonella*-specific CD4+ T cells and antibodies coincides with the decline in *S*. Typhimurium bacteraemia cases in older children. As much as 47% of Malawian children (aged 6-18 months) are exposed to *Salmonella* at least once within the gastrointestinal tract (GIT). Natural *Salmonella*

exposure within the GIT is associated with development of potentially protective SBA in children. Invasive *Salmonella* infection elicits an increase in generation of *Salmonella*-specific CD4+T cells, IgG and IgA antibody secreting cells (ASC). Oral Ty21a vaccination (model of natural *Salmonella* infection) did not elicit an increase in generation of both CD4+Cytokine+ and CD8+Cytokine+ T cells in the peripheral blood and gut mucosa compartments at day 11, and day 18 post vaccination.

Conclusion: Young children (<2 years of age) are more vulnerable to invasive *Salmonella* infection. *Salmonella* exposure within the GIT and peripheral blood compartments tissues facilitates acquisition of robust immunity (mediated by antibodies and T cells) in children and these might provide protection to subsequent *Salmonella* infection. Public health interventions are urgently required in SSA including vaccination with cross-protective *Salmonella* vaccine, improvements in sanitation, access to clean and safe water and food hygiene.

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CARTA also tremendously contributed to refining research study ideas and analysis of the data through joint advanced seminars (JAS).

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ABBREVIATIONS

AE	Adverse effect
AF700	Alexa Fluor 700
APC	Allophycocyanin
APC	Antigen presenting cell
ART	Antiretroviral therapy
ASC	Antibody secreting cells
BCA	Bicinchoninic Acid
BCIP	Bromo-4-chloro-3-indolyl-phosphate solution
BMP	Blantyre Malaria Project
BSA	Bovine serum albumin
BSI	Blood stream infection
CD	Cluster of differentiation
CFTR	Cystic fibrosis transmembrane receptor
CFU	Colony forming units
CGD	Chronic granulomatous disease
CI	Confidence interval
CM	Central memory
COMREC	College of Medicine Ethics Committee
CPT	Cotrimoxazole prophylactic therapy
CR	Complement receptor

CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
DHO	District health office
Dot EIA	Dot enzyme immunosorbent assay
EDTA	Ethylenediaminetetraacetic acid
EID	Early infant diagnosis
ELISA	Enzyme-linked-immunosorbent assay
ELISpot	Enzyme-Linked ImmunoSpot
EM	Effector memory
FcR	Fc receptor
FDC	Follicular dendritic cells
FITC	Fluorescein isothiocyanate
FMO	Fluorochrome Minus One
FSC	Forward scatter
GALT	Gut Associated Lymphoid Tissue
GCP	Good Clinical Practice
GIT	Gastro intestinal tract
GMMA	Generalized Modules from Membrane Antigens
GPS	Global Positioning System (GPS)
H	Flagellin H antigen
H2S	Hydrogen sulphide
HIV	Human Immunodeficiency Virus

HLA	Human leukocyte antigen
HPF	High power field (HPF)
ICS	Intra-cellular cytokine staining
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
ION	Ionomycin
LB	Luria Bertani
LOD	Limit of detection
LP	Lamina propria
LPS	Lipopolysaccharide
LSTM	Liverpool School of Tropical Medicine
M cells	Microfold cells
MAC	Complement membrane attack
MAC	MacConkey
MAdCAM1	Mucosal vascular address in cell adhesion molecule 1
MBP	Mannose binding protein
MDR	Multi-drug resistant
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node

MLST	Multilocus sequence type
MLW	Malawi Liverpool Wellcome Trust
MMNC	Mucosal mononuclear cells
MP	Membrane proteins
MUAC	Mid-Upper Arm Circumference
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NBCS	Newborn calf serum
NBT	Nitro blue tetrazolium
NHC	Ndirande Health Centre
NK	Natural killer
NLF	Non-lactose fermenters
Nramp1	Natural resistance-associated macrophage protein one
NTS	Nontyphoidal *Salmonella*
O	Somatic O antigen
OMP	Outer membrane protein
OR	Odds ratio
p	Probability value
pag	PhoP activated genes
PAMPS	Pathogen associated molecular patterns
PB	Pacific blue
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PCV	Packed cell volume
PE	Phycoerythrin
PLN	Peripheral Lymph Node
PMA	Phorbol myristate acetate
PMN	Polymorph nuclear cell
PP	Peyer's patches
prg	PhoP-repressed genes
PRR	Pathogen recognition receptors
PSO	Polysaccharide O antigen
QECH	Queen Elizabeth Central Hospital
rck	Resistance to complement killing
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
RT	Room temperature
RT PCR	Real time PCR
SBA	Serum bactericidal activity
SEB	Staphylococcal enterotoxin B
SIV	Simian immunodeficiency virus
SPI	*Salmonella* Pathogenicity Islands
SSA	sub-Saharan Africa
SSC	Side scatter
SW	Soche Ward

TCR	T cell receptor
TD	Thymus dependent
TGF-β1	Transforming growth factor beta
Th1	T helper 1
Th17	T helper 17
Th2	T helper 2
TI	Thymus independent
TLR	Toll Like Receptor
TNF-α	Tumour necrosis factor alpha
TSI	Triple sugar iron
TTSS or T3SS	Type three secretion system
URT	Upper respiratory tract
Vi	Capsular Vi antigen
Vi CPV	Vi capsular polysaccharide vaccine
WB	Whole blood
WHO	World health organization
XLD	Xylose lysine deoxycholate
ZHC	Zingwangwa Health Centre

CHAPTER 1: INTRODUCTION

1. THEME AND OVERVIEW

This thesis focusses on a number of cross-cutting issues including natural and vaccine induced immunity to *Salmonella* in humans. Investigations into natural immunity aimed at characterizing the development of antibody and T cell immunity to *Salmonella* in healthy children and children with *Salmonella* bacteraemia during the acute and convalescent phases, and the relationship between *Salmonella* exposure events within the gastrointestinal tract (GIT) and the development of *Salmonella*-specific serum immunity in healthy children. Investigations into oral Ty21a vaccine induced immunity aimed modelling natural *Salmonella* infection immunizing events occurring within the gut associated lymphoid tissues (GALT) and secondary lymphoid tissues (SLTs) in healthy adults from the UK.

1.1 THE *SALMONELLA* BACTERIUM

Salmonella are Gram-negative, facultative and rod-shaped bacteria belonging to Enterobacteriaceae family. Members of *Salmonella* genus are mainly motile, aerogenic, non-lactose fermenting, oxidase negative, urease negative, citrate-utilizing and produce hydrogen sulphide.

1.2 NOMENCLATURE

The genus name *Salmonella* was adopted in honour of an American scientist Dr Daniel Elmer Salmon whose assistant researcher Theobald Smith discovered *Salmonella* from the intestine of a pig in 1884 (Su & Chiu, 2007, Schultz, 2008). Salmonellae are widely distributed in nature and cause gastroenteritis and bacteraemia in both humans and animals. The nomenclature of *Salmonella* has been the subject of scientific debate for some time because historically the taxonomy of *Salmonella* genus was based on names according to epidemiology, clinical conditions, host range, biochemical reactions and surface antigenic patterns (Su & Chiu, 2007, Agbaje*, et al.*, 2011). These early taxonomy approaches proved to be inconsistent in dividing the genus into species and serovars. The advent of nucleotide technology in early 1970s was the turning point for *Salmonella* genus nomenclature (Crosa*, et al.*, 1973). Nucleotide sequence relatedness as demonstrated by DNA-DNA hybridization experiments uncovered that typical salmonellae were closely related and could be considered a single species with the exception of *Salmonella bongori* which was shown to have a distinct nucleotide sequence (Le Minor*, et al.*, 1982, Reeves*, et al.*, 1989). In 1986, a proposal to designate *Salmonella enterica* as the only species was recommended by Le Minor and Popff and received overwhelming acceptance by the subcommittee of Enterobacteriaceae of the International Committee on Systematic Bacteriology at the XIV International Congress of Microbiology (Su & Chiu, 2007). In 1989, Reeves and colleagues published and upgraded *Salmonella enterica* subsp. Bongori from subspecies to *Salmonella bongori* species (Reeves*, et al.*, 1989). The current nomenclature used by the Centre for Disease

Control and Prevention is borne out of recommendation from World Health Organization's (WHO) collaborating centre based on the two-species *Salmonella enterica and bongori* with each species containing multiple serovars (Agbaje*, et al.*, 2011). Currently, more than 2,500 *Salmonella* serovars have been identified based on antibody reaction with surface antigens: O (somatic), H (flagellin) and Vi (Vi or capsular polysaccharide) as described by Kauffman and White. The Kauffman-White scheme is now re-designated as the White Kauffman-Le Minor Scheme and this document has a list of all identified *Salmonella* serovars (Agbaje*, et al.*, 2011). The current nomenclature is summarized in the Figure 1.1.

All salmonellae of direct relevance to human infection fall within the subspecies *enterica.* Note that, for example, the full written designation of *Salmonella enterica*, subsp. *enterica* Typhimurium is commonly shortened to *Salmonella* Typhimurium or *S.* Typhimurium, and this modification will be followed in this thesis.

S. Typhi, *S.* Paratyphi A and B are all human host restricted *Salmonella* serovars while *S.* Typhimurium and *S*. Enteritidis have a wider host range and spread between humans and domestic animals such as chicken, goat and cattle.

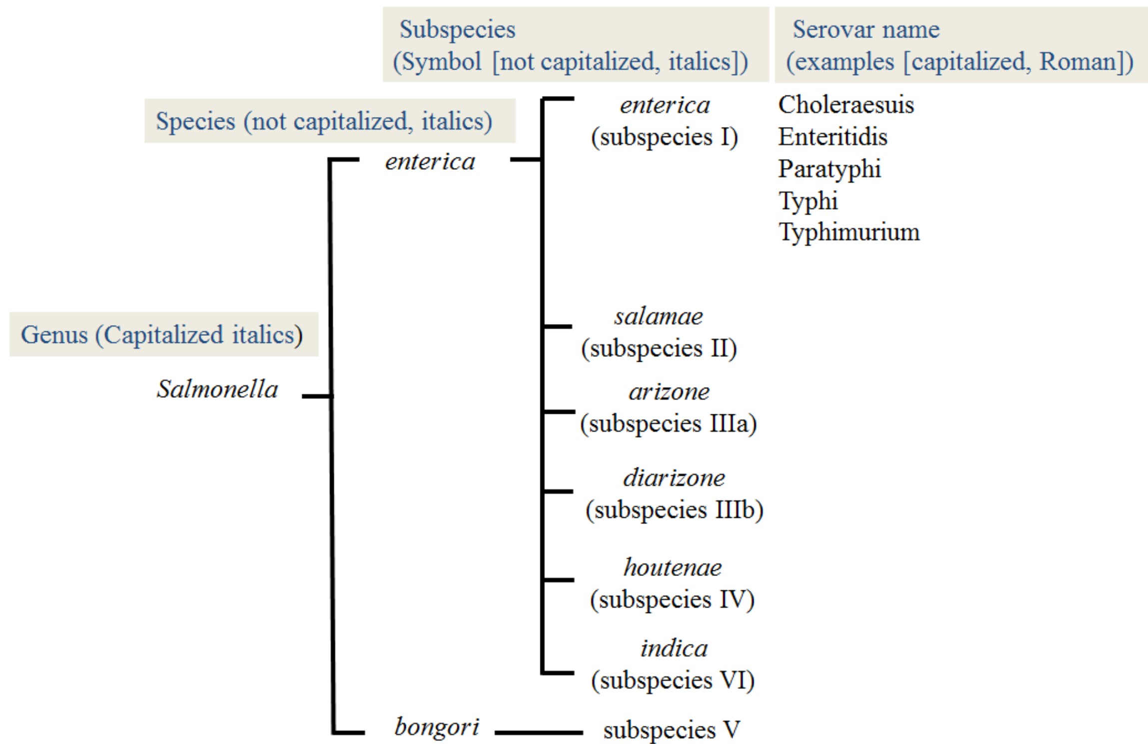

Figure 1. 1: Current *Salmonella* nomenclature

In this introductory chapter we focus on *S.* Typhimurium and also provide comparative background with *S.* Typhi and *S.* Enteritidis.

1.3 BURDEN OF *SALMONELLA* INFECTIONS IN HUMANS

1.3.1 Geographical distribution and epidemiology of *S*. Typhi

Over the last century, cases of enteric fever (typhoid fever) caused by *S*. Typhi have tremendously reduced in the developed countries (Clark, *et al.*, 2010). In the developing countries particularly in Eastern Asia, the sub-continent of India and Africa, where access to clean water is low and people live in poor sanitary conditions, enteric fever is still endemic (Figure 1.2) (Crump, *et al.*, 2004, Crump & Mintz, 2010). It is estimated that 21.7 million people worldwide contract typhoid infection annually and about 217,000 of these typhoid cases die (assuming a conservative case fatality rate of 1%) (Crump, *et al.*, 2004, Crump & Mintz, 2010). In the year 2004, in south Asia, southeast Asia and the Indian Subcontinent, the overall incidence rate of typhoid fever was as high as >100/100,000 cases per year (Crump, *et al.*, 2004). Toddlers, preschool children, school going children and young adults are at risk of typhoid disease (Pasetti, *et al.*, 2011)

In sub-Saharan Africa (SSA), estimating the true burden of typhoid fever has been challenging because data is patchy and confined largely to sentinel facility-based surveillance (Crump & Heyderman, 2014). In Kenyan urban slums, the incidence of *S*. Typhi bacteraemia was 247 cases per 100,000 person-years of observation among children <10 years and this was similar to incidence of *S*. Typhi bacteraemia in Asian urban slums (Breiman, *et al.*, 2012).

In SSA, multi-drug resistant (MDR) *S*. Typhi isolates have increased in the recent years (Kariuki*, et al.*, 2010, Lutterloh*, et al.*, 2012). In Kenya, MDR *S.* Typhi isolates appear to belong to a single haplotype H58. This haplotype H58 MDR *S.* Typhi was first identified in Southeast Asia suggesting intercontinental spread of a single MDR clone (Kariuki*, et al.*, 2010). In Zambia, between 2010 and 2012 the outbreak of typhoid affected about 2,000 with case fatality rate of 0.5% (Hendriksen*, et al.*, 2014). A majority of *S*. Typhi isolates in Zambia are MDR (83%) and belong to MLST ST1 and haplotype H58B (Hendriksen*, et al.*, 2014).

In Malawi and Mozambique border MDR *S.* Typhi associated with neurological manifestation were reported in 2009 (Lutterloh*, et al.*, 2012, Sejvar*, et al.*, 2012). Out of 214 suspected cases, 47 deaths were registered, representing case fatality rate of 5% (Lutterloh*, et al.*, 2012). In Malawi, between 2011 and 2014, through blood stream infection surveillance that Malawi Liverpool Wellcome Trust (MLW) has undertaken at a referral hospital in Blantyre, approximately 2,000 *S*. Typhi were isolated in blood and 95% of these isolates are MDR (unpublished data). MDR isolates from Blantyre, Malawi also belong to haplotype H58 (unpublished data).

Since 2008, in Uganda outbreaks of typhoid associated with increased intestinal perforation in the districts of Kasese (ranging 40-80%) and Bundibugyo (more than 20%) were reported (Neil*, et al.*, 2012, Walters*, et al.*, 2014). Case fatality rate for *S.* Typhi bacteraemia was 8.1% (47 deaths/577 cases) in the district of Kasese (Neil*, et al.*,

2012). Estimated *S.* Typhi incidence in the community survey is considerably high at 8,092 cases/100,000 persons (Neil*, et al.*, 2012).

In Dzivaresekwa and Kuwadzana suburb of Harare City, Zimbabwe, typhoid outbreak involving approximately 2,600 confirmed and suspected-cases were reported in 2011 (Muti*, et al.*, 2014, Polonsky*, et al.*, 2014). These outbreaks were attributed to environmental sources (Centers for Disease & Prevention, 2012). Taken together these reports demonstrate that the burden of typhoid fever in the developing countries is considerably high and requires public health interventions including vaccination and improvements in food hygiene and sanitation.

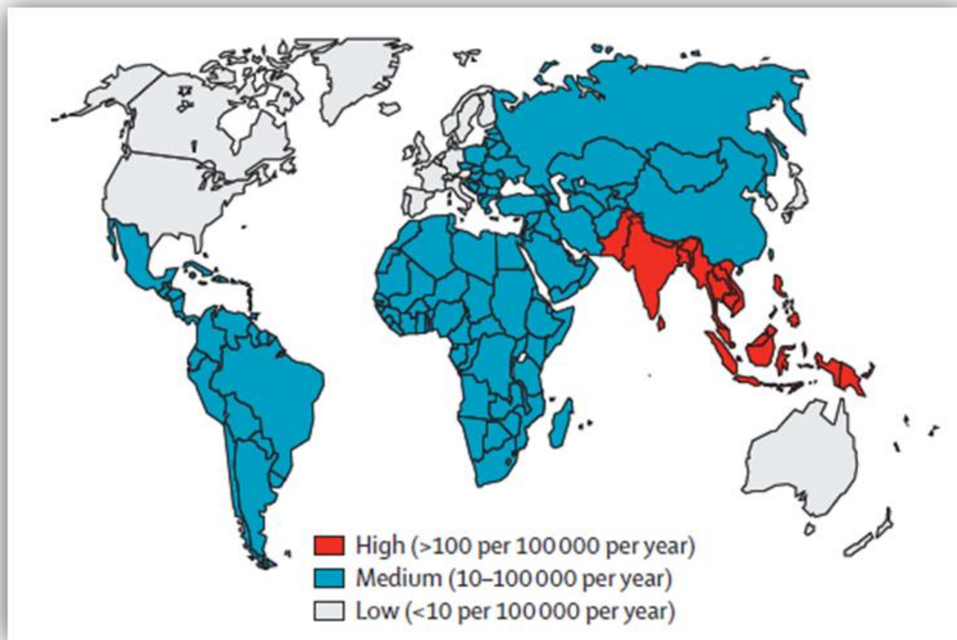

Figure 1. 2: Geographical distribution of typhoid fever

(Adapted from Bull World Health Org 2004:82:351)

1.3.2 Geographical distribution and epidemiology of nontyphoidal *Salmonella*

Nontyphoidal *Salmonella* (NTS), principally *Salmonella enterica* serovars Typhimurium (*S*. Typhimurium) and Enteritidis (*S*. Enteritidis) causes gastroenteritis worldwide but commonly cause life-threatening bacteraemia in SSA (Figure 1.3) (Reddy*, et al.*, 2010, Feasey*, et al.*, 2012). NTS bacteraemia is particularly common in children below 3 years of age and HIV infected individuals (Gordon*, et al.*, 2008). Other important NTS bacteraemia comorbidities in children from SSA include malaria, malnutrition, anaemia and sickle cell (Gordon*, et al.*, 2008).

A meta-analysis demonstrated that about 10% of all infections in Africa are blood stream infection (BSI) (Figure 1.3) (Reddy*, et al.*, 2010). The important bacterial pathogens causing BSI in Africa include; *Salmonella*, *Streptococcus pneumoniae, Staphylococcus aureus* and *E. coli*. Approximately 90% of *Salmonella* BSI are caused by NTS (Reddy*, et al.*, 2010). Fever surveillance in 11 sites located in 7 countries across SSA (Tanzania, Mozambique, Malawi, Kenya, Ghana, Gabon and Burkina Faso) during the RTS,S/AS01 malaria vaccine phase 3 trials demonstrated NTS bacteraemia incidence of 478 cases/100,000 patients per year in children aged 5 and 17 months (Agnandji*, et al.*, 2011). NTS case fatality in children, exceed 20%, even with appropriate antimicrobial treatment (Gordon*, et al.*, 2002, Gordon*, et al.*, 2008).

Over the past 6 years NTS bacteraemia have been decreasing in SSA and this has been attributed in part to the decline in malaria cases (important risk factor in young children)

and introduction of antiretroviral therapy (ART) (HIV is an important risk factor) (Kariuki*, et al.*, 2006, Mackenzie*, et al.*, 2010, Feasey*, et al.*, 2014). In Blantyre, Malawi, between 1997 and 2008 blood stream infections (BSI) declined from 16% of suspected cases to 10% (a majority of these BSIs were caused by NTS) (Feasey*, et al.*, 2014). Prior to the introduction of free antiretroviral therapy (ART) and cotrimoxazole prophylactic therapy (CPT) in Malawi public hospitals case fatality of NTS bacteraemia was more than 40% in HIV infected individuals. BSI case fatality rate has reduced from 40% to 14% following the roll out of antiretroviral therapy (ART) and cotrimoxazole prophylactic therapy (CPT) (Feasey*, et al.*, 2014).

Taken together, although NTS bacteraemia cases has fallen in the recent years, the burden of remain considerably high (cases of NTS bacteraemia and related case fatality rate remain high). Public health interventions including vaccination and improvements in food hygiene and environmental sanitation are required.

1.3.3 Invasive NTS molecular genetics

Evidence from whole genome sequencing of *S*. Typhimurium strain D23580, the commonest NTS serovar isolated in Malawi and a representative invasive NTS strain in SSA, uncovered that it has a particular multilocus sequence type (MLST), ST313 which is distinct from the classical *S*. Typhimurium sequence type ST19 associated with gastroenteritis in other parts of the world (Kingsley*, et al.*, 2009, de Jong*, et al.*, 2012). High throughput genome sequencing of *S.* Typhimurium strain D23580 has also revealed

loss of genes implicated in virulence such as ssel, ratB, and the accumulation of 44 novel pseudogenes or deletions relative to *S*. Typhimurium strain LT2 (Kingsley*, et al.*, 2009, de Jong*, et al.*, 2012). Taken together, ST313 appear to have undergone genomic degradation similar to *S.* Typhi which suggests possible loss of an enteric lifestyle and possible human-host adaptation (Msefula*, et al.*, 2012, Okoro*, et al.*, 2012) and this have not been thoroughly investigated.

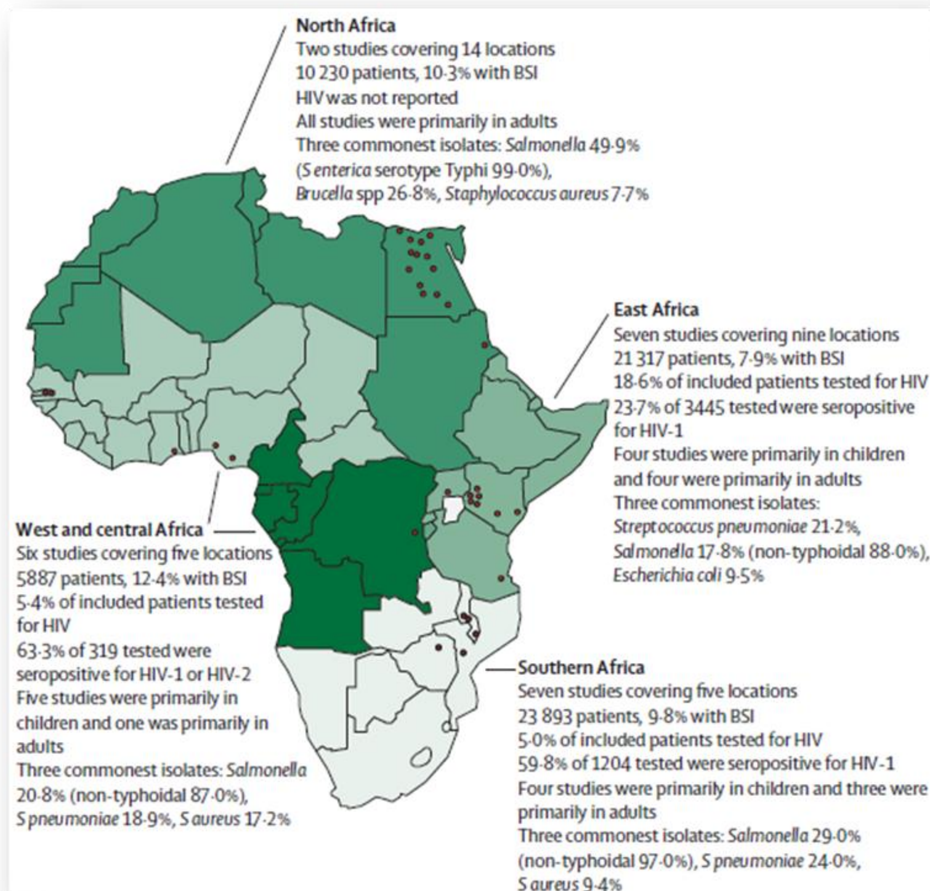

Figure 1. 3: Community acquired-blood stream infections in Africa

(Adapted from Reddy EA Lancet Infect Dis 2010:10:417-32)

1.3.4 Environmental risk factors

1.3.4.1 Food and water

Salmonella infection is contracted through oral-faecal route. In many developing countries (particularly in Eastern Asia, the sub-continent of India and Africa) food hygiene practices, sanitary conditions remain poor and access to clean water is still low (Crump & Mintz, 2010, Kariuki*, et al.*, 2010, Breiman*, et al.*, 2012). These inefficacies hugely contribute to the risk of contracting *Salmonella* infections. This is further supported by evidenced of seasonal peaks of NTS bacteraemia during the rainy season (Kariuki*, et al.*, 2006, Gordon*, et al.*, 2008, Morpeth*, et al.*, 2009). In Africa, it has been demonstrated that enteric organisms such as *Salmonella* are found at highest concentrations in drinking water source at the onset of the wet season (Wright, 1986). In the developed countries food such as meat, eggs and daily products have all been implicated as the vehicles of NTS transmission (Crump*, et al.*, 2002). Similarly NTS have also been isolated from cattle, goats, sheep and pigs in Africa (Morpeth*, et al.*, 2009). However in Africa, there is no proven link of invasive NTS isolates from animals and humans (Kariuki*, et al.*, 2006, Feasey*, et al.*, 2012).

1.3.4.2 **Transmission of *Salmonella***

S. Typhi, *S.* Paratyphi A and B are all human host restricted while NTS have a wider host range and spread between humans and domestic animals. Children may excrete NTS in their stool for some weeks after recovering from enteric infection (Morpeth*, et al.*, 2009).

Asymptomatic NTS carriers have previously been demonstrated in Africa (Nkuo-Akenji, *et al.*, 2001). Duration of NTS excretion and carriage could form the basis of *Salmonella* transmission from human to humans and animals to humans. Contact with domestic animals, particularly chickens is a well-established risk factor of contracting NTS infection (Crump*, et al.*, 2002). Kariuki *et al* in Kenya, demonstrated carriage of identical NTS strains in stool of children with invasive disease and human household contacts, while there was lack of identical NTS strains in children with invasive disease and isolates from households domestic animals and environment (Kariuki*, et al.*, 2006). These findings suggested anthroponotic transmission (human-host restricted) and not zoonotic transmission (Kariuki*, et al.*, 2006). *S*. Typhimurium strain D23580 has recently been shown to cause invasive disease in chickens (Parsons*, et al.*, 2013), but whether chicken could be the vehicle for transmission of invasive NTS strain from animals to humans in Africa has not been shown. Taken together, at the moment it is not clear whether transmission of invasive NTS isolates in Africa occur from human to humans and also animal to humans.

1.4 CLINICAL FEATURES AND MANAGEMENT OF INVASIVE *SALMONELLA* DISEASE

1.4.1 Clinical features of typhoid fever

Typhoid fever is an invasive, systemic clinical syndrome that is characterized by high fever. Typhoid patients also presents with bradycardia, malaise, headache, cough and

abdominal pains during the first week of infection. During the second week typhoid patients may present with rose spots, hepato-splenomegaly, diarrhoea and constipation. During the third week typhoid patients may develop complications including sepsis and shock, gastrointestinal bleeding or perforation, encephalopathy, and focal metastatic complications such as cholecystitis or hepatitis (Weening, *et al.*, 2005, Crump, *et al.*, 2008, Gordon, 2008, Nambiar, *et al.*, 2009, Feasey, *et al.*, 2012).

1.4.2 Clinical presentation of invasive NTS disease (NTS bacteraemia)

NTS bacteraemia clinical presentation is poorly defined especially in young children and exhibit clinical overlap with the presentations of both pneumonia and malaria (Graham, *et al.*, 2000, Graham & English, 2009, Feasey, *et al.*, 2012). Invasive NTS disease typically presents as a febrile systemic illness similar to enteric fever (Graham, *et al.*, 2000, Gordon, *et al.*, 2002, Feasey, *et al.*, 2012). Adults with *Salmonella* bacteraemia commonly presents with a combination of high fever and splenomegaly (Peters, *et al.*, 2004). NTS also causes meningitis in children and adults and this is associated with poor health outcomes (Molyneux, *et al.*, 2009). Case fatality of NTS meningitis is 52% in young children and 80% in adults (Molyneux, *et al.*, 2009).

1.4.3 **Treatment of typhoid fever**

Emergence of MDR *S.* Typhi resistant to ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, amoxicillin and ciprofloxacin commonly used for typhoid treatment

has been reported in Africa and Asia (Kariuki*, et al.*, 2010, Zaki & Karande, 2011). Ciprofloxacin resistance is an increasing problem, especially in the Indian subcontinent and Southeast Asia (Rowe*, et al.*, 1997, Kariuki*, et al.*, 2010). In-case of ciprofloxacin resistance, azithromycin is the recommended first line treatment and it performs better at treating uncomplicated enteric fever than both fluoroquinolone drugs and ceftriaxone (Crump & Mintz, 2010, Aggarwal*, et al.*, 2011, Zaki & Karande, 2011). Azithromycin significantly reduces relapse rates compared to ceftriaxone (Effa & Bukirwa, 2011).

Current treatment regime for typhoid fever is associated with better clinical outcomes compared to the past. For instance, during three episodes of *S.* Typhi outbreak between 1948-1990, Van den Bergh *et al* demonstrated that typhoid patient's treated with full dose of chloramphenicol cleared high fever in 6 days, 13% of cases developed complications and mortality was 5% while typhoid patients that were not treated, fever cleared after 16 days, 38% developed complications and mortality was 26% (van den Bergh*, et al.*, 1999). A recent review by Parry and colleagues, reported that typhoid patients treated with fluoroquinolones on average fever-clearance time is less than four days, and the cure rates exceed 96 percent (Parry*, et al.*, 2002). They also reported that less than 2 percent of treated patients become persistent faecal carriers or relapse occurs (positive faecal culture at the end of treatment or recurrence of symptoms with a positive blood or bone marrow culture after hospital discharge) (Parry*, et al.*, 2002). These reports show that clinical outcome of treated typhoid patients are generally good.

1.4.4 Treatment of NTS bacteraemia

In Malawi and SSA the epidemics of MDR invasive NTS have emerged (Gordon*, et al.*, 2008). MDR are defined as resistance to ampicillin, chloramphenicol, and co-trimoxazole (Gordon*, et al.*, 2008). Currently NTS sepsis is treated with the third generation cephalosporins and fluoroquinolones such as ciprofloxacin (Feasey*, et al.*, 2012). Azithromycin is alternative antimicrobial drug while ceftriaxone is the preferred first line intravenous treatment for patients unable to take oral dugs (Feasey*, et al.*, 2012).

1.5 *S.* TYPHIMURIUM AND *S.* TYPHI AS PATHOGENS AND HOST ADAPTIVE DIFFERENCES

This section provides a description of *Salmonella* bacterium structure to appreciate their importance in disease pathogenesis.

1.5.1 Flagella

Flagella are long helical filaments attached to rotary motors embedded with the membrane and are essential for motility during infection (Figure 1.4) (de Jong*, et al.*, 2012). The main subunit of *Salmonella* flagella is encoded by fliC or fljB and these correspond to H1 and H2 variants of the H antigen, respectively (Silverman & Simon, 1980). Interestingly, only one type of flagellin can be expressed at a specific time through a phase variation mechanism (Simon*, et al.*, 1980). In contrast to *S*. Typhimurium where variation of flagellin types has been observed, *S*. Typhi are mostly monophasic since they lack the fljB locus (Frankel*, et al.*, 1989). Parkhill *et al* have demonstrated that fljB is a

pseudogene in *S*. Typhi which may explain this phenomenon (Parkhill, *et al.*, 2001). During the early phase of *S*. Typhimurium infection within the intestine, flagellin has been implicated in up-regulation of pro-inflammatory cytokines (IFN-γ, TNF-α, IL-1β) and the chemo-attractant (IL-8), and results in the influx of neutrophils into the gut mucosal tissue (Zeng, *et al.*, 2003). In-contrast, *S*. Typhi, through TviA (*S*. Typhi specific DNA region preventing the production of IL-8 during invasion of intestinal epithelial cells) repress flagellin, therefore avoiding toll like receptor 5 (TLR-5) signalling and subsequent migration of neutrophil through IL-8 secretion (Raffatellu, *et al.*, 2005, Winter, *et al.*, 2008).

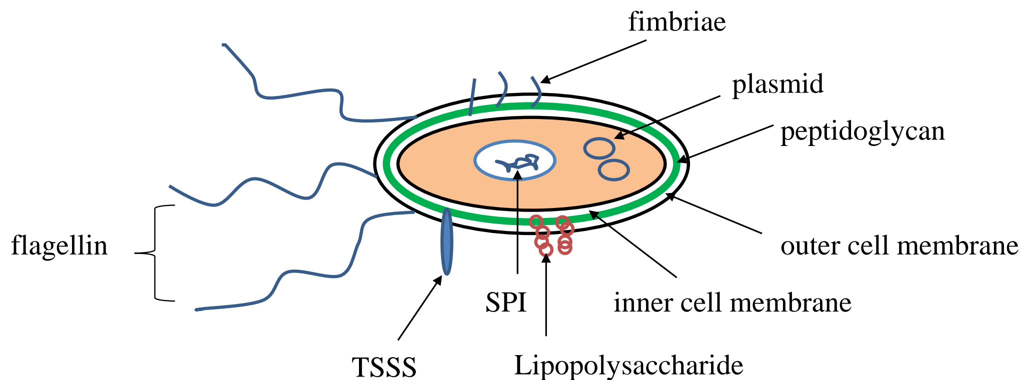

Figure 1. 4: Structure of *S*. Typhimurium

1.5.2 Vi capsule

The Vi antigen is a polysaccharide capsule and is present in only three *Salmonella* serovars; *S*. Typhi, *S*. Paratyphi C and *S*. Dublin (Pickard, *et al.*, 2003). Vi has important roles in *S*. Typhi virulence and is regulated by the two loci: viaA and viaB (Kolyva, *et al.*,

1992). It is thought that Vi presence increases disease infectivity and severity (Wain*, et al.*, 2005). Contradicting these thoughts, in human volunteers exposed to Vi capsule negative *S*. Typhi mutants exhibit a typhoid-like disease (Zhang*, et al.*, 2008). These observations suggest that Vi is not absolutely required for *S.* Typhi disease infectivity and severity. In addition, Vi capsule is associated with resistance to serum bactericidal activity and intracellular survival (Looney & Steigbigel, 1986, Miyake*, et al.*, 1998). Vi capsule prevents recognition of *S.* Typhi lipopolysaccharide (LPS) by the host TLR-4 and inflammation in the intestinal mucosa (Sharma & Qadri, 2004, Wilson*, et al.*, 2008). Together these observations indicate that the Vi capsule facilitates immune-modulatory activities in the intestinal mucosa by limiting *S*. Typhi LPS and TLR-4 interaction (limits pro-inflammatory response and influx of neutrophils), while at the same time promoting dissemination of *S*. Typhi to distant tissues (escape serum bactericidal activity and promoting survival inside the phagocytes).

1.5.3 Lipopolysaccharide

Lipopolysaccharide (LPS) is the main cell wall component of all Gram negative bacteria and is comprised of three structural regions; O antigen, core and lipid A (Ernst*, et al.*, 2001) (Figure 1.4 and 1.5). Both O antigen and core are made up of polysaccharide chains while lipid A is made up of the fatty acid and phosphates substitutes bound to a central glucosamine dimer. In addition, lipid A comprises the outer leaflet made of the outer membrane lipid bilayer, while the inner leaflet is a phospholipid. Primarily LPS prevents bacteria from complement attack. Assembly of membrane attack complex

(MAC) is affected by the chemistry of the O polysaccharide, the length of O polysaccharide chain and relative amounts of long chain smooth LPS (Rautemaa & Meri, 1999, Murray*, et al.*, 2006). MacLennan *et al* demonstrated previously that NTS strains causing invasive NTS disease in Malawi had long-chain lipopolysaccharide and *rck* gene (MacLennan*, et al.*, 2008), features that resist direct complement-mediated killing (Heffernan*, et al.*, 1992, Heffernan*, et al.*, 1992). Lack of *ex vivo* NTS killing and complement deposition in serum from Malawian children (< 16 months of age) lacking anti-*Salmonella* antibody strongly suggest that long-chain lipopolysaccharide and *rck* confer protection against complement in the absence of specific IgG or IgM antibody in humans (MacLennan*, et al.*, 2008). The O-specific chain is very variable even within the Gram-negative species and this form the chemical basis for serological classification of *Salmonella*. In addition, LPS components; O-antigen, core polysaccharide and lipid A activates cellular immune system through interactions with TLR-4 and its accessory protein termed MD-2 (Beutler, 2000, Kaisho & Akira, 2000).

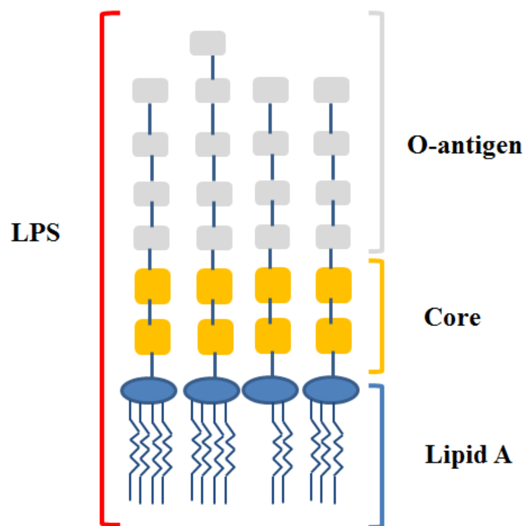

Figure 1. 5: Chemical structure of Gram-negative lipopolysaccharide

1.5.4 *Salmonella* Pathogenicity Island and Type Three Secretion System

Salmonella Pathogenicity Islands (SPI) are genetic structures where virulence genes cluster in a localized region of the chromosome (Figure 1.4) (Groisman & Ochman, 1996). *Salmonella* acquire SPI virulence genes through horizontal gene transfer (Groisman & Ochman, 1996). There are up to 21 SPIs known to date, but only SPI-1 and SPI-2 have been extensively studied (Sabbagh, *et al.*, 2010). For delivery of virulence proteins, SPIs utilize the type three secretion system (TTSS or T3SS). TTSS are specialized virulence devices capable of modifying host cell function (signal transduction, cytoskeletal architecture, membrane trafficking and cytokine gene expression) through the direct translocation of bacterial virulence proteins into the host cells cytoplasm (Hueck, 1998, Ohl & Miller, 2001). Unique sets of virulence proteins

contribute to the distinct virulence phenotype of each *Salmonella* serovar (Ohl & Miller, 2001).

Salmonella enterica contains two TTSS gene clusters encoding a secretion apparatus that functions like a molecular syringe (de Jong*, et al.*, 2012). SPI-1 harbours the genes for TTSS-1 which are essential during the early phase of *Salmonella* infection for invasion of non-phagocytic cell like intestinal epithelial cells (M cells) and induction of intestinal secretory and inflammatory responses (Watson*, et al.*, 1995, Galan, 1999). In-vitro models uncovered that *Salmonella* mutants lacking a functional SPI-1 TSSS are unable to invade epithelial cells or trigger cytokine production (Watson*, et al.*, 1995). These attributes permits *Salmonella* (typically *S.* Typhimurium) to invade the intestinal mucosa barriers, trigger pro-inflammatory responses, neutrophils influx and but may fail to establish system infection when overcome by host resistance at this early phase of infection.

TTSS-2 encoded on SPI-2 is essential during the second phase of *Salmonella* infection, for replication inside the macrophages and establishment of systemic infection (Hensel*, et al.*, 1998) (Forest*, et al.*, 2010). TTSS-2 is activated in the phagosome and it translocate effector proteins from phagosome into macrophage cytosol and prevents co-localization of *Salmonella* containing vacuoles (SCV) with NADPH dependent oxidase that catalyses production of reactive oxygen species within the phagocyte (Uchiya*, et al.*, 1999, Ohl & Miller, 2001). These attributes permit *Salmonella enterica* (typically *S.* Typhi) to survive and replicates within the host macrophage and establish systemic infection.

1.5.5 PhoP/PhoQ regulator

PhoP/PhoQ regulators are two component regulators that regulate simple signal transduction systems of bacterial gene expression in response to environmental cues (Miller, *et al.*, 1989, Ohl & Miller, 2001). PhoP/PhoQ regulators are found in many Gram-negative bacteria including *Salmonella* (Groisman, 2001). PhoP/PhoQ regulation involve triggering expression of the PhoP activated genes (pag) and repressing expression of genes termed PhoP-repressed genes (prg) (Miller, *et al.*, 1989, Ohl & Miller, 2001). PhoP-activated genes are expressed (SPI-2 TSSS) for intra-cellular survival while PhoP regressed genes (SPI-1 TTSS) are switched off in the phagosome (Pegues, *et al.*, 1995). Using PhoP null and PhoP mutant's experiments, it has been shown that timely regulation of the PhoP/PhoQ regulator is essential for *Salmonella* survival (Miller & Mekalanos, 1990). At least in part, PhoP- activated gene promotes resistance to antimicrobial peptides by catalysing covalent modification of the lipid A component of LPS (Guo, *et al.*, 1998, Ohl & Miller, 2001). PhoP regulated lipid A modification also promotes a bacterium LPS molecule to avoid pro-inflammatory response (Guo, *et al.*, 1997). These attributes allow *Salmonella* (typically *S*. Typhi) to survive and replicate within the host macrophage and establish systemic infection.

1.6 IMMUNITY TO *SALMONELLA* INFECTION

In this section we discuss immunity to *Salmonella*. Human studies on immunity to *Salmonella* are generally lacking and much of what is known about immunity to *Salmonella* comes from murine studies. Since *S*. Typhi is human host restricted and cannot cause disease in mice, therefore immunity to *S.* Typhi is actually investigated in *S*. Typhimurium murine models. It therefore possible that what is true for immunity to *S*. Typhimurium could not be true for immunity to *S.* Typhi.

1.6.1 Spread of ingested *Salmonella* to distant tissues via the GIT

To fully understand the human host response to *Salmonella* infection it is important to appreciate the steps that lead to tissue invasion. Human beings ingest *Salmonella* from contaminated food, water and fomites (Kariuki*, et al.*, 2006). *Salmonella* reaches the distal ileum or caecum, following the survival of host protective mechanisms that include competition with normal flora and gastric acid toxicity (Mastroeni, 2003, Tam*, et al.*, 2008). Murine models reveal that Peyer's patches (PP) and epithelial covering villi are the key sites where *Salmonella* penetrates the gut epithelial barrier (Tam*, et al.*, 2008) *Salmonella* invades the PP specialised epithelial cells known as Microfold cells (M cells) through SPI-1 (Monack*, et al.*, 2004) (Figure 1.6). *Salmonella* are also transported from the gastrointestinal tract to the blood stream by CD18 expressing phagocytes (Vazquez-Torres*, et al.*, 1999). *Salmonella* also penetrates the gut epithelial barrier through dendritic cells (DC). DCs extend their dendrites between epithelial cells, overlying villi

and capture gut luminal *Salmonella*. DCs then translocate the captured *Salmonella* into the lamina propria (LP) by retracting its dendrites (Rescigno, *et al.*, 2001). *Salmonella* that penetrate the PP, directly access the mesenteric lymph node (MLN) while LP *Salmonella* finds their way to the MLN through the lymphatic drainage system (Monack, *et al.*, 2004). Resident macrophages ingest *Salmonella* in the PP and MLN, to control *Salmonella* infection. However, virulent *Salmonella* may evade macrophage immunity by inducing macrophage cell death through SPI-1 encoded Sip B protein which activates caspase 1 (Monack, *et al.*, 2004). Depending on *Salmonella* virulence and host immunological factors, *Salmonella* can remain restricted to the MLN or disseminate via the thoracic duct to systemic organs including peripheral blood, spleen, liver and bone marrow (Mastroeni, 2003, Sansonetti, 2004).

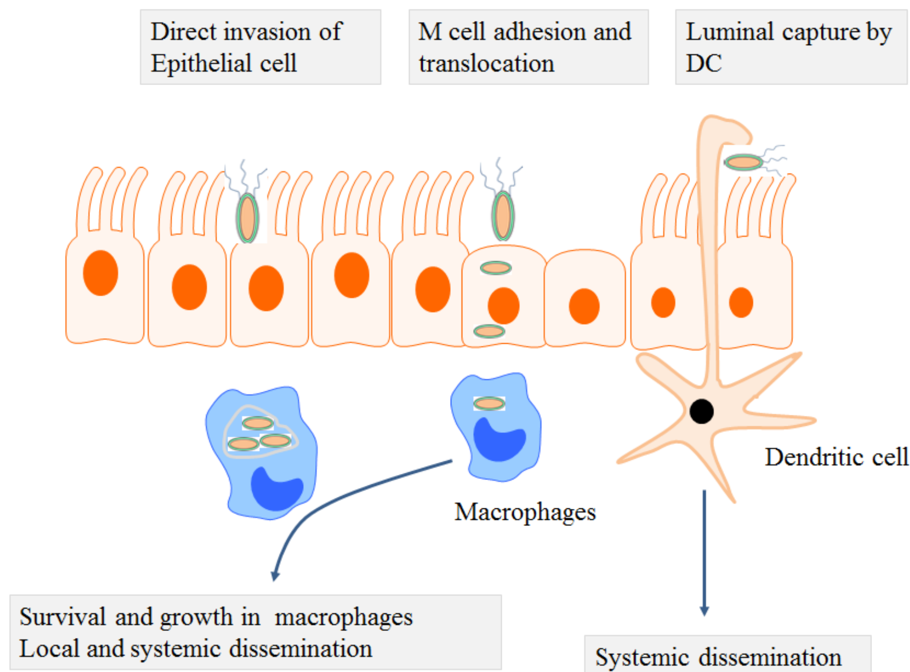

Figure 1. 6: *Salmonella* ingestion and invasion of the gut mucosal barrier

At the gut lumen *Salmonella* evade epithelial through direct invasion (depend on the virulence of *Salmonella* serovar) of the epithelial cells, through the M cells and DC capture of the *Salmonella* from the gut lumen. In the gut mucosal *Salmonella* can be killed by innate immune cells (neutrophils and macrophages) and adaptive immune cells (B cells and T cells) or survives and disseminate to distance tissues.

1.6.2 Innate immunity in response to *Salmonella* infection

In addition to gastric pH, innate immunity provides the first line of defence against *Salmonella* infection. It comprises the cellular components such as macrophages and neutrophils and the soluble components such as complement and cytokines.

1.6.2.1 **Activation of innate immune responses to *Salmonella* infection**

Innate cells (neutrophils and monocytes) are primarily activated through pathogen recognition receptors (PRR) expressed on cell surfaces such as toll like receptor-4 (TLR-4) when they recognise pathogen associated molecular patterns (PAMPS) such as lipopolysaccharide (LPS) expressed by infecting pathogens. Important specific PAMPs and TLRs interactions in *Salmonella* and innate cells involves; LPS and TLR-4, flagellin and TLR-5 and lipopeptide and TLR-2/6 (Srinivasan & McSorley, 2006, Tam*, et al.*, 2008). For instance, TLR-4 and LPS interactions result in signal transduction that culminates into activation of NFκB and leads to the transcription of pro-inflammatory cytokines such as TNF-α. The importance of macrophage TLR-4 has been shown in LPS *d/d* mice (TLR-4 defect mice). LPS *d/d* mice are hypo responsive to *Salmonella* LPS and

fail to control *Salmonella* growth compared to wild type mice that efficiently control *Salmonella* growth (Hormaeche, 1990, Mastroeni, 2002).

1.6.2.2 Phagocytosis and intracellular killing by phagocytes

Monocytes and neutrophils are important for controlling *Salmonella* during the early phase of infection (Fierer, 2001, Cheminay*, et al.*, 2004, Tam*, et al.*, 2008). Mice rendered neutropenic by administration of granulocyte depleting monoclonal antibodies, are more susceptible to *Salmonella* infection compared to wild type mice, indicating neutrophils importance in controlling early *Salmonella* infection (Conlan, 1997). Neutrophils and monocytes efficiently ingest *Salmonella* opsonised by complement factor C3b, through surface membrane complement receptor 3 (CR3) (van Bruggen*, et al.*, 2007). However, non-opsonised *Salmonella* can also be ingested by macrophages and neutrophils through CD14 and LPS interactions (Heale*, et al.*, 2001).

Killing of engulfed *Salmonella* is achieved through metabolic reactions within the phagosome membrane and cytosol (Mastroeni, 2003). Phagocytes killing mechanisms include; acidification (pH ranging 5-4.5) through glucose consumption, generation of phagolysosomes, generation of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) termed respiratory burst (Mastroeni, 2003). In mice, *Salmonella* growth in the tissues is controlled by macrophage associated Nramp1 (natural resistance-associated macrophage protein one) gene (also known as *Slc11a1*) during the first few days of infection (Hormaeche, 1990). Nramp1 encodes divalent metal (Fe^{2+}, Zn^{2+} and

Mn^{2+}) pump phosphoglycoproteins, which are recruited to *Salmonella* containing phagosome and these metals are required for efficient bactericidal functions (Vidal*, et al.*, 1993, Mastroeni, 2003). In humans, polymorphism of the Nramp1 gene has been associated with susceptibility to intracellular bacteria including Mycobacterium tuberculosis infection (Vidal*, et al.*, 1993). Furthermore, importance of macrophages in controlling *Salmonella* infection is shown in chronic granulomatous disease (CGD) patients. CGD patient's macrophages exhibit defective respiratory burst and this permits intracellular bacteria invasion (Mouy*, et al.*, 1989).

1.6.2.3 Phagocytes killing and the role of cytokines

Innate immune cells such as macrophages, neutrophils and natural killer cells (NK) generate specific-cytokines that allow co-ordination of immune responses and subsequent *Salmonella* killing. To ensure efficient immune response, macrophages and neutrophils at the site of infection generate chemokines IL-8 and MIP-1 respectively (Tam*, et al.*, 2008). IL-8 and MIP-1 allow recruitment of more leukocytes to the site of infection. TNF-α generated by macrophages promotes transmigration of leukocytes to site of infection, in addition to generation of organised lesions at the infected tissues. Importance of TNF-α has been shown in mice treated with anti-TNF-α antibodies or TNFR 55 knockout mice prior to *Salmonella* oral challenge, these mice fail to restrict bacterial growth and exhibit poor organised lesions in the infected tissues (Mastroeni, 2002). IFN-γ is also important for macrophages efficient killing of intracellular

Salmonella (Yrlid*, et al.*, 2000, Tam*, et al.*, 2008). NK cells and gamma-delta T cells provide the early source of IFN-γ, (Lalmanach & Lantier, 1999, MacLennan*, et al.*, 2004, Nyirenda*, et al.*, 2010). Early source of IFN-γ generated by NK cells has been shown in *Rag 1* knockout mice (lack mature CD4+ T cells, CD8+ T cells and B cells). *Rag 1* knockout mice can produce IFN-γ in response to *Salmonella* infection, indicating that NK cells is the source of this IFN-γ (Ramarathinam*, et al.*, 1993).

1.6.2.4 Complement mediated immunity to *Salmonella*

Complement is comprised of more than 20 proteins present in plasma and on cell surfaces. Activation of complement leads to formation of a cascade and these proteins are essential for specific biological functions including pathogen killing through complement membrane attack (MAC), opsonisation of pathogen and recruitment of inflammatory cells (Krushkal*, et al.*, 2000). Complement cascade can be activated in three pathways through C3; the classical pathways requires antibody and bacteria complexes for activation and these binds to C1 C4 C2 complexes, lectin binding pathway requires mannose binding protein (MBP) binding on bacteria surface for activation and this binds to C4 and the alternative pathway requires non-specific bacteria surface and factors B D P and these binds to C3 (Figure 1.7). Complement involvement in controlling *Salmonella* infection is shown in mice with C1q deficiency (Warren*, et al.*, 2002). C1q deficient mice are more susceptible to infection with *S.* Typhimurium compared to strain-matched control mice, indicating the importance of C1q complement classical pathway in

protection against *Salmonella* infection (Warren, *et al.*, 2002). Sickle cell patients are also more susceptible to *Salmonella* bacteraemia compared to healthy controls (Calis, *et al.*, 2008). This susceptibility to *Salmonella* bacteraemia has been attributed to reduced serum bactericidal activity as a result of defective function of alternative complement pathway and low concentration of C3 (Hand & King, 1977) and also impaired splenic function (Booth, *et al.*, 2010).

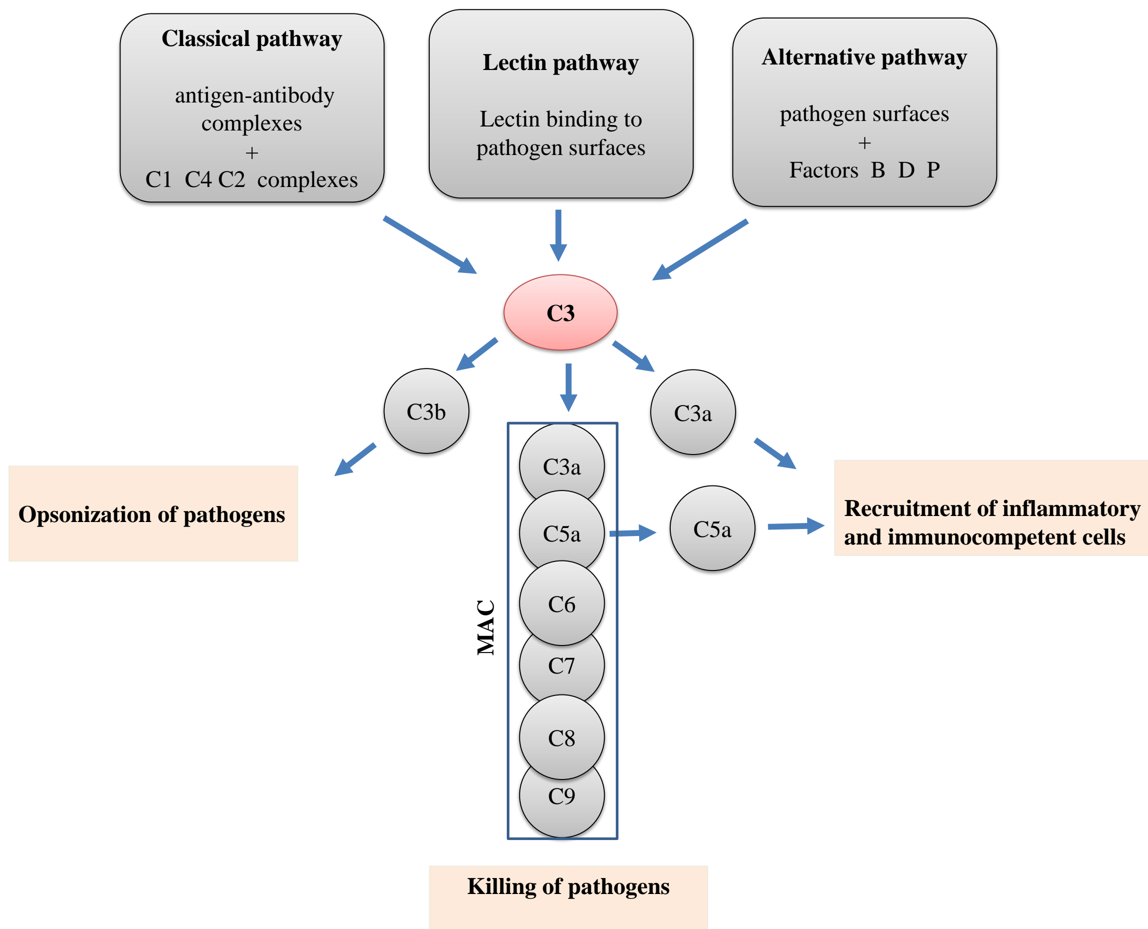

Figure 1. 7: Complement pathways

1.6.3 Adaptive immunity to *Salmonella*

1.6.3.1 *Salmonella* antigen processing and presentation by the APC

Mature dendritic cells (DCs) and macrophages that ingest *Salmonella* at the site of infection migrate to lymph nodes, to prime *Salmonella*-specific T cells and B cells (Yrlid, *et al.*, 2000). DC can either directly (upon uptake and processing of *Salmonella*) or indirectly (by bystander mechanisms, including cross-presentation) present *Salmonella* peptide antigens (Sundquist, *et al.*, 2004). *Salmonella* antigens are mainly processed through exogenous pathway and peptides are presented by antigen presenting cell (APC) to CD4+ T cell through APC MHC-II and CD4+ TCR interaction (Yrlid, *et al.*, 2000) (Figure 1.8). *Salmonella* antigens could also be presented by APC to CD8+ T cell through APC MHC-I and CD8+ TCR interaction, in a process referred to as cross-presentation (Salerno-Goncalves & Sztein, 2009). In cross-presentation, APC take up antigens and process these antigens through MHC Class II pathway and later processed antigens are directed to their own MHC I pathway and peptides are subsequently presented to naive CD8+ T-cells (Heath, *et al.*, 2004, Rock & Shen, 2005, Salerno-Goncalves & Sztein, 2009). Furthermore, Sztein reported that CD8+ T cells play a role in controlling *Salmonella* infection and killing is mediated by both MHC-Ia restricted and non-classical HLA-E restricted cytotoxic T cell response in Ty21a vaccinated humans (Sztein, 2007).

In MHC-II antigen presentation pathway, phagocytised *Salmonella* are delivered to specialised antigen processing endosome compartments (Wolf & Ploegh, 1995). The

endosome compartments contain MHC-II molecules in complex with the invariant chain inserted in its membrane (Wolf & Ploegh, 1995). Proteases are activated by acidic conditions within the endosome compartments, which allow the generation of *Salmonella*-specific peptides and degradation of invariant chain (Wolf & Ploegh, 1995, Jensen*, et al.*, 1999). HDA-DM catalyses the removal of invariant chain and peptides loading into the MHC-II grooves (Wolf & Ploegh, 1995, Jensen*, et al.*, 1999). The peptide MHC-II complex is then transported to the surface of antigen presenting cell (APC) for CD4+ T cell activation through peptide MHC-II complex and TCR interaction (Wolf & Ploegh, 1995, Jensen*, et al.*, 1999).

Efficient T cell activation however, requires co-stimulatory signals in additional to T cell TCR and APC peptides MHC complex interactions. Co-stimulatory signals involve interactions between APC via CD80 and CD86 (B7.1/B7.2) with T cell CD28 and CTLA-4 (Mittrucker*, et al.*, 1999, McSorley*, et al.*, 2002). CD28 mediates positive T cell co-stimulatory signal while CTL-4 mediates T cell inhibitory signal (Mittrucker*, et al.*, 1999, McSorley*, et al.*, 2002). It has been demonstrated that CD28 knockout mice fail to mount efficient T cell immunity against *S.* Typhimurium, indicating the role of CD28 in providing T cell positive co-stimulatory signal (Mittrucker*, et al.*, 1999). The recently activated *Salmonella* specific CD4 T cells leave the lymph nodes to the infected tissues, to mount *Salmonella*-specific immune response.

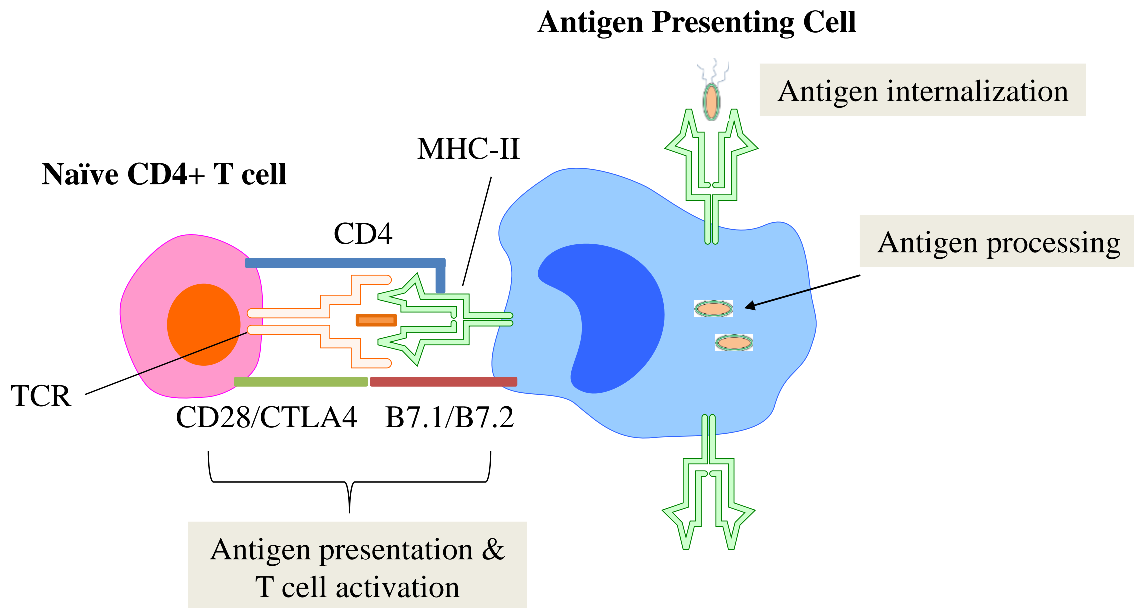

Figure 1. 8: APC antigen processing and presentation to CD4+ T cell

Naïve CD4+ T cells are primed by the APCs in the lymphoid tissues (including GALT, PLN) through the interaction of MHC-II and specific-peptide complex (APC) and TCR (CD4+ T cell). Mature APC internalise *Salmonella* and processed *Salmonella*-specific peptides are presented to naïve CD4+ T cells through MHC-II. Primed CD4+ T cells migrate to the site of infection of effector functions.

1.6.3.2 **Polarisation of CD4+ T helper immunity to *Salmonella***

Polarisation of activated CD4+ T cells towards CD4+ T helper 1 (Th1), CD4+ T helper 2 (Th2) and CD4+ T helper 17 (Th17) are dictated by factors including infecting organisms and cytokines (Figure 1.9). CD4+ Th1 immunity to *Salmonella* is the dominant form of

immunity. CD4+ T cell production of IFN-γ and TNF-α significantly increases during acute and convalescent phases, in *Salmonella* infected patients compared to healthy controls (Stoycheva & Murdjeva, 2004, Thompson*, et al.*, 2009, Charles*, et al.*, 2010, Khoo*, et al.*, 2011). *Salmonella* infections stimulates macrophage to produce cytokines; IL-12 and IL-23 and these cytokines drives the polarization of recently activated CD4+ T cells towards CD4+ T helper 1 cells (MacLennan*, et al.*, 2004). In a feedback fashion CD4+ T cell generated IFN-γ and TNF-α provides help to macrophages effector functions. Patients with defects in IL-12/IFN-γ axis are more susceptible to *Salmonella* indicating the importance of CD4+ Th1 immunity in controlling *Salmonella* infection (Sharifi Mood*, et al.*, 2004). Lin *et al,* demonstrated that human-host defence against *Salmonella* infection (during the acute phase) is associated with these increase in CD4+ T helper 1 transcriptional factor Tbet and IL-2, while CD4 T helper 2 transcriptional factor Gata 3 and IL-4 are reduced, indicating the importance of CD4+ T helper 1 response in controlling *Salmonella* infection (Lin*, et al.*, 2008).

Polarisation of CD4+ T cells towards CD4+ T helper 2 cells in *Salmonella* infection is dictated by IL-4 and IL-10 (Ramarathinam*, et al.*, 1993). A study by Srinivasan *et al*, showed that intestinal environment (immune regulatory) may also promote APCs to signal recently activated CD4+ T cells towards CD4+ T helper 2 cells (Srinivasan & McSorley, 2006). IL-4 and IL-10 play an inhibitory role of CD4+ T helper 1 mediated response against *Salmonella* (Ramarathinam*, et al.*, 1993). IL-4 knockout mice efficiently control *Salmonella* infection compared to wild type following *Salmonella* oral challenge, suggesting that IL-4 promote salmonellosis progression (Ramarathinam*, et al.*, 1993).

Emerging evidence from animal studies suggests that CD4+ T helper 17 (Th17) cells have a role in controlling *Salmonella* infection (Raffatellu*, et al.*, 2008). In simian immunodeficiency virus (SIV) infected macaques (human HIV infected model), CD4+ Th17 cells in the ileal mucosa of rhesus macaques are depleted and this impairs mucosal barrier functions (blunted Th17 responses) resulting in increased systemic dissemination of *S.* Typhimurium from the gut (Raffatellu*, et al.*, 2008). Furthermore, Th17 cells have been implicated in controlling extracellular bacterial infection in the gut thereby avoiding systemic dissemination of the pathogen (Raffatellu*, et al.*, 2008, Blaschitz & Raffatellu, 2010). Cytokines; IL1, IL-6, IL-23 and TGF-β1 and transcriptional factor Roγt are required for CD4+ T cell differentiation towards Th17 cells and their subsequent expansion (Chen & O'Shea, 2008). The contribution of Th17 immunity in controlling NTS bacteraemia in humans has not been investigated.

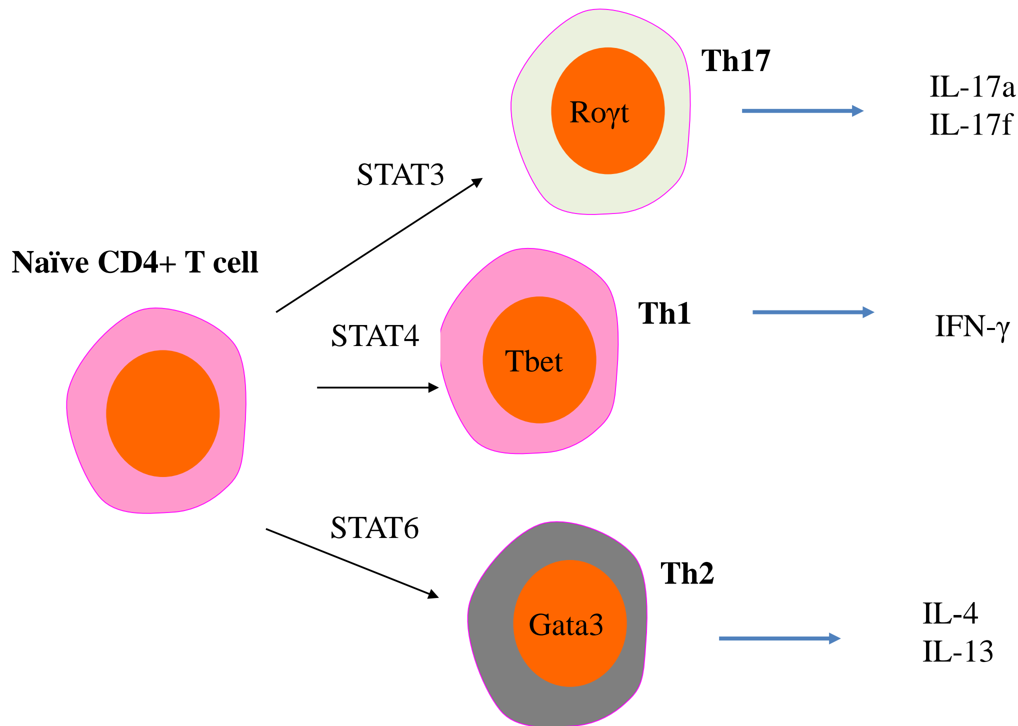

Figure 1. 9: CD4+ T cell plasticity in response to *Salmonella* antigens

The polarization of Th1, Th2 and Th17 immunity is dictated by several factors including the infecting pathogen (*Salmonella*) or components (LPS, flagellin and OMP), cytokines profile generated by naïve cells including macrophage and natural killer cells (IL-12 drive Th1 while IL-6, IL-1 and TNF-α drive Th2). In turn- CD4+ T cells generate pro-inflammatory; IFN-γ and IL-2 (Th1 immunity) or anti-inflammatory; IL-4, IL-6 and IL-10 (Th2 immunity) that up regulates and down regulates macrophages bactericidal machinery respectively. IL1, IL-6, IL-23 and TGF-β1 are required for CD4 T cell differentiation to Th17 cells and their subsequent expansion

1.6.3.3 B cell immunity to *Salmonella* infection

B cells play an important role in protection against *Salmonella*. Antibody production is the dominant function of B cells, but they are also involved in antigen presentation to T cells. *Salmonella* are facultative intracellular organism and are capable of surviving in both the extracellular and intracellular space. It is thought that extracellular *Salmonella* are mainly controlled by antibody mediated immunity (MacLennan, *et al.*, 2008). *Salmonella* in the extracellular space might have result from escaping neutrophil and macrophage mediated immunity. Opsonic IgG or IgM antibodies specific for *S*. Typhimurium control *Salmonella* bacteraemia by activating complement cascade through the classical pathway which are ultimately killed through membrane attack complex (MacLennan, *et al.*, 2008). Opsonic IgG or IgM antibodies specific for *S*. Typhimurium also control *Salmonella* bacteraemia by facilitating efficient neutrophils and monocytes phagocytosis through their surface membrane FcR (Uppington, *et al.*, 2006) and the engulfed *Salmonella* are killed by respiratory burst (Gondwe, *et al.*, 2010).

MacLennan *et al* showed in Malawian children that antibody mediated serum killing of invasive NTS strain occurs in children >16 months of age and not in younger children (MacLennan, *et al.*, 2008), indicating the requirement of mature NTS-specific antibodies (IgG and IgM) in this immunity (MacLennan, *et al.*, 2008). Interestingly, a similar trend (age related development) of antibody mediated serum immunity to *S*. Typhi was reported in children from Nepal (Pulickal, *et al.*, 2009). Furthermore, Xu and colleagues showed positive correlation of *Salmonella*-specific antibody titres with resistance to lethal challenge with *S*. Typhimurium in vaccinated genetically susceptible C57BL/BL

mice (Xu, *et al.*, 1993). These findings support the exploration of an antibody-based vaccine for NTS bacteraemia.

The production of *Salmonella*-specific IgG, IgM and IgA antibodies are increased during *Salmonella* disease. Anti-IgG titres to *Salmonella* LPS, flagellin and membrane proteins (MP) are higher in *Salmonella* infected patients compared to healthy controls (Lee, *et al.*, Choo, *et al.*, 1997, Strid, *et al.*, 2007). Limited studies have explored the longevity of immune responses triggered naturally by *Salmonella* infection, to determine whether or not protection to subsequent infection is conferred. Longevity of IgG-antibodies targeting *S.* Typhi membrane protein (MP) in Malaysian children with typhoid fever (clinical typhoid fever, blood or stool culture positive or Widal test positive) 21 months into the recovery period was explored. At 6 months into the recovery period 50% of typhoid cases had detectable IgG antibody responses-specific for MP (positive dot enzyme immunosorbent assay [dot EIA]) and these responses declined to 30% at 12 months (Choo, *et al.*, 1997). These findings suggest the longevity of these IgG-specific antibody responses triggered by natural *Salmonella* infection might mimics those induced by vaccination. Evidence on protection conferred by natural *Salmonella* exposure is elusive.

1.6.3.4 **B cell and T cell interactions in response to *Salmonella* infection**

Cellular interactions between B cells and T cells are important for development of robust immunity to *Salmonella* and other pathogens (Mastroeni & Menager, 2003). During secondary humoral response, B cells also function as APCs. This is evidenced by reduced

ability to produce IFN-γ and IL-2 by CD4+ T cells isolated from B-cell-deficient *Igh-6*$^{-/-}$ mice immunized with live attenuated *Salmonella* (Mastroeni*, et al.*, 2000). Immunized *Igh-6*$^{-/-}$ mice also fail to control the growth of virulent *Salmonella* in secondary infection (Mastroeni*, et al.*, 2000, McSorley & Jenkins, 2000, Mittrucker*, et al.*, 2000). On the other hand, B cells generation of class switched and high affinity antibodies is dependent on T cells help (Figure 1.11) (Mittrucker*, et al.*, 1999). This is proved in T cell deficient mice (athymic *nu/nu* mice), when vaccinated with live attenuated *Salmonella* fail to generate high affinity antibodies including IgG1, IgG2A and IgG2B instead generate low affinity antibodies such as IgM and IgG3 against *Salmonella* LPS (Mastroeni & Menager, 2003). Essentially, interaction between CD4+ T cells and B cells is important for establishment long-term and robust CD4+ T cells and antibody immunity to *Salmonella* infection (Mastroeni, 2002, Takemori*, et al.*, 2014).

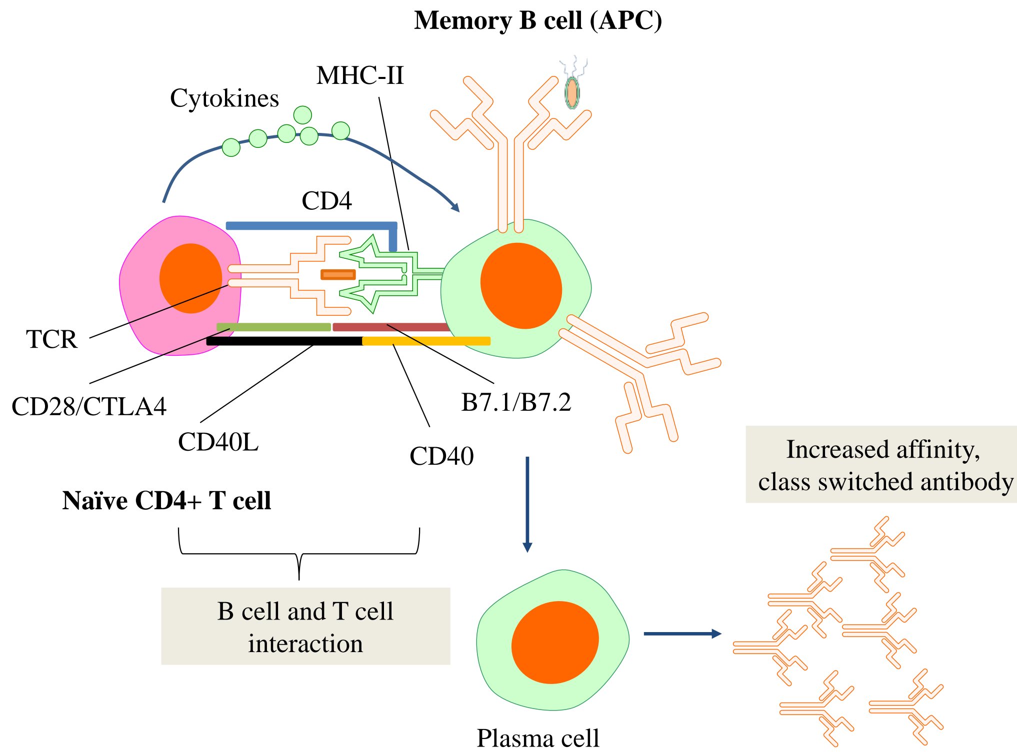

Figure 1. 10: Generation of high affinity and class-switched antibodies through B cell and T cells interaction

Primed B cells also function as APC to CD4+ T cells. Primed CD4+ T cells provide help primed B cells to generate memory response, class-switch antibodies and high affinity antibodies specific for *Salmonella*.

1.6.4 Clinical features and immunity to *Salmonella* exposure within the GIT

1.6.4.1 Normal flora resistance to enteric pathogens

Normal flora in the gastrointestinal tract (GIT) is present in large numbers particularly in the colon and lower intestine (Hooper & Gordon, 2001, Sekirov*, et al.*, 2010). Normal flora provides colonisation resistance of the intestinal lumen to enteric pathogens including *Salmonella* (Endt*, et al.*, 2010, Stecher & Hardt, 2011). Three mechanisms of intestinal microbiota-mediated colonisation resistance have been clearly defined including direct inhibition of the pathogen by antimicrobial effector molecules (bacteriocins, metabolic by-products), efficient competition for nutrients by shutting down all potentially available nutrient niches for the pathogen and indirect inhibition by stimulating the host's antimicrobial defence system (defensins, mucin and secretory IgA) (Stecher & Hardt, 2011). In the event that antibiotics (particularly broad-spectrum antibiotics) are administered the protective role of the normal flora is compromised (Miller*, et al.*, 1954). Antibiotics can kill large numbers of commensal gut bacteria and thereby favouring an ecological niche of opportunistic pathogen such as *Salmonella.*

1.6.4.2 Symptomatic *Salmonella* exposure: diarrhoea

Enteric pathogen exposure within the GIT may be accompanied by gastrointestinal symptoms such as diarrhoea and this may be followed by carriage. The mechanisms underlying *Salmonella* diarrhoeal disease have not been clearly elucidated in humans. Generally the presence or absence of symptoms following *Salmonella* exposure within

the gut lumen may be dictated by the host immune response, pathogen virulence and the infecting dose. This has been demonstrated by modelling estimates of dose and illness from *Salmonella* outbreaks (Bollaerts*, et al.*, 2008) and streptomycin mouse model for *Salmonella* diarrhoea (Kaiser*, et al.*, 2012). Diarrhoea occurs when there is an altered movement of electrolytes and water that follows an osmotic gradient within the gut lumen, and this alterations is driven by enteric pathogens including *Salmonella* (Hodges & Gill, 2010). *Salmonella* are among the enteric pathogens that cause inflammatory diarrhoea and this primarily target the lower bowel, particularly the distal ileum and the colon (Navaneethan & Giannella, 2008). *Salmonella* causes inflammatory diarrhoea and is characterised by presence of neutrophils (Zeng*, et al.*, 2003, Raffatellu*, et al.*, 2005). Neutrophils regulate absorption through cytokine secretion and generation of precursors such as adenosine and secretagogue that activates the cystic fibrosis transmembrane receptor (CFTR), a Cl^- channel (Navaneethan & Giannella, 2008, Hodges & Gill, 2010) (Figure 1: 11). Taken together *Salmonella* causes inflammatory diarrhoea and this is characterised by influx of neutrophils, increased secretion of Cl^- in the gut lumen following CFTR activation and the general reduced absorption.

1.6.4.3 Duration of NTS shedding

Evidence of *Salmonella* exposure to the GIT relies on stool cultures. Several factors are thought to govern the duration of *Salmonella* shedding including age, clinical presentation, antibiotic treatment and the anatomical site of carriage. In patients with

NTS infection within the intestinal tract, *Salmonella* faecal shedding is usually short, ranging from 4 to 8 weeks in children and adults (Buchwald & Blaser, 1984). However the duration of NTS shedding is normally longer in children less than five years compared to older children and adults (Buchwald & Blaser, 1984). Antimicrobial treatment of acute *Salmonellosis* has been implicated to prolong the duration of faecal *Salmonella* shedding (Dixon, 1965, Levine*, et al.*, 1982). Persistent NTS shedding for a period longer than 1 year (formal definition chronic carrier) is very rare (might occur in <1%) (Musher & Rubenstein, 1973, Buchwald & Blaser, 1984). The location of persistent foci in NTS infection is unknown, although cholelithiasis has been documented in about 40% of *Salmonella* carriers (Musher & Rubenstein, 1973). Taken together, these findings show that NTS chronic carriage is rare, but shedding of NTS even for shorter duration could be a way of transmitting NTS infection in animals and humans.

1.6.4.4 Evidence of immunity to *Salmonella* disease within the GIT

Exposure of *Salmonella* within the GIT is likely an immunizing event. Diarrhoea resulting for enteric pathogen exposure in naïve children is typically severe and faecal shedding lasts longer compared to subsequent diarrhoea episode (Buchwald & Blaser, 1984, Pitzinger*, et al.*, 1991, Steffen, 2005). Similarly travellers' diarrhoea is usually severe with longer duration of faecal shedding in visitors from enteric pathogens non-endemic countries compared to diarrhoea in native subjects from enteric pathogens endemic countries (Steffen, 2005). These observations support the notion that *Salmonell*a exposure within the GIT does confer protection to subsequent specific infection within

the GIT. Whether this protection induced by *Salmonella* exposure within the GIT can provide protection against *Salmonella* blood stream infection (BSI) is a key question.

Furthermore, it was demonstrated in the mouse model of diarrhoea that perioral treatment with ciprofloxacin of *S.* Typhimurium a disrupts adaptive immunity to *S.* Typhimurium while parenteral ceftriaxone does not disrupt development of adaptive immunity (Endt, *et al.*, 2012). Selection of antibiotics for intestinal localised *Salmonella* infections must take into account the impact on establishment of natural immunity and sensitivity of *Salmonella* to the antibiotics. This area has received limited attention and need further investigations to ascertain the impact of drug selection for treatment of *Salmonella* and subsequent establishment of natural immunity.

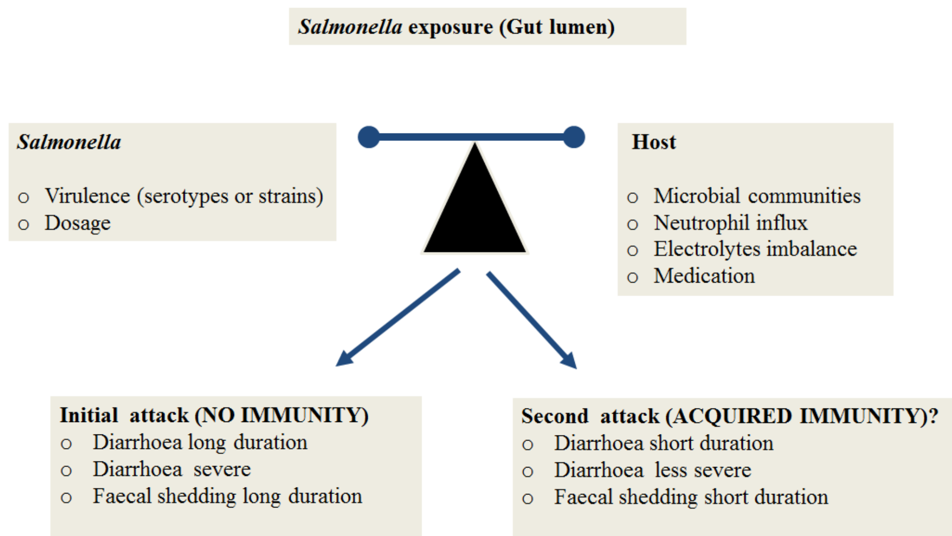

Figure 1. 11: Protective immunity following *Salmonella* exposure within the GIT

Low dosage of NTS in the gut lumen and the balance between NTS and normal flora might dictate asymptomatic colonisation. This balance might however permit NTS to exist in the gut lumen for longer periods and faecal shedding could be detected. The imbalance between the NTS and normal flora dictate symptomatic colonisation of the gut lumen which is associated with the influx of the neutrophils and electrolyte imbalance. These processes result in diarrhoea with or without detectable faecal shedding. During the initial NTS attack the diarrhoea is usually severe and the duration of shedding and diarrhoea is longer. While during the second NTS attack the diarrhoea is less severe and the duration shedding and diarrhoea is shorter, perhaps due to the protective immunity induced during the previous attack.

1.6.5 Immunological lessons drawn from humans that are vulnerable to iNTS

1.6.5.1 Malnutrition is commonly associated with NTS bacteraemia

NTS bacteraemia is commonly associated with malnutrition in SSA (Graham*, et al.*, 2000, Babirekere-Iriso*, et al.*, 2006). The mechanism underlying susceptibility to NTS bacteraemia in malnourished children has not been elucidated in humans. Since malnourished children exhibit a leaky gut (Reynolds*, et al.*, 1996), it is possible that impaired gut integrity in malnourished children permit the spread of *Salmonella* from the gut lumen through the gut mucosa and submucosa, and subsequently spread to systemic circulation (Reynolds*, et al.*, 1996). Rehabilitation of malnourished children may restore

the gut mucosa barrier and restricts spread of enteric pathogens including *Salmonella* into systemic circulation, but this is unproven.

1.6.5.2 Malaria and *Salmonella* bacteraemia co-infection infection (Neutrophils and monocytes)

Both malaria and *Salmonella* bacteraemia remain important causes of death among under-five children in SSA (Guinovart, *et al.*, 2006, Reddy, *et al.*, 2010). NTS bacteraemia is commonly associated with malaria infection, particularly severe malarial anaemia (Bronzan, *et al.*, 2007). Case fatality rate is higher in children presenting to hospital with severe malaria anaemia and bacteraemia co-infection compared to malaria infection alone (Bassat, *et al.*, 2009). This is in part due to indistinguishable clinical presentation of NTS bacteraemia, and lack of diagnostics in poor resource settings. Children are diagnosed and treated for malaria infection while NTS bacteraemia is unattended. The association between malaria infection and NTS bacteraemia in Africa was first reported in the 1920s (Graham, 2010). Biggs *et al* recently demonstrated NTS bacteraemia and malaria infection co-infection was common among febrile paediatric inpatients (aged 2 months to 13 years) from low altitude and high malaria transmission area compared to those from high altitude and low malaria transmission area in Tanzania (Biggs, *et al.*, 2014). In contrast to NTS bacteraemia pattern, *S.* Typhi bacteraemia was uncommon in febrile paediatric inpatients from low altitude and high malaria

transmission area (Biggs, *et al.*, 2014). These findings underlie the differences in the burden and mode host susceptibility of *S.* Typhi and NTS among children from SSA.

The mechanism underlying association between NTS bacteraemia and malaria co-infection is not clear. It has been shown that the reduction in neutrophil oxidative burst activity, reduction in IL-12 levels and increased levels of IL-10 in malaria infected mice play a role in impairing immunity to NTS (Roux, *et al.*, 2010, Cunnington, *et al.*, 2012, Maclennan, 2014). Together these studies suggest that compromised neutrophil immunity against malaria and increased inhibitory cytokines favours NTS growth and perhaps dissemination to systemic organs. These mechanisms have not been investigated thoroughly in humans.

1.6.5.3 Cellular and humoral immune responses in HIV infected individuals

NTS bacteraemia is common in HIV infected individuals worldwide. Impaired antibody and cellular immunity have been implicated. MacLennan *et al* demonstrated that HIV infected adults exhibit significantly high levels of anti-*Salmonella* IgG titres targeting *S.* Typhimurium-LPS O antigen compared to healthy adults and these antibodies were inhibitory as they block bactericidal anti-*Salmonella* IgG antibodies targeting *S.* Typhimurium-outer membrane protein (OMP) (MacLennan, *et al.*, 2010). Whether or not *S*. Typhimurium-specific IgG antibodies in HIV infected individuals are class switched or high affinity antibodies is not clear.

Gordon *et al* demonstrated that low absolute CD4+ T cells in HIV infected individuals correlate independently with viable NTS in peripheral blood, suggesting failure to clear intracellular infection in advanced HIV (Gordon, *et al.*, 2010). In keeping with these observations, Raffatellu *et al* demonstrated that simian immunodeficiency virus (SIV) infection results in depletion of Th17 cells (subset of CD4+ T cells) in the ileal mucosa of rhesus macaques, thereby impairing mucosal barrier functions to *S*. Typhimurium and this subsequently promotes *S*. Typhimurium spread to distant tissues (Raffatellu, *et al.*, 2008).

A recent transcriptome study in HIV infected adults with NTS bacteraemia showed attenuated NFќB inflammation while NFќB inflammation was normal in controls with other common extracellular bacteraemia (Schreiber, *et al.*, 2011). NFκB signalling pathway plays a key role in signalling downstream activities including cell proliferation and pro-inflammatory cytokine generation (including IFN-γ). In keeping with these findings, Gordon *et al* demonstrated dysregulation of pro-inflammatory cytokine (including IL-12) production by macrophages in adults with advanced HIV in responses to *Salmonella* (Gordon, *et al.*, 2007) and also impaired NTS internalisation and killing by the macrophages even in the presence of exogenous IFN-γ (Gordon, *et al.*, 2007).

Furthermore, immunohistochemistry studies have revealed that the architecture of lymphoid tissues are disorganised in HIV infected subjects (Alos, *et al.*, 2005), suggesting disorganisation of the lymphoid tissues underline compromised immunity to pathogens including *Salmonella* in HIV infected subjects, since mounting of effective adaptive immunity is dependent on proper machinery of the lymphoid tissue. There is

evidence that ARV therapy restores the loss of CD4+ T cells to relatively normal counts (Guadalupe*, et al.*, 2003) and cases of BSI including *Salmonella* bacteraemia have reduced tremendously in HIV infected Malawian adults following the roll out of Antiretroviral therapy (ART) and Cotrimoxazole preventative therapy (CPT) (Feasey*, et al.*, 2014). Whether or not ART therapy restores the organisation of lymphoid tissues and function of the adaptive immunity to *Salmonella* infection in HIV infected individuals is not clear. A study on Malawian children who were chronically infected with HIV receiving ART showed delayed restoration of memory B cells compared to CD4+ T cells and B cell function specific for pneumococcal protein antigen was also delayed (Iwajomo*, et al.*, 2014). Another study on Malawian adults infected with HIV receiving ART showed that recovery of pneumococcal CD4+ T cells at 12 months following ART initiation and incomplete recovery of CD4+ T cell function (CD154 expression and production of antigen specific IFN-γ) (Sepako*, et al.*, 2014). Together these findings suggest that ART therapy incompletely restores B cell and T cell immunity in HIV infected individuals.

1.7 **VACCINES**

In this section, discussion focusses on vaccine development status for both *S*. Typhi and NTS bacteraemia. Two currently licenced vaccines (Oral Ty21a and Vi CPS) for *S*. Typhi and also new generation vaccines are reviewed. No human vaccine is licenced for NTS and discussion dwells on progress made in exploring NTS vaccine candidates.

1.7.1 Oral Ty21a vaccine

Oral Ty21a is a vaccine for typhoid fever and was generated in the 1970s following chemical mutagenesis of the wild type strain *S*. Typhi Ty2 and does not express the Vi polysaccharide and *galE* gene (Germanier & Fuer, 1975, Guzman*, et al.*, 2006). Ty21a (Vivotif) contains attenuated *S.* Typhi Ty2 in lyophised form, and each capsule contains no less than $2x10^9$ viable cells (Guzman*, et al.*, 2006). Oral Ty21a vaccine is administered in children above 6 years and adults. Three doses of Ty21a vaccine are recommended worldwide, but other countries including Canada and USA opt to provide four doses (Guzman*, et al.*, 2006). One capsule is taken on days 1, 3 and 5 with lukewarm water at least 1 hour before meal (Pasetti*, et al.*, 2011). Clinical trials for oral Ty21a vaccine showed that it is extremely safe and well tolerated (Olanratmanee*, et al.*, 1992). Common adverse effects (AEs) reported include mild gastrointestinal disturbances and fever (Guzman*, et al.*, 2006). A meta-analysis showed overall three years cumulative protective efficacy of 51% (Fraser*, et al.*, 2007). Furthermore, immunogenicity studies demonstrated that oral Ty21a vaccine induces both antibody and T cell mediated immunity. However oral Ty21a vaccine requires multiple doses to elicit a protective response, even after booster doses the vaccine confers incomplete protection. To address oral Ty21a vaccine shortfalls a number of novel live attenuated *S.* Typhi vaccine candidates have been developed including Ty800, CVD 908, CVD 908-htrA, CVD 909 and M01ZH09 (Tacket*, et al.*, 2000, Tacket & Levine, 2007, Wahid*, et al.*, 2011). Unfortunately none of these new live attenuated vaccines are currently licensed as typhoid vaccines (Table 1-B).

1.7.2 Vi capsular polysaccharide vaccine

Vi capsular polysaccharide vaccine (Vi CPV) for typhoid fever was licenced in 1994. Vi CPV is now available in >90 countries and is administered as a single or multiple doses intramuscularly (Martin, 2012). The vaccine induces acquisition of serum anti-Vi IgG and these have been shown to correlate with efficacy (Hessel*, et al.*, 1999). Vi CPV confers about 70% protection for up to 3 years in children aged >2 years and adults (Tacket*, et al.*, 1986). Interestingly, in populations where Vi CPV have been implemented, cases of typhoid fever have been reduced (Khan*, et al.*, 2010). In Kolkata, among children aged 2 and 5 years, a cluster randomised trial of Vi CPV demonstrated a low transmission and herd immunity amongst controls (Sur*, et al.*, 2009). The pitfalls of Vi CPV include; lack of immunogenicity in children below 2 years, shorter duration of protection and the need for repeated doses (Robbins & Robbins, 1984, Tacket*, et al.*, 1988, Hessel*, et al.*, 1999). Furthermore, Vi CPV lack memory response and even with repeated doses, immune responses are not boosted (Kantele*, et al.*, 2012). In pursuit of better vaccine for typhoid fever, glycoconjugates vaccines appear to be promising. Glycoconjugate vaccines induces T cell immune responses that provides help to antibodies against Vi and O-antigens through covalent linkage to protein carrier molecules (Maclennan*, et al.*, 2014). The glycoconjugate strategy for generation of new *Salmonella* vaccines is principally an antibody approach and to date a number of the vaccine have been generated including Vi-TT, Vi-rEPA, Vi-CRM and Vi-DT (Maclennan*, et al.*, 2014). Importantly, the Vi-TT and Vi-rEPA are already licenced for

in-country use in India and China respectively (Maclennan*, et al.*, 2014) (Table 1-A and Table 1-B).

1.7.3 Vaccine candidates for *Salmonella* bacteraemia

No human vaccine is currently available for *S.* Typhimurium, *S.* Enteritidis, and *S.* Paratyphi A and B. Efficacy for two licenced vaccines for *S.* Typhi (Vi CPV and Ty21a) are poor and it is not known whether they could provide cross-protection to NTS. Antibodies to *Salmonella* targeting moieties on the outer surface including Vi, O and H antigens of *Salmonella* are thought to mediate protection (Maclennan*, et al.*, 2014). These antigens (Vi, O and H antigens) are highly immunogenic and have been proposed as vaccine candidates (Brenner*, et al.*, 2000, Maclennan*, et al.*, 2014).

S. Typhimurium LPS has considerable potential as a vaccine target and is being develop as a conjugate vaccine to overcome short-lived T cell independent immunity elicited by LPS alone (MacLennan, 2013). In mice OMP and FliC induce both T cell and antibody immunity and are also being investigated as vaccine candidates (Simon*, et al.*, 2011, Simon & Levine, 2012). Whether or not OMP or FliC co-administered or incorporated into conjugate vaccine would consolidate the protective efficacy is not known.

A number of challenges have been noted in developing vaccines for NTS. For instance, flagellin is not constitutively expressed by *S.* Typhimurium during infection due to its phase variable expression and this might cause problems as a vaccine candidate (Maclennan*, et al.*, 2014) . Immunisation with OmpC and OmpF and OmpD confers

protection in mice challenge studies (Cunningham*, et al.*, 2007, Gat*, et al.*, 2011) but the production of purified Omp antigens is complicated by multiple spanning domains (Maclennan*, et al.*, 2014).

Generalized Modules from Membrane Antigens (GMMA) technology provide an innovative strategy (purification is straight forward and economical) to maintain the conformation integrity of *Salmonella* antigen (Maclennan*, et al.*, 2014). GMMA vaccines can deliver both surface polysaccharides and outer membrane proteins to the immune system and are more immunogenic compared to the glycoconjugate vaccine in mice (Maclennan*, et al.*, 2014). GMMA reactogenicity and immunogenicity in humans are not known.

In the recent years protein arrays have enabled the screening of sera for antibodies targeting thousands of *Salmonella* proteins. Lee *et al* recently identified potential vaccine candidates including SseB by uncovering overlapping antibodies targeting protein antigens in serum from mice immunised with live attenuated *Salmonella* and children convalescing from invasive NTS disease (Lee*, et al.*, 2012). Furthermore, important *Salmonella* protein antigens including PagC, OmpA, Hly-E and GroEL that are targeted by the host immunity were uncovered in a study of Bangladeshi patients with acute typhoid and those with febrile illness due to other causes (Charles*, et al.*, 2010). These antigens are now being explored to evaluate their potential to induce protection, particularly as subunit vaccine in pre-clinical phase (Lee*, et al.*, 2012). Protein arrays approach in the discovery of candidate vaccine targets will help in refining the current vaccines and generation of better novel vaccines for *Salmonella*.

As part of vaccine broader strategy in development of *Salmonella* vaccines, it will be important to ensure that the immunity generated by a candidate vaccine is cross-protective (targeting *S*. Typhi, *S*. Paratyphi, *S*. Typhimurium, and *S.* Enteritidis, *S.* Dublin and *S.* Stanleyville). O-antigen based conjugated vaccines might offer cross-protection against other non-encapsulated serovars within the same group. Other groups have proposed to generate a multivalent vaccine made of 5-6 conjugates covering all invasive *Salmonella* disease (Simon & Levine, 2012, Maclennan*, et al.*, 2014)

Table 1-A: Licensed vaccine for *S.* Typhi

Vaccine type	Vaccine	Description	Advantage	Disadvantage
Live attenuated	Ty21a (Licenced for adults and children > 5 yrs) (Germanier & Fuer, 1975, Guzman*, et al.*, 2006)	*S.* Typhi Ty2 mutation in Vi polysaccharide and galE gene	Some cross protection against *S.* paratyphi B	Not licenced for infants and low efficacy
Vi	Vi CPV (Licenced for adults and children > 2 yrs) (Khan*, et al.*, 2010)	*S.* Typhi purified Vi	Single dose and low reactogenicity	Not licenced for infants. Low efficacy. Lack of memory and affinity maturation. Only protects against *S.* Typhi
Vi conjugate	Vi-EPA (Licenced in China) (Szu, 2013) Vi-tetanus toxoid (Licenced in India) (Szu, 2013)	*S.* Typhi Vi conjugated to recombinant P. aeruginosa exoprotein A *S.* Typhi Vi conjugated to tetanus toxoid	T dependent antibody response for memory induction and affinity maturation. Low reactogenicity	Only protects against *S.* Typhi

Table 1-B: Vaccines under development targeting *Salmonella* serovars

Type	Vaccine	Description	Advantage	Disadvantage
New live attenuated oral	M01ZH09 (Hindle*, et al.*, 2002, Kirkpatrick*, et al.*, 2006)	Mutation in *phoP*/*pho*Q	B and T cell immunity	Breadth of coverage may be limited by insufficient expression of key antigens
	Ty800 (Hohmann*, et al.*, 1996)	Mutation in *aroC*, *ssaV*	Reduced need for multiple dosing	
	CVD 908-*htrA* (Tacket*, et al.*, 2004)	Mutation in *aroC*, *aroD*, *htrA*		
	CVD 909 (Wahid*, et al.*, 2007)	Mutation in *aroC*, *aroD* and *htrA*, constitutively expresses Vi		
Vi conjugate	Vi-diphtheria toxoid (Szu, 2013)	Vi conjugated to diphtheria toxoid	T dependent antibody response. Low reactogenicity	Only protects against *S.* Typhi
	Vi-CRM (van Damme*, et al.*, 2011, Bhutta*, et al.*, 2014)	Citrobacter Vi conjugated to diphtheria toxoid		
Combination conjugate	O-polysaccharide conjugate plus Vi-conjugate (Simon & Levine, 2012)	Paratyphi A O-polysaccharide conjugate to carrier protein formulated with Vi-conjugated	Broader coverage with a single vaccine	
Recombinant proteins	OmpC, OmpF and OmpA(Toobak*, et al.*, 2013)		B and T cell immunity	Antigen conformation may limit ability to induce B cell immunity
Proteins purified from whole *Salmonella*	OmpC and OmpF (Salazar-Gonzalez*, et al.*, 2004, Secundino, *et al.*, 2006), OmpD (Cunningham*, et al.*, 2007)		B and T cell immunity. Potential for pan immunity	Purification of integral membrane proteins difficult
GMMA	*S.* Typhimurium and *S.* Enteritidis GMMA (MacLennan, 2013)	*S.* Typhimurium and *S.* Enteritidis GMMA	Potential for pan immunity. Easy to manufacture and at low cost	Reactogenicity and immunogenicity in man not known

1.8 RATIONALE

NTS and *S*. Typhi bacteraemia remain important public health problem in Malawi and elsewhere in Africa and Asia. Currently, there's no vaccine for NTS bacteraemia and the vaccines available for *S.* Typhi cannot be used in children in under 2 years. There's is a knowledge gap as to what naturally constitutes protective immunity to invasive *Salmonella* disease and how this develops in children from *Salmonella* endemic regions to inform the development of an effective vaccine. This thesis investigates the following;

1. Development of antibody and T cell immunity to *Salmonella* in healthy and invasive *Salmonella* infected Malawian children.
2. Relationship between *Salmonella* exposure within the GIT and development of *Salmonella*-specific serum immunity in healthy Malawian children.
3. Model natural *Salmonella* infection immunizing events within the gut mucosa by oral Ty21a typhoid vaccination in healthy adults from UK.

The hypotheses investigated in this thesis are;

1. Gut localized or systemic *Salmonella* exposure induces the development of antibody and T cell immunity that has the potential to protect against subsequent *Salmonella* infection and particularly invasive *Salmonella* disease.
2. Oral Ty21a vaccination induces the development of *Salmonella*-specific T cells and these might provide insight into natural immunizing events occurring within the gut mucosal compartment.

CHAPTER 2: STUDY DESIGNS, PARTICIPANTS, MATERIALS AND METHODS

2.1 DEVELOPMENT OF ADAPTIVE IMMUNITY TO NONTYPHOIDAL *SALMONELLA* IN MALAWIAN CHILDREN

2.1.1 Study design and participants

A prospective cross-sectional study was conducted among healthy Malawian children aged 0 to 60 months to characterise the acquisition of *S*. Typhimurium-specific T cell and antibody immunity with age. Study participants were children attending vaccination clinic, health check clinics and newborns in the maternity ward at Ndirande Health Centre (NHC). Furthermore adults were recruited in this study as controls. Following study approval by the College of Medicine Ethics Committee (COMREC) (section 2.1.6), the study team (including myself as the study investigator, research nurse and the MLW science communication team) conducted the study specific sensitization meetings with NHC healthy workers (Clinicians, Nurses and Healthy Surveillance Assistants) and Ndirande community leaders (headmen and religious leaders). These sensitization meetings were aimed at bringing awareness of the research study at NHC and focussed on discussing information contained in the study information sheets. Study specific sensitization meetings are useful as they help to eliminate misinformation about the research study objectives, rationale and specific procedures. The recruitment process was conducted by the study research nurse. Recruitment process began with group

sensitisation talk given to parents and guardians attending NHC. Children's parents and guardians who were keen to participate in the study were sensitised individually and their child recruited into the study provided they consented. Study participants were recruited consecutively provided they were meeting entry criteria including the specific age group (Figure 2.1 and section 2.1.3). Study specific samples were collected by the research nurse and transported to the laboratory for immunological investigations (section 2.1.7).

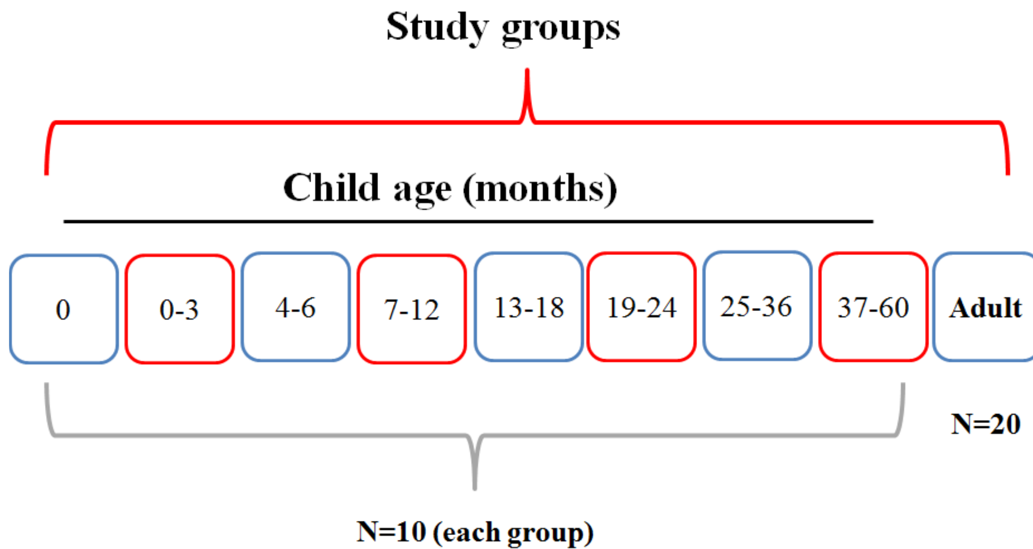

Figure 2. 1: Study design; Development of adaptive immunity to NTS

Eighty healthy Malawian children of varying ages (0-60 months) as indicated in figure 2.1, were recruited. Twenty healthy adults were also recruited in the study as controls. 5ml venous blood was collected from all children except for 0 month group (newborns), where blood was collected from the umbilical cord of the placenta post-delivery. Blood samples were collected and processed as described in methods section 2.1.7.

2.1.2 Sample size

When this study was being conducted there were no data on how variable the responses would be, and how they might vary with age. To illustrate the power of the study to detect an effect the situation that the relationship of immune response (*Salmonella*-specific T cells producing cytokines) to age is linear was considered. With 80 subjects whose ages ranged from 0 to 60 months (resulting in a standard deviation for age (years) of about 1.4) there is 80% power to detect that the slope of the line differs from zero at the 5% significance level if the slope is at least 0.21 standard deviation / year of age.

2.1.3 Inclusion and exclusion criteria

Children aged between 0 and 60 months were recruited provided they were medically well. Study exclusion included; children presenting to NHC with fever >38°C, severe malnutrition (weight-for-age<60% or weight-for-height<80%), malaria parasitaemia, date of birth (exact age) not known, known HIV-positive (documented in child health passport) or clinical HIV/AIDS and those that tested positive using rapid HIV tests (children aged <18 months) (who tested positive were referred to early infant diagnosis (EID) clinic for HIV-DNA confirmatory test). HIV exposed children (born from HIV infected mothers), either uninfected or infected were also excluded from the study.

2.1.4 Study location

Participants were recruited at NHC (vaccination clinic, under-five clinic and maternity ward) in Blantyre district. NHC provide primary health care within the Blantyre city. According to Blantyre district health office (DHO) and QECH 2010/2011 annual report, it was projected that NHC to have catchment population (those receiving service from the health facility) of about 221,217 in the year 2012 (MCI, 2013). Blood was collected from study participants at NHC and transported at room temperature (RT) to Malawi Liverpool Wellcome Trust Laboratories (MLW) located at Queen Elizabeth Central Hospital (QECH), Blantyre for immunological investigations.

2.1.5 Blood stream infection surveillance

Queen Elizabeth Central Hospital (QECH) is a 1,250 bed teaching hospital and the largest government hospital in Malawi, providing free health care to Blantyre district (population approximately 1 million). QECH is the only inpatient paediatric facility for non-fee paying patients in Blantyre. MLW has been conducting routine blood stream infection (BSI) surveillance of febrile children presenting to QECH since 1997. MLW team (including laboratory technicians, research nurses and clinicians) performed these experiments. Blood-cultures are obtained from febrile children who are thick-film-negative for malaria parasites or critically ill, irrespective of malaria infection. Blood-culture is undertaken using a paediatric bottle BacT/Alert® PF (BioMerieux, UK) and isolates identified using standard techniques (Gordon, *et al.*, 2002).

2.1.6 Ethical consideration

The study was compliant with Good Clinical Practice (GCP) regulations and conducted in accordance with the 1996 ICH GCP guidelines and the 2000 Declaration of Helsinki. Ethical approval (protocol number P.08/09/815) was granted by the College of Medicine Ethics Committee (COMREC). Informed consent was obtained from a parent or guardian of each participating child.

2.1.7 Laboratory methods

2.1.7.1 Collection of peripheral blood sample

Venous blood samples were collected by an experienced study research nurse. A tourniquet was applied and the site for venipuncture sterilized with alcohol prep. The needle was inserted into the selected vein and a total of 5 ml of blood was drawn from each child. Blood was transferred into appropriate tubes i.e. 1ml BD vacutainer® serum tube, 3ml BD vacutainer® sodium heparin and 1ml BD microtainer® ethylenediaminetetraacetic acid (EDTA) (all Becton Dickinson, USA) and transported to the laboratory for testing.

2.1.7.2 Collection of umbilical cord blood samples

The umbilical cord blood specimen was collected by experience study nurse *ex-utero* as previously described (Pafumi, *et al.*, 2011) with minor modifications. The placenta was placed on a sterile cloth and the umbilical cord was allowed to hang down over the side.

The umbilical cord and the blood vessel were cleaned and disinfected with betadine solution and alcohol. 5ml of cord blood was drawn from several places where necessary using a sterile needle and syringe. Blood was transferred into appropriate tubes i.e. 1ml BD vacutainer® serum tube, 3ml BD vacutainer® sodium heparin and 1ml BD microtainer® EDTA (all Becton Dickinson, USA) and transported to the laboratory for testing.

2.1.7.3 HIV testing

Children with unknown HIV status were tested using HIV rapid test kits, according to national guidelines. Finger prick blood was collected using an EDTA coagulated capillary tube (Becton Dickinson, USA). Two rapid HIV test kits were used in parallel; Determine™ HIV1/HIV2 (Abbott Laboratories, Japan) and Unigold™ (Trinity Brotch, Dublin). A third test SD™ Bioline HIV1/2 3.0 (Standard Diagnostics, Korea) was used as a tie-breaker in case of discrepant results. Testing was performed, according to the manufacturer's instructions.

2.1.7.4 Malaria testing

Research study nurse collected blood from the finger prick using EDTA coagulated capillary tube (Becton Dickinson, USA) and prepared a thick smear for malaria parasite slides (MPS) by transferring a drop of blood from the capillary tube onto a clean labelled slide. A drop of blood on the slide was spread and air dried for 10 minutes at room

temperature. These slides were transported to Blantyre Malaria Project (BMP) Laboratory at NHC for malaria testing. The BMP laboratory team (Laboratory Technicians) stained air dried slides with field stain A and B, air dried for 10 minutes at room temperature and examined on high power field (HPF) microscope as previously described (Bailey*, et al.*, 2013), with minor modifications. Parasite examination was verified by another laboratory technician before results were released.

2.1.7.5 Preparation and storage of serum

A total of 1ml blood was collected in BD vacutainer® serum tube and transported to the laboratory within 4 hours. Cloated whole blood was centrifuged at 500g for 10 min and serum isolated. 200µl aliquots of serum were stored at -70°C until the day of testing.

2.1.7.6 Preparation of live *S.* Typhimurium for SBA

A well characterised *S*. Typhimurium strain D23580 (MacLennan*, et al.*, 2008, Kingsley*, et al.*, 2009) was grown on a Luria Bertani (LB) agar (Sigma Aldrich, USA) plate overnight and a single colony was selected and inoculated into 10ml Luria Bertani (LB) broth (Sigma Aldrich, USA). This is a clinical strain isolated from a HIV negative child in 2004, at the peak of a Blantyre multidrug-resistant (MDR), invasive NTS epidemic (MacLennan*, et al.*, 2008, Kingsley*, et al.*, 2009). The inoculated broth container was loosely capped to permit gas exchange and incubated at 37^{0}C for 18-24 hours to achieve

stationary growth phase. The culture suspension was briefly vortexed and 100µl transferred into pre-warmed fresh 10ml LB broth, tightly-capped (to avoid spillage) and incubated at 37^{0}C on a rocker plate (Stuart Scientific, Belgium) at 20 rpm for 2 hours, to achieve a log growth phase. Two 1.5ml aliquots of *S.* Typhimurium strain D23580 in log growth phase were made in 2ml micro tubes (Eppendorf, Hamburg, Germany) and centrifuged at 6000rpm for 5 minutes and the supernatants were discarded. *S.* Typhimurium strain D23580 pellets were washed thrice with 1ml sterile phosphate buffered saline (PBS) at 6000 rpm. *S.* Typhimurium strain D23580 pellets were re-suspended in 500µl of PBS, and pooled to achieve approximately $1x10^{8}$ cfu/ml (Miles*, et al.*, 1938). Viable *S.* Typhimurium strain D23580 colony forming units were confirmed using the Miles and Misra as described before (Miles*, et al.*, 1938). *S*. Typhimurium D23580 was diluted with PBS in 96 micro-well plates (Sarstedt, Germany), in 10-fold dilutions. LB agar plates were divided into 8 sections and in each section triplicates of 10µl (*S*. Typhimurium strain D23580 preparation) from 96 micro-well plate were inoculated to the corresponding sections and aerobically incubated at 37^{0}C for 24 hours. *S.* Typhimurium strain D23580 colony forming units (CFU) per ml were calculated as follows: CFU per ml = average number of colonies at each dilution x 100 (volume factor) x dilution factor.

2.1.7.7 Preparation of *S.* Typhimurium homogenate for T cell stimulation

A single colony of *S.* Typhimurium strain D23580, from an LB agar plate, was inoculated into 10ml LB broth and incubated at 37^{0}C for 18-24 hours. Two 5ml aliquots were centrifuged at 4,000rpm for 5 minutes and the supernatants were discarded. *S.* Typhimurium strain D23580 pellets from both tubes were re-suspended in 2.5ml of PBS and pooled in a single tube. Two 1ml aliquots were transferred in 2ml screw cap micro tube (Sarstedt, Germany) and topped up with 100µm beads (Biospec products, USA). *S.* Typhimurium strain D23580 aliquots were bead beaten using the Mini-Bead beater™ (Biospec products, USA) thrice, on high setting, for 120 seconds. The sample was inverted several times between each bead beating, to allow cooling. Samples were allowed to separate for 5 minutes and the bead-free supernatant portions were transferred into fresh 2ml screw cap micro tubes (Sarstedt, Germany*)*. Protein concentration of S. Typhimurium strain D23580 homogenate and protein standards were determined using Thermo Scientific™ Bicinchoninic Acid (BCA) Protein assay (Thermo Scientific, UK). A standard curve was used to compute the *S*. Typhimurium strain D23580 homogenate protein concentration. The protein concentration was adjusted using sterile PBS, and *S*. Typhimurium strain D23580 homogenate aliquots at 100µg/ml were stored -20^{0}C.

2.1.7.8 Intracellular cytokine staining for detection cytokine producing T cells

Intra-cellular cytokine staining (ICS) is an immunological technique that allows simultaneous detection and quantification of cytokine producing cytokine cells (T cells) at single cell level and detection of cellular phenotype (memory T cell subsets, CD4+ T cells). In addition this assay can detect cell viability and also proliferation. ICS for antigen specific T cells has been used as a marker of immune memory following natural infection and also in vaccine studies (De Rosa, 2012, Saade*, et al.*, 2012, Freer & Rindi, 2013). Taken together ICS for antigen specific T cells is both a functional and phenotype assay and can be used to evaluate immunogenicity, poly-functionality and memory profile.

Whole blood (WB) T cell ICS assay was performed as previously described (Nyirenda*, et al.*, 2010) with minor modifications. WB for ICS assay was collected in 3ml BD vacutainer® sodium heparin (Beckon Dickson, UK). 450µl aliquots of fresh WB were stimulated, on the day of collection with 50µl of bead-beaten *S*. Typhimurium strain D23580 at a final protein concentration of 1µg/ml, or with both phorbol myristate acetate (PMA) 1µg/ml and ionomycin (ION) at 10µg/ml (all Sigma Aldrich, USA). Furthermore, all conditions were co-stimulated with anti-CD28/49d (Becton Dickinson, USA), for 6 hours at 37°C in aerobic incubator. At 2 hours, intra-cellular cytokine release was inhibited with 1µl of BD GolgiStop™ (Becton Dickinson, USA) at 10^6 cells/mL. At 6 hours, cells from 200µl of each sample were lysed with 2ml of 1X FACs lysing solution

(Becton Dickinson, USA) then permeabilised with 500μl of 1X permeabilising solution (Becton Dickinson, USA). Cells were washed with PBS/0.5% bovine serum albumin (BSA) buffer (Sigma Aldrich, USA) and stained with 3μl each of surface antibodies: CD3-APC Cy7, CD4-PB (all Becton Dickinson, USA) and 5μl each of intra-cellular cytokine antibodies: IFN-γ-PE, TNF-α-FITC, and IL-2-APC (all Becton Dickinson, USA). Cells were fixed in 200μl 1% formaldehyde/PBS and 20,000 events on CD4+ T lymphocyte gate were acquired immediately using a CyAN ADP^{TM} flow cytometer (Beckman Coulter, USA). CD3+CD4+ T cells producing IFN-γ, TNF-α and IL-2 were defined as CD3+CD4+IFNγ+, CD3+CD4+TNFα+, and CD3+CD4+IL2+ (Figure 2.2). Further analysis for polyfunctional CD4+ T cells producing single, double and triple cytokines were further analysed by Boolean gates using Flow Jo^{TM} version 7.6.5 software (Tree star, USA).

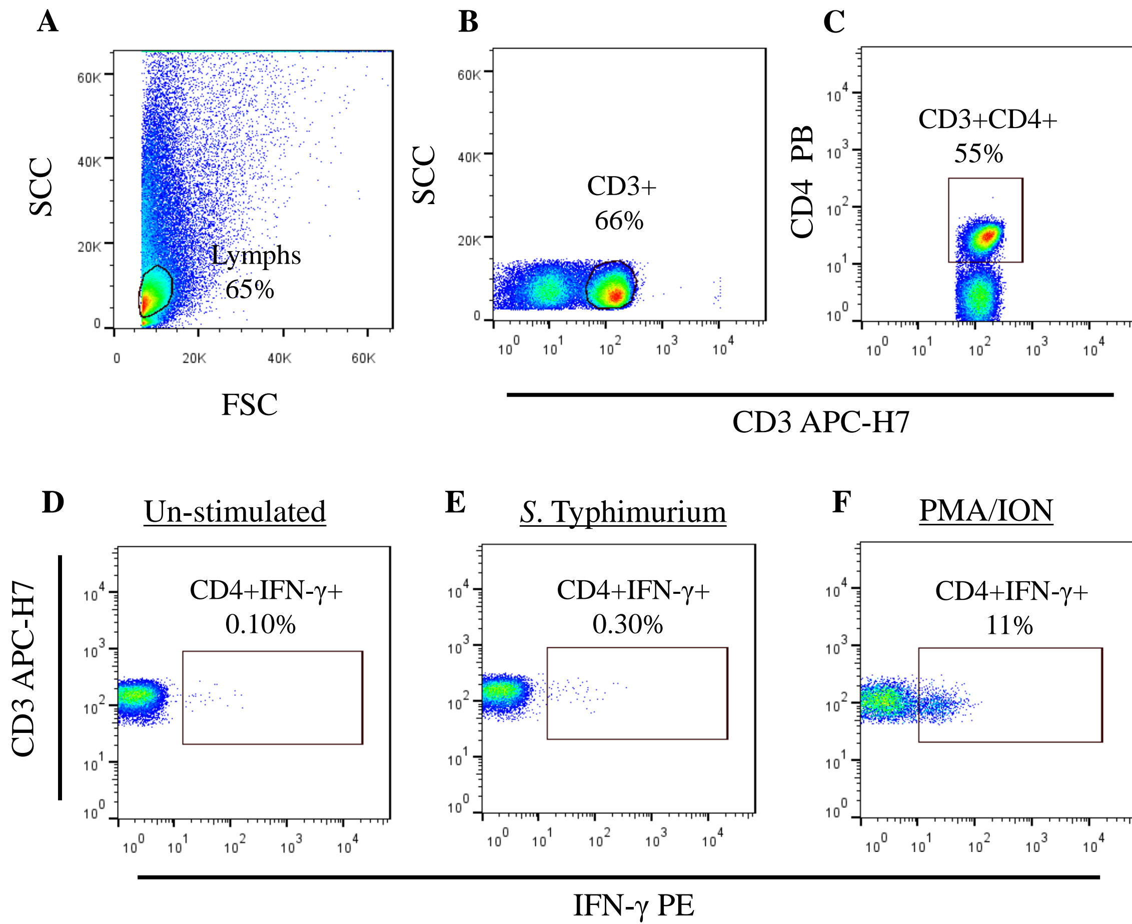

Figure 2. 2: Gating strategy for CD4+ T cell producing cytokines

Lymphocytes were gated using side scatter (SSC) and forward scatter (FSC) characteristics (Figure 2.2A). Lymphocytes expressing CD3 were gated from the lymphocytes gate (Figure 2.2A, 2.2B). CD3+CD4+ T cells were gated from the CD3+ lymphocyte gate (Figure 2.2B, 2.2C). CD3+CD4+ T cells producing IFN-γ were gated from CD3+CD4+ T cell gate (Figure 2.2C-F). Representative un-stimulated, *S.* Typhimurium strain D23580 and PMA/ION stimulated plots are shown (Figure 2.2D-F).

2.1.7.9 Immunopheno-typing for quantification of CD4+ memory T cell subsets

Immuno-phenotyping (IPT) is an immunological technique used to detect and quantify cellular proteins (such as CD3) which are bound by flourochrome conjugated antibodies or marker (such as CD3-APC) and these are read out by flow cytometry. IPT is commonly used to detect and quantify memory CD4+ T cell subsets including effector memory (CD45RO+CCR7-), central memory (CD45RO+CCR7+) and naïve (CD45RO-CCR7+) (Mackay*, et al.*, 1990, Bunce & Bell, 1997). Whole blood (WB) for immuno-phenotyping was collected in 1ml BD microtainer® ethylenediaminetetraacetic acid (EDTA) (all Becton Dickinson, USA). 200µl of WB was stained with antibodies: CD3-APC, CD4-PB, CD45RO-FITC and CCR7-PE (all Becton Dickinson, USA) and RBCs lysed with 2ml of 1X FACS lysing solution (Becton Dickinson, USA). Cells were washed with PBS (Sigma Aldrich, USA) and fixed in 200µl (1% formaldehyde/PBS). Up to 20,000 events on CD4+ T lymphocyte gate were acquired immediately on CyAN ADP™ flow cytometer (Beckman Coulter, USA) and analysed using FlowJo™ version 7.6.5. Lymphocytes were gated by their forward scatter (FSC) and side scatter (SSC) characteristics. Naïve T cells were defined as CD4+CD45RO-CCR7+, effector memory (EM) T cells as CD4+CD45RO+CCR7- and central memory (CM) T cells as CD4+CD45RO+CCR7+ (Figure 2.3) (Mackay*, et al.*, 1990, Bunce & Bell, 1997).

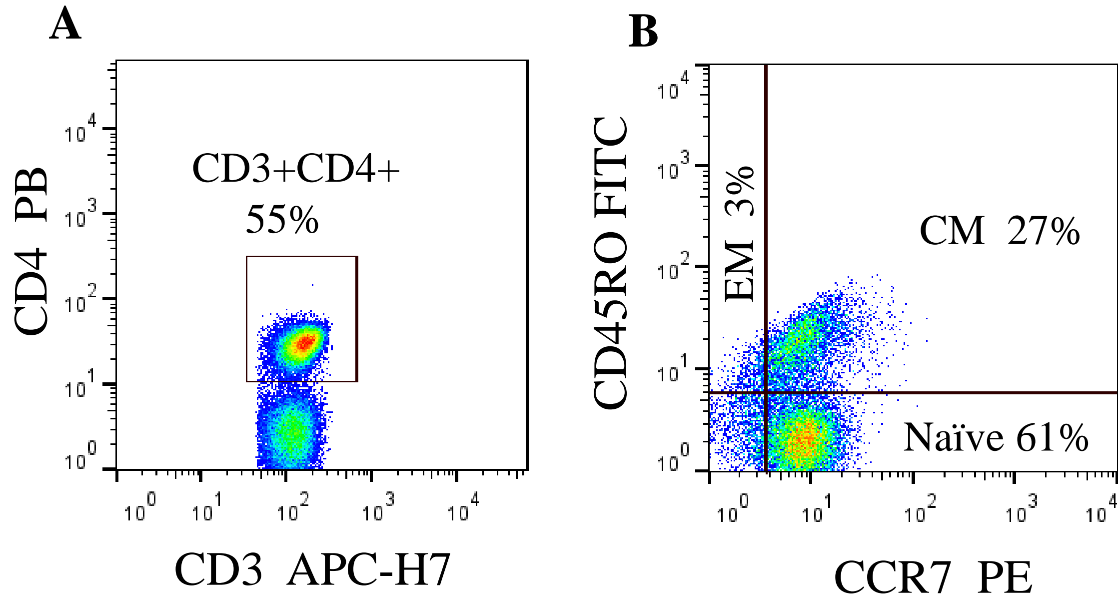

Figure 2. 3: Gating strategy for CD4+ memory T cells

From the CD3+CD4+ T cell gate (Figure 2.3A) CD4+ memory T cells were defined by the expression of CD45RO and CCR7 as shown (Figure 2.3B). We defined naïve T cells as CD4+CD45RO-CCR7+, effector memory (EM) T cells as CD4+CD45RO+CCR7- and central memory (CM) T cells as CD4+CD45RO+CCR7+.

2.1.7.10 Serum bactericidal assay for quantification of antibody immunity

Serum bactericidal activity (SBA) assays were performed as previously described (MacLennan, *et al.*, 2008). Serum or PBS was mixed with *S.* Typhimurium strain D23580 at $1.0x10^6$ cfu/ml (10µl of the prepared viable bacteria at 1 $x10^7$ cfu/ml were added to 90µl of undiluted freshly-thawed serum), prepared as described in section 2.1.7.6. Test samples were placed in a 37°C aerobic incubator for 180 minutes. Test samples were

serially diluted and plated in triplicate on Luria Bertani (LB) agar. Viable colony count of *Salmonella* was done after 24 hours of incubation as described in section 2.1.7.6. Log10 change in *S*. Typhimurium cfu/ml from the baseline (PBS condition) was reported. As previously described (MacLennan*, et al.*, 2008).

2.1.7.11 Serum ELISA for quantification of anti-*S.* Typhimurium-IgG antibody

Enzyme-linked immunosorbent assay (ELISA) is an immunological technique that has been applied for detection of memory B cell secreted antibodies to a specific antigen including *Salmonella* in serum or plasma. ELISA for detection of antibodies to specific pathogen has been used to evaluate immune responses elicited by a vaccine or natural infection, longevity of antibody responses, diagnosis of infection and surveillance (Carlsson*, et al.*, 1975, Beasley*, et al.*, 1981, Strid*, et al.*, 2007). ELISA for detection of *Salmonella* specific antibodies were performed as previously described (Cunningham*, et al.*, 2007) with minor modifications. Nunc-Immuno™ MicroWell™ 96 well solid plates (Sigma Aldrich, USA) were coated overnight using 100µl of carbonate-bicarbonate buffer (Sigma Aldrich, USA) per well containing antigens adjusted to 5µg/ml: *S.* Typhimurium O;4,5-LPS (ALEXIS Biochemicals), *S*. Typhimurium-OMP, *S.* Typhimurium-FliC (MacLennan*, et al.*, 2010) (*S*. Typhimurium-OMP and *S*. Typhimurium-FliC were generated and kindly donated by Adam Cunningham laboratory [University of Birmingham]) and *E. coli*- LPS 0127:B8 (Sigma Aldrich, USA). Further description of these antigens is provided in Table 2-A below. Plates were washed with

wash buffer (PBS plus 0.05% Tween 20) and blocked with 200µl/well blocking buffer (PBS plus 1% BSA) for 1 hour at 37°C. Test serum at 1:20 in dilution buffer (PBS plus 0.05% Tween 20 plus 1% BSA) was serially diluted 3-fold and incubated at 37°C in aerobic incubator for 1 hour. After washing, 100µl of 1:2000 secondary Goat Anti-human IgG-AP antibodies (Southern Biotech, USA) were added and incubated for 1 hour at 37°C in aerobic incubator. Finally, after washing, 100µl of SIGMAFAST™ p-Nitrophenyl phosphate substrate (Sigma Aldrich, USA) was added to each plate and the plate was read after 30 minutes, using a Bio Tek™ reader ELx800 (Bio Tek Instruments, USA) at 405nm. Specific antibodies were determined using arbitrary measurements (Figure 2.4).

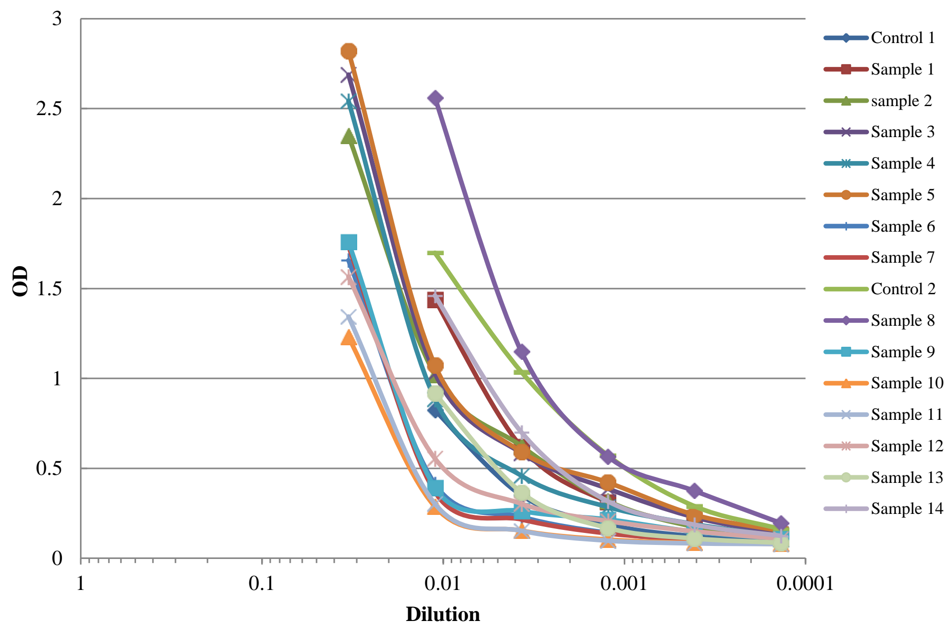

Figure 2. 4: Quantifying anti-*S.* Typhimurium O; 4,5 LPS IgG antibody in serum

Antibody titres targeting *S.* Typhimurium LPS in serum were determined by arbitrary measurements; 1 (cut off absorbance) was divided by the dilution factor for each sample.

Table 2- A: *S.* Typhimurium antigens used in antibody ELISA experiments

***S.* Typhimurium antigens**	**Description of antigens**
1. *S*. Typhimurim LPS	Commercial *S*. Typhimurium 0;4,5 antigen (ALEXIS Biochemicals)
2. *S*. Typhimurim OMP	Crude outer membrane protein generated from *S*. Typhimurium
3. *S*. Typhimurim FliC	FliC filament protein of flagellin generated from *S*. Typhimurium
4. *E*. *coli*-LPS 127:B8	Commercial *E. coli*-LPS 127:B8 (Sigma Aldrich, USA) was used as control

2.2 *SALMONELLA* EXPOSURE AND DEVELOPMENT OF SPECIFIC IMMUNITY IN MALAWIAN CHILDREN

2.2.1 Study design and participants

A prospective longitudinal cohort study was conducted among healthy children and their mothers to determine the relationship between *Salmonella* exposure within the gastrointestinal tract (GIT) or oropharynx and development of serum immunity to *Salmonella*. Mothers were recruited to determine whether maternal breast milk bactericidal activity is associated with prevention of *Salmonella* colonization of the GIT among breastfed children (Figure 2.5). Children were recruited from 6 months of age and not from birth because there was limited time to recruits and follow study participants for a period longer than 18 months. However, Chapter 3 describes that at 6 months children exhibit poor SBA to *Salmonella* as passively acquired maternal antibodies decline in blood circulation hence limiting this immunity as the confounder at baseline (study entry).

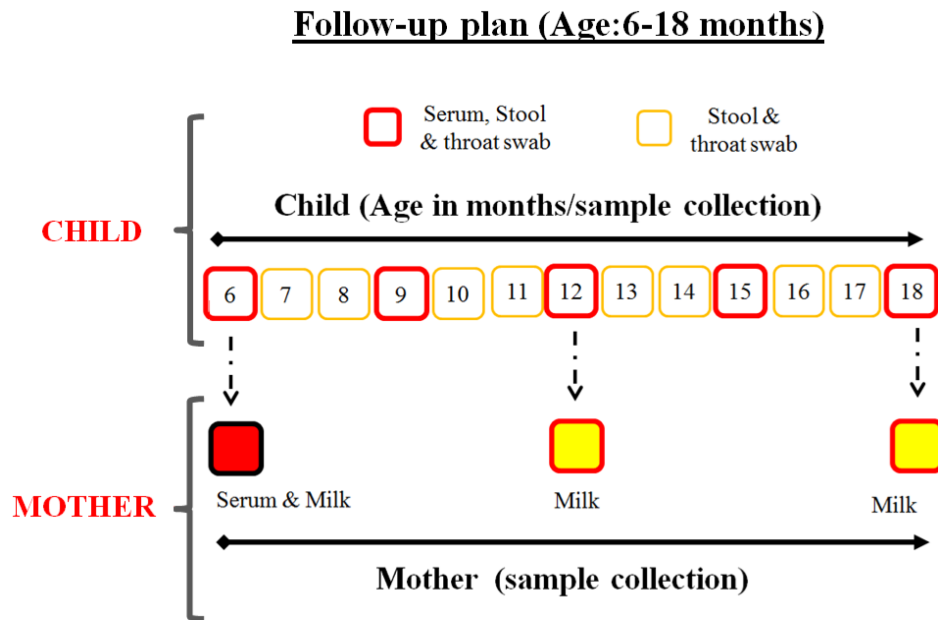

Figure 2. 5: Study design; *Salmonella* exposure and development of immunity

A total of 60 healthy children and their mothers were recruited when children were aged 6 months and followed up to until when they were 18 months old. Study participants were followed at monthly intervals (children) and six-month intervals (mother) for study specific procedures as in indicated. Stool, serum and oropharynx swab were collected from each children while serum and breast milk were collected from mothers during follow-up (Figure 2.5). All samples collected and processed as described in laboratory methods section 2.2.8.

Following the study approval by COMREC (see below), the study team (including myself as the study investigator, research nurse and the MLW science communication

team) conducted the study specific sensitization meetings with ZHC healthy workers (Clinicians, Nurses and Healthy Surveillance Assistants) and Zingwangwa community leaders (village headmen and religious leaders). These sensitization meetings aimed at bringing awareness of the research study at ZHC and focussed on discussing information contained in the study information sheets. The recruitment process was conducted by the study research nurse. Study recruitment began with group sensitisation talk that was given to the parents and guardians attending ZHC. Children's mothers who were keen to participate in the study were sensitised individually and their child recruited into the study provided they consented. Study participants were recruited consecutively provided they were meeting entry criteria including the specific age group (section 2.2.3). Study specific samples were collected by the research study nurse and transported to the laboratory for microbiological and immunological investigation (section 2.2.8).

2.2.2 Sample size

When this study was being conducted, the incidence of *Salmonella* exposure in healthy children for our study population was not known. However, a study with a similar design in Mexico showed that 40% of children were colonized or had minimally symptomatic infection with *Salmonella* during the first year of life (Cravioto, *et al.*, 1990). A ratio of 1.5 (60% unexposed/40% exposed) was assumed, percentage of unexposed with outcome 10% (serum bactericidal activity) and percentage of exposed with outcome 50%. To detect 40% difference in serum bactericidal activity between *Salmonella* exposed and unexposed among children aged between 6 and 18 months with 80% power at the 0.05

significance level and allowing for 20% loss to follow up, a total 60 children aged 6 months were recruited.

2.2.3 Inclusion and exclusion criteria

Study participants were only recruited as pairs (child and mother). Healthy children attending Zingwangwa Health Centre (ZHC) vaccination clinic aged 6 months were recruited. Mothers to these children were also recruited provided they were medically well. Children exclusion criteria included: children born preterm (before 38 weeks), known HIV-positive (documented in child health passport) or clinical HIV/AIDS and those that tested positive using rapid HIV tests (aged < 18 months were referred to early infant diagnosis (EID) clinic for HIV-DNA confirmatory test). HIV infected mothers and HIV exposed children were also excluded from the study. Children presenting with acute illness including fever >38°C, and residence outside the geographical wards that are situated within 5 km from ZHC were also excluded from the study.

2.2.4 Study location

Participants were recruited into the study at ZHC (under-five clinic) in Blantyre district. ZHC provide primary health care within the Blantyre city. According to Blantyre district health office (DHO) and QECH 2010/2011 annual report, it was projected that ZHC to have catchment population (those receiving service from the health facility) of about 147, 676 in the year 2012 (MCI, 2013). Study specific samples were collected at ZHC and

transported to MLW for microbiological and immunological investigations as described in laboratory methods section 2.2.8.

2.2.5 Tracing and mapping of study participants homes (Follow up)

During the first study specific visit, the research study nurse or study field worker obtained sketch maps and contact details from each study participant mother to locate their home. Study field worker, used these sketch maps and contact details to visit each study participant home and obtain exact location GPS co-ordinates. This information was used to trace study participants in case of missing their scheduled visit, and the GPS co-ordinates were used to investigate *Salmonella* epidemiology (i.e. *Salmonella* transmission pattern).

2.2.6 Study participants feeding practices and medical history

At each study specific visit, a questionnaire was administered to mothers to explore child feeding practices. Mothers were also asked to provide medical history for their child.

2.2.7 Ethical consideration

The study was compliant with Good Clinical Practice (GCP) regulations and conducted in accordance with the 1996 ICH GCP guidelines and the 2000 Declaration of Helsinki.

Ethical approval (P.01/13/1327) was granted by COMREC. Informed consent was obtained from a parent or guardian of each participating child and mothers.

2.2.8 Laboratory methods

2.2.8.1 Collection of peripheral blood sample

Blood samples were collected as described in section 2.1.7.1 at all scheduled visits (Figure 2.6).

2.2.8.2 Preparation and storage of serum

Serum samples were prepared and stored as described in section 2.1.7.5.

2.2.8.3 Serum Bactericidal Assay for quantification of *S.* Typhimurium specific antibody mediated immunity

Quantification of serum bactericidal activity (SBA) were performed as described in section 2.1.7.10

2.2.8.4 Collection of breast milk and storage

Breast milk from mothers was collected to investigate whether breast milk kills *S*. Typhimurium *ex vivo* and speculate whether breast feeding prevents colonisation of *Salmonella* in the GIT of breast fed babies. To ensure that breast milk was properly collected by study participating mothers. At the clinic mothers were asked to wash their hands with soap and running water before expressing milk. Mothers were asked to sit somewhere comfortable, relax, have their baby close by and if necessary warm their breasts to express milk. Breast milk was self-expressed by mothers. A total of 5ml breast milk was collected in a sterile universal container, and transported to the laboratory within 4 hours of collection. At the laboratory, milk samples were stored at 4º C until processing on the same day of collection. Milk was centrifuged at 400g for 20 minutes and the fatty layer was discarded. Breast milk fluid (supernatant) was aspirated and aliquoted in 2ml volumes and stored at -70º C until when required for testing (Shapiro*, et al.*, 2007).

2.2.8.5 Breast milk bactericidal assay for quantification of antibody immunity

A milk bactericidal assay was developed by optimizing the previously described SBA (MacLennan*, et al.*, 2008). *S.* Typhimurium strain D23580 was adjusted to $1.0x10^6$ cfu/ml and incubated at 37°C for 180 minutes with 3 test samples: milk which was non-heated, milk which had been heated at 72°C for 30 minutes (preliminary experiments showed

that breast milk has factors that promoted *Salmonella* growth, therefore heated condition was considered as a baseline condition) and PBS (Table 2-B).

Table 2- B : Experimental conditions used for evaluation milk bactericidal activity

Experimental condition	Objective
1. PBS plus *Salmonella*	Confirm baseline concentration of *Salmonella*
2. Milk plus *Salmonella*	Determine milk bactericidal activity
3. Heated milk at 72°C plus *Salmonella*	Inactivate complement and other bactericidal peptides

Test samples were then serially diluted and plated in triplicate on Luria Bertani (LB) agar. A colony count of *Salmonella* was also done after 24 hours of incubation as described in 2.1.7.6. Log10 change in *Salmonella* cfu/ml from the baseline (heated at 72°C for 30 minutes) was reported.

2.2.8.6 Collection of stool specimens

Mothers were provided in advance with stool collection containers (Sarstedt, Germany), and advised how to collect stool specimens from their children in sterile containers on the day they were due to attend study clinic. The spoon in the lid of the container was used to collect stool. Stool was collected up to a marked line on the tube and transported to the clinic within 8 hours of sample collection.

2.2.8.7 Stool processing storage and culture for selective *Salmonella*

Stool specimens were stored in the refrigerator at 4ºC and processed for isolation of *Salmonella* on the same day. Stool specimens for DNA extraction were stored as whole stool for long term at -70°C. Matchstick head-size stool specimen was inoculated directly on selective media xylose lysine deoxycholate (XLD) agar plate (Oxoid, UK) and into 10ml Selenite F broth (Oxoid, UK) and incubated aerobically at 37°C for 18-24 hours (Figure 2.6).

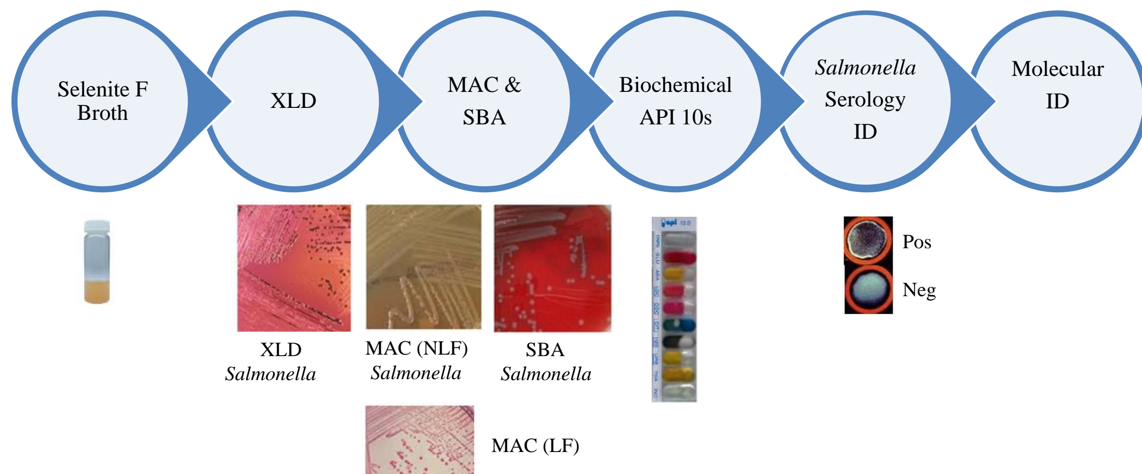

Figure 2. 6: Overview, isolation and identification of *Salmonella* from stool sample

The process of isolation and identification of *Salmonella* from stool is shown. Representative images *Salmonella* colonies on differential culture media are shown.

Selenite F Broth was used to enrich *Salmonella* from stool which may be in small numbers while reducing the growth of faecal coliform, particularly *E. coli* (Leifson, 1939, Wain & Hosoglu, 2008). The mechanism of how selenite F broth inhibit faecal coliforms

is not clear but selenium toxicity has been implicated that it reacts with sulphur and sulphydral groups in critical bacteria cell components (Weiss, 1965). The inoculated Selenite F broth was sub-cultured onto an XLD agar plate, and incubated aerobically at 37°C for 18-24 hours, and 1ml of inoculated Selenite F broth was transferred into 2ml micro tube (Eppendorf, Hamburg, Germany) and this was stored at -70°C for *Salmonella* detection using real time PCR (rt-PCR). Presumptive *Salmonella* colonies on XLD agar (selective and differential media allows preferential growth of *Salmonella* and *Shigella*) appear pink, with or without a black centre (Park, *et al.*, 2012). However other enteric bacteria including *Citrobacter, Proteus* and *Serretia* also grow on XLD and these may resemble *Salmonella* as they also appear pinkish and exhibit black centre due to hydrogen sulphide (H2S) production (Park, *et al.*, 2012). Presumptive *Salmonella* colonies were then sub-cultured onto sheep blood agar (SBA) and MacConkey (MAC) agar plates (both Oxoid, UK) (spreading both plates for single colonies) and incubated aerobically at 37°C for 18-24 hours. Suspected *Salmonella* colonies on MAC agar plate are non-lactose fermenters (NLF), and these appear colourless. Proteus species swarm on SBA agar plate and is distinguished from *Salmonella* and other enteric bacteria such as *Citrobacter* and *Serretia* that do not swarm on SBA. Presumptive *Salmonella* were then distinguished from other enteric bacteria (i.e *Citrobacter* and *Serretia*) using biochemical tests including triple sugar iron (TSI) agar (Oxoid, UK) and Urea agar (Oxoid, UK). TSI agar is used for the determination of carbohydrate fermentation and hydrogen sulphide (H2S) production in the identification of Gram-negative bacilli (Hajna, 1945). *Salmonella* are urea negative and TSI acid but with or without gas, and alkaline slope with or without

H2S production (Yong, *et al.*, 1985). Further *Salmonella* identification was determined using API® 10S (bio Merieux, France) according to the manufacturer's instructions. *Salmonella* serovar identification was then determined using standard serological grouping (Kauffman-White scheme). This allows identification of *Salmonella* serovars using antisera reactions according to their antigenic properties O (somatic antigen), H (flagellin antigen) and Vi (capsular polysaccharide antigen). *Salmonella* isolates with incomplete serological identification (Table 2-C) were archived for molecular identification and characterization. All *Salmonella* isolates were stored at -70 ^{o}C in microbank™ (Pro-lab Diagnostic, UK).

Table 2- C: *Salmonella* serological tests

	***S.* Enteritidis**	***S.* Typhimurium**	***S.* Typhi**	***Salmonella species***
PSO	+	+	+	+
PSH	+	+	+	+
O9 antigen	+	-	+	-
O4 antigen	-	+	-	-
Vi antigen	-	-	+	-
HG,m antigen	+	-	-	-
Hi antigen	-	+	-	-
Hd antigen	-	-	+	-
H1,2 antigen	-	+	-	-
O5 Antigen	-	+	-	-

2.2.8.8 Development of real time PCR for *Salmonella* detection in stool specimen and other fluids

To determine the pattern of *Salmonella* exposure within the GIT and the oropharynx both stool and oropharyngeal swabs were collected from each child at 1 month intervals. Currently, stool culture remain gold standard test for the detection of *Salmonella* within the GIT (WHO, 2003). However, stool culture is less sensitive (< 50%) (Hoffman*, et al.*, 1984, Matheson*, et al.*, 2010) and time consuming (take no less than 3 days)(Wain*, et al.*, 2001). This study therefore also aimed to develop real-time PCR (rt-PCR) for *Salmonella* detection in faecal specimens. In Malawi and elsewhere in SSA the commonly isolated *Salmonella* serovars in blood include *S*. Typhimurium, *S.* Enteriditis and *S*. Typhi. To begin with, this study aimed at detecting the frequency of *S.* Typhimurium and *S*. Typhi using rt-PCR, therefore DNA primers sequences for the following genes: invaA (targeting all *Salmonella* serovars), FliC (targeting *S.* Typhimurim), LPXO (targeting *S.* Typhimurium ST313 strain) and fimbriae (targeting *S*. Typhi) were generated.

DNA primers were generated using NCBI tool Primer Blast. Table 2-D describes design properties of DNA primers generated. Specificity of primers was checked using a BLAST search and primer secondary structures with the potential to hinder annealing were identified using mfold software version 3.6 (Washington University). Furthermore, melting temperature matches and checking for any hairpins was performed using the online tool (www.basic.northwestern.edu). Table 2-E and 2-F provides the list of DNA primers and probes generated.

Table 2- D: Primer design properties

1. Product size ranging from 80-150bp.
2. Having a GG, CC, GC or CG ('G/C-clamp') at the 3'end is preferable: 2 Gs or Cs in the last 5 bases, 1 G or C in the last 3 bases, and an A or T at the 3'end.
3. Melting temperature 55-60°C, only 1-2°Cs difference between forward and reverse primers.
4. GC content of 50-60%.
5. Runs of same bases were avoided, especially stretches of >3 Gs or Cs at 3' end. These results in polymerase slippage.
6. Self-complementarity at 3' end was avoided.
7. Any 3'dimer formed by primer annealing to itself or partner must be non-existent or very weak (deltaG should be > -2 kcal). Any primer with both terminal delta G> -2 and an extendable 3'end (5'overlap) should be avoided. The strongest overall dimer should be unstable as well, delta G> -6.

Table 2- E: List of primers

Primer name	**Primer direction**	**Primer code**
Flic-STm 1	Forward	TGCTGATTTGACAGAGGCTAAA
Flic-STm 1	Reverse	TCGCCTACCTTAACTGCTAAAC
Flic-STm 2	Forward	GGGAACTGGTAAAGATGGCTATTA
Flic-STm 2	Reverse	TTCACATCCTCAGTTGCTGTC
LPXO-ST313 1	Forward	TAGTCGAAGATGACGGCTTTG
LPXO-ST313 1	Reverse	CGGTTCAGTACGTTACCATCTT
LPXO-ST313 2	Forward	ACCTCCTATTTCCAGCGAGA
LPXO-ST313 2	Reverse	CTCGCCGTGGAATGGTTT
INVA 1	Forward	AGCGTACTGGAAAGGGAAAG
INVA 1	Reverse	CACCGAAATACCGCCAATAAAG
INVA 2	Forward	TCATCGCACCGTCAAARGA
INVA 2	Reserve	CGATTTGAARGCCGGTATTATT
Fimbriae-ST 1	Forward	CCGACCAAGTTCCAGATCAA
Fimbriae-ST 1	Reverse	GTTGGTTAGTAGCGAGGTGTT
Fimbriae-ST 2	Forward	CGCGAAGTCAGAGTCGACATAG
Fimbriae-ST 2	Reverse	AAGACCTCAACGCCGATCAC

Table 2- F: List of probes

Probe name	Probe description
Flic-STm 1	Fam-TGTTACCGGCACAGCATCTGTTGT-BHQ1
Flic-STm 2	Fam-CCAGCAAGAGTCACCTCACCGTTC-BHQ1
LPXO-ST313 1	Vic-AGCTATGGCGCTGTCGATCAACTT-BHQ1
LPXO-ST313 2	Vic-ACTGCCGTCGCTAAGAAACTGCTT-BHQ1
INVA-1	Fam-TTACGGTTCCTTTGACGGTGCGAT-BHQ1
INVA-2	Fam-ACGCTTCGCCGTTCRCGYGC-BHQ1
Fimbriae-ST 1	Vic-TGGCCAGTAATAATGTCGGGACGA-BHQ1
Fimbriae ST 2	Vic-CATTTGTTCTGGAGCAGGCTGACGG-BHQ1

2.2.8.9 Extraction of DNA from stool for PCR testing and sequencing

To extract DNA for detection of *Salmonella* and other enteric bacteria in stool using real time PCR and sequencing of gut microbial communities, DNA extraction methods that allow optimum detection of both Gram positive and Gram negative bacteria were adopted. Approximately 200mg of stool was suspended in 500µl of PBS. Freshly prepared, 50ul lysozyme (10mg/ml) (Sigma Aldrich, USA) was added to each sample and incubated aerobically at 37 °C for 1 hour. Approximately 300 mg of 0.1mm diameter silicon beads (Biospec products, USA) were added to samples to disrupt bacteria at 2100rpm for 1 minute. Both the bead beating step and addition of lysozyme steps were aimed at enhancing the extraction of DNA from Gram positive and Gram negative bacteria. 1.2 ml lysis buffer ASL (Qiagen, Netherlands) was added to each sample and

thoroughly homogenized by vortexing. Samples were heated for 5 minutes at 70°C, vortexed for 15 seconds and centrifuged at 16,000rpm for 1 min to pellet stool particles. An InhibitEX tablet (Qiagen, Netherlands) was added to 1.2 ml supernatant to remove PCR inhibitors from DNA extraction preparations, vortexed continuously for 1 minute then incubated for 1 minute at room temperature. Samples were centrifuged twice at 16,000rpm for 3 minutes to pellet inhibitors bound to InhibitEX matrix. 15µl QIAGEN Proteinase K (Qiagen, Netherlands), 200µl supernatant and 200µl Buffer AL (Qiagen, Netherlands) were mixed and incubated at 70°C for 10 minutes. 200µl of ethanol (96–100%) was added to the lysate and the mixture was transferred into QIAamp spin column (Qiagen, Netherlands) placed in a 2 ml collection tube and centrifuged at 16,000rpm for 1 minute. QIAamp spin column was placed on new 2ml collection tube, and tube containing the filtrate was discarded. 500µl Buffer AW1 (Qiagen, Netherlands) was added to the QIAamp spin column and centrifuged at 16,000rpm for 1 minute. 500µl Buffer AW2 (Qiagen, Netherlands) added to QIAamp spin column and centrifuged twice at 16,000rpm for 3 minutes. 200µl Buffer AE (Qiagen, Netherlands) was added directly onto the QIAamp membrane incubated for 1 minute at room temperature, then samples were centrifuged at 16,000rpm for 1 minute to elute DNA. Eluted DNA was stored at –20°C.

2.1.1.1 Real time PCR for detection of *Salmonella* in stool specimens

Previously optimised PCR protocol at MLW molecular laboratory was adopted. Master-mix for rt-PCR was prepared using pre-defined quantities. A total of 20µl master-mix

was comprised of the following:12.5µl Platinum® Quantitative PCR Super Mix-UDG (Life Technologies, USA), 0.10µl specific forward primer, 0.10 specific reverse primer, 0.10 specific probe (all primers and probes at 200nM), 0.05µl ROX reference dye (Life Technologies, USA) at 50nM final concentration, and 7.15µl nuclease-free water. This mixture was transferred to appropriate 96-well plate PCR wells. Five µl of test DNA, controls DNA, negative control (UV treated water) were added to appropriate well (with master-mix). Rt-PCR was run using Applied Biosystems® 7500 Real-Time PCR Systems (Life Technologies, USA) under appropriate reporter dye settings. The threshold was set in the lag phase, controls were checked for correctness, and then test samples CT values were registered (Figure 2.7).

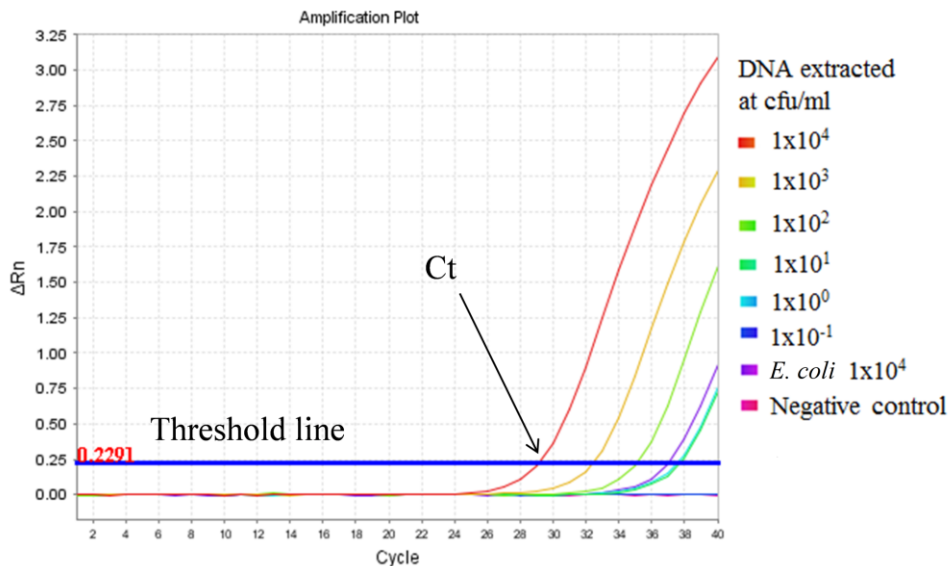

Figure 2. 7: Representative real time PCR read out

2.2.8.10 Limit of detecting of *Salmonella* using real time PCR

The level of yielding positive *Salmonella* result using stool culture is low (< 50% true positive stool samples are detected) (Hoffman*, et al.*, 1984, Matheson*, et al.*, 2010). To determine a method that would yield superior limit of detecting *Salmonella* in stool samples, a number of methods were compared; standard stool culture method (selenite broth and XLD) with rt-PCR on spiked stool and rt-PCR on spiked stool (PCR method 3) and treated with selenite broth (PCR method 4) (Figure 2.8). *Salmonella* was adjusted to 10^4 – 10^1 cfu/ml using Miles and Misra technique and DNA extracted using method 3 or method 4 (Figure 2.8). DNA extraction was performed as described section 2.2.8.9, but without treatment with lysozyme and bead beating step.

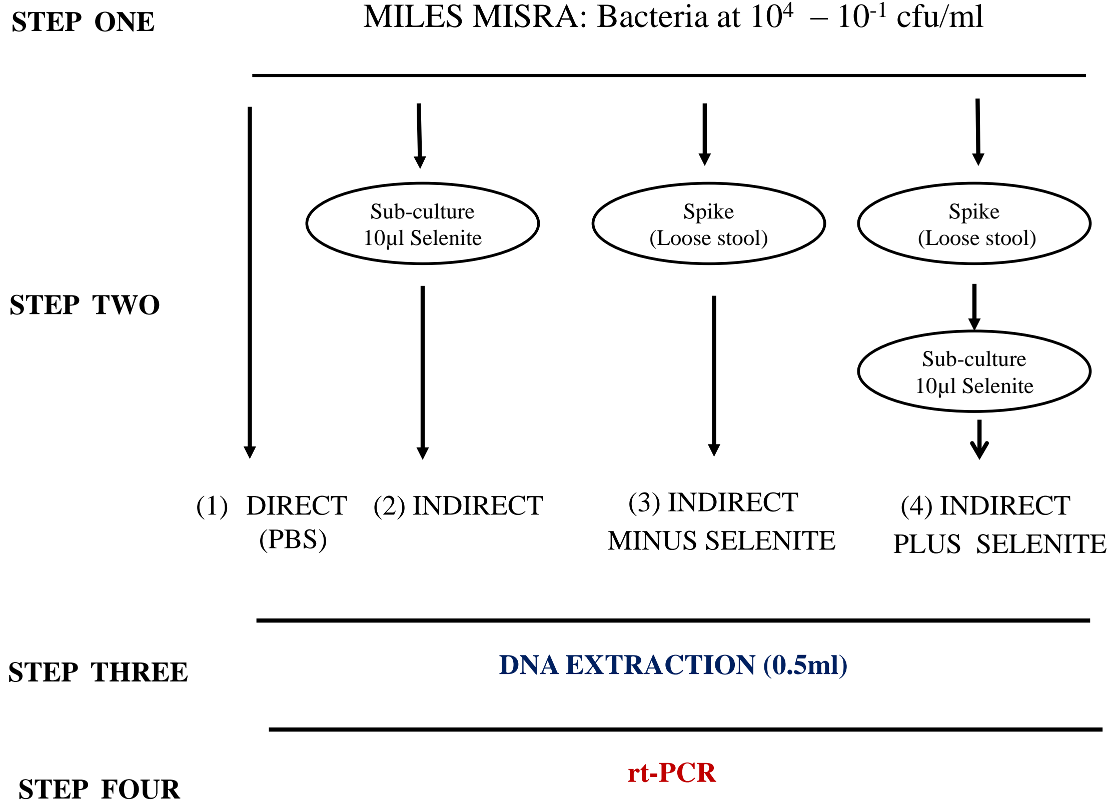

Figure 2.8: Methods of detecting *Salmonella* in stool using PCR

Limit of detecting (LOD) *S.* Typhimurium in stool using the standard stool culture ranged between 10^3 and 10^4cfu/ml, while LOD *S.* Typhimurium in stool ranged between 10^1 and 10^3 cfu/ml using PCR method 3 (Figure 2.9A). Observations were made that addition of Selenite F broth (PCR method 4) improved the LOD *Salmonella* in stool using rt-PCR (from LOD ranging 10^1-10^3 cfu/ml, [CT 30-35] using PCR method 3 to LOD ranging $<10^1$-10^1 cfu/ml, [CT <25] using PCR method 4) (Figure 2.9B).

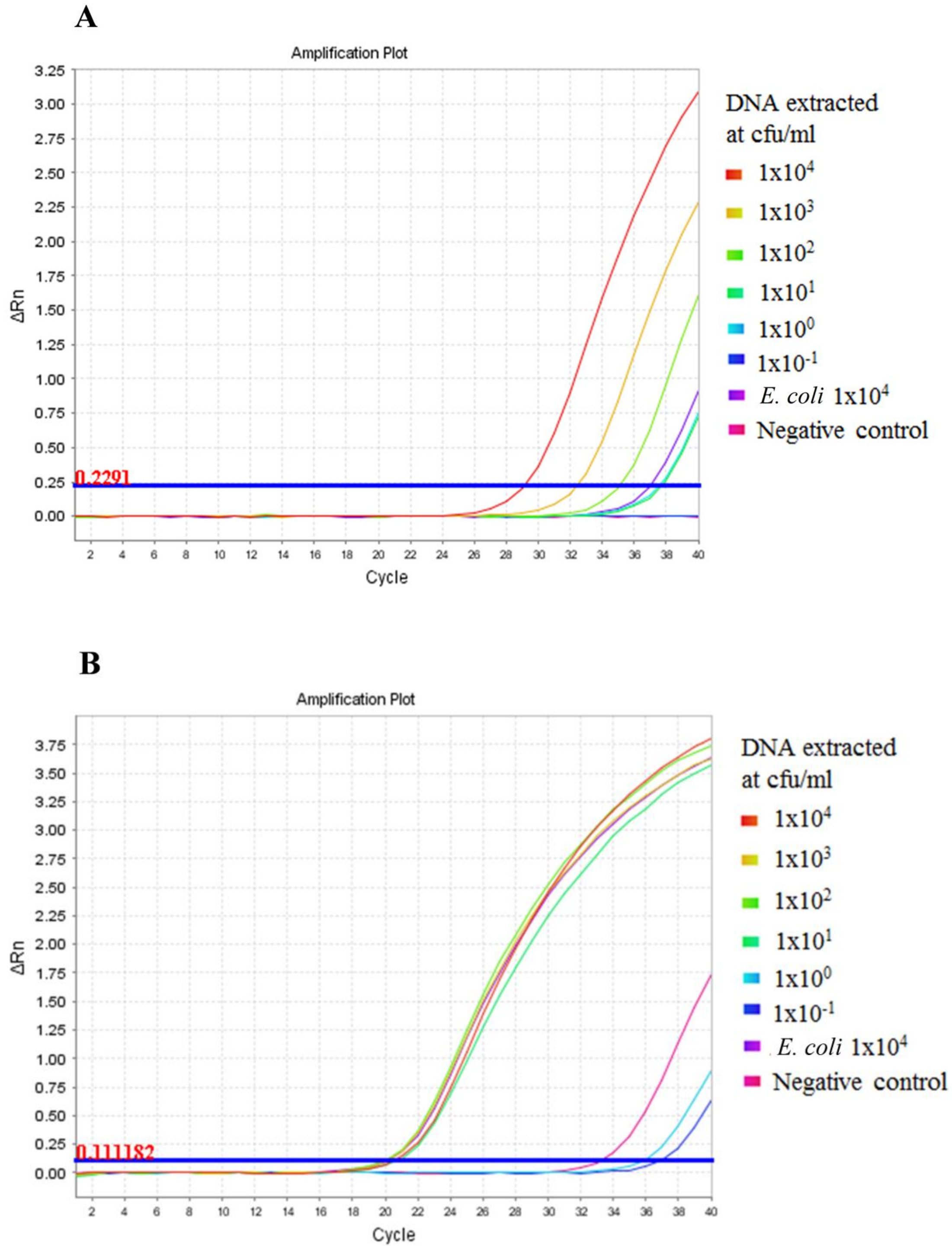

Figure 2. 9: Development of real time PCR for detection of *Salmonella* exposure in stool

Representative diagram showing direct rt-PCR results (Figure 2.9A) and indirect (selenite enriched) rt-PCR results (Figure 2.9B). DNA was prepared at varying concentration as indicated.

2.2.8.11 Specificity and sensitivity of PCR for *Salmonella*

Stool samples harbours a huge amount of closely related (genetically) enteric bacteria(Fanaro*, et al.*, 2003). These possess a challenge as regards to generation of highly specific and sensitive primers for rt-PCR assay. Newly generated DNA primers specificity was tested against a panel of Gram negative including *Proteus*, *E. coli*, *Citrobacter* and *Klebssiella* and Gram positives including *Staphylococus aureus* and *Streptococcus pneumoniae*. Both InvaA DNA primer and LPXO DNA primer were not specific for *Salmonella* (Proteus and *E. coli* DNA tested positive) (Table 2-E and 2-F). FliC and Fimbriae DNA primers were therefore considered for use in successive experiments. It's possible that these DNA primers could be specific against enteric bacteria but fail to distinguish DNA from *Salmonella* serovars. Intra-*Salmonella* specificity and sensitivity of the DNA primers were investigated. Fimbriae DNA primer sensitivity was 100%, 95% CI (19.2-100) and specificity was 100%, 95% CI (75.1-100) (Table 2-G). FliC-1 DNA primer sensitivity was 100%, 95% CI (58.3-100), while its specificity was 37.5%, 95% CI (8.7-75.3) (Table 2-G). FliC-2 DNA primer sensitivity was 83%, 95% CI (36 -97.2) and sensitivity 100%, 95% CI (58.9-100) (Table 2-H and I). Taken together sensitivity and specificity for fimbriae DNA primer was excellent while specificity and sensitivity were poor for FliC-1 DNA primer and moderately good for

FliC-2 DNA primer. Fimbriae DNA primer was used for detection of *S*. Typhi in stool specimens. FliC-1 and FliC-2 DNA primers were used for screening and confirmation of *S*. Typhimurium detection in stool specimens.

Table 2- G: Specificity and sensitivity for Fimbriae DNA primer (*S*. Typhi)

	Present	**n**	**Absent**	**n**	**Total**
Positive	TP	2	FP	0	2
Negative	FN	0	TN	13	13
Total		2		13	15

Sensitivity= 100%, 95% CI (19.2-100) **Specificity** = 100%, 95% CI (75-100)

TP refers to true positive

FN refers to false negative

FP refers to false positive

TN refers to true negative

Table 2- H: Specificity and sensitivity for FliC-1 DNA primer (*S*. Typhimurium)

	Present	n	Absent	n	Total
Positive	TP	7	FP	5	12
Negative	FN	0	TN	3	3
Total		7		8	15

Sensitivity= 100%, 95% CI (58-100) **Specificity** = 37.5%, 95% CI (8.7-75.3)

TP refers to true positive

FN refers to false negative

FP refers to false positive

TN refers to true negative

Table 2- I: Specificity and sensitivity for FliC-2 DNA primer (*S*. Typhimurium)

	Present	n	Absent	n	Total
Positive	TP	5	FP	0	5
Negative	FN	1	TN	7	8
Total		6		7	13

Sensitivity= 83.3%, 95% CI (36-97.2) **Specificity** = 100%, 95% CI (58.9-100)

TP refers to true positive

FN refers to false negative

FP refers to false positive

TN refers to true negative

2.3 DEVELOPMENT OF T CELL AND ANTIBODY MEDIATED IMMUNITY IN RESPONSE TO INVASIVE *SALMONELLA* INFECTION

2.3.1 Study design and participants

A prospective longitudinal cohort study was conducted comprising of children presenting to hospital with invasive *Salmonella* (index cases). To evaluate T cells and antibodies immune response, index cases were recruited when *S*. Typhimurium or *S*.Typhi was isolated in blood cultures as part of routine surveillance at Queen Elizabeth Central Hospital (section 2.1.5). Family members of index cases were also recruited to evaluate T cells and antibodies immune response and further explore *Salmonella* immune-epidemiology. Blood samples were collected from study participants for immunological investigations (Figure 2.10).

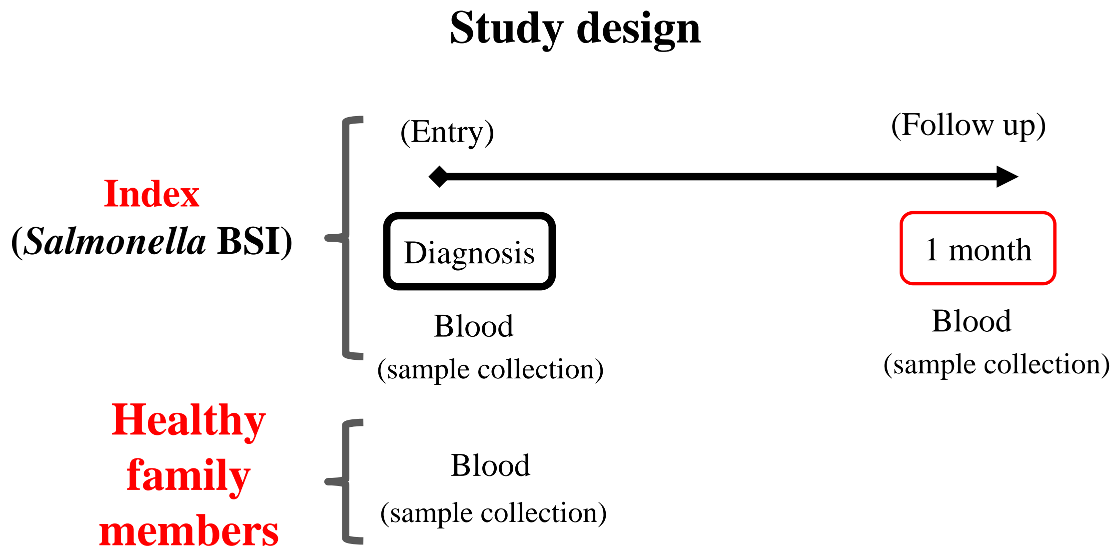

Figure 2. 10: ***Salmonella*** **bacteraemia and development of cellular and immunity**

A total of 20 children with *Salmonella* bacteraemia and their family members were recruited. Only index cases were prospectively followed at 1 month into the recovery period. A blood sample was collected from study participant as indicated (Figure 2.10). All blood samples were processed as described in section 2.3.6.

2.3.2 Sample size

A total of 20 children (less than 15 years of age) were recruited presenting to hospital with *Salmonella* bacteraemia and their family members. This was a pilot study and power calculations were not undertaken.

2.3.3 Inclusion and exclusion criteria

Children recruited were only those that had microbiologically confirmed *Salmonella* bacteraemia, among febrile paediatric admissions to Queen Elizabeth Central Hospital (QECH) and who were domiciled in Blantyre district. Family members of these children were also recruited. Children with conditions that could potentially interfere with the evaluation of the study objectives were excluded such as terminally illness and upper GI surgery. Children with HIV infection and severe malnutrition were not included.

2.3.4 Study location

Children and family members participating in the study were recruited and blood samples collected at QECH (paediatrics and child health ward and outpatient department). QECH has been described in section 2.1.5. Blood samples were transported to MLW for immunological investigations within 4 hours of collection.

2.3.5 Ethical consideration

The study was compliant with Good Clinical Practice (GCP) regulations and conducted in accordance with the 1996 ICH GCP guidelines and the 2000 Declaration of Helsinki. Ethical approval (P.02/11/1040 and P.08/12/1265) was granted by COMREC. Informed consent was obtained from a parent or guardian of each participating child and adult family members.

2.3.6 Laboratory methods

2.3.6.1 Collection of peripheral blood sample

Blood samples were collected as described in section 2.1.7.1 at all study specific scheduled visits (Figure 2.10).

2.3.6.2 Preparation and storage of serum

Serum samples were prepared and stored as described in section 2.1.7.5

2.3.6.3 Intracellular cytokine staining for detection of CD4+ IFN-γ+ T cells

Whole blood (WB) for intra-cellular cytokine staining (ICS) assay was collected in 1ml BD vacutainer® sodium heparin and the assay was performed as described in section 2.1.7.8 with minor modifications. Briefly 450µl blood was stimulated with (*Salmonella enterica* Generalized Modules for Membrane Antigens expressing O antigen), GMMA- (*Salmonella enterica* Generalized Modules for Membrane Antigens not expressing O antigen), FliC (*S*. Typhimurium flagellin protein) and OMP (*S*. Typhimurium outer membrane protein) [all adjusted to 10µg/ml] were all generated and kindly donated by Calman MacLennan laboratory (Novartis Vaccine Institute for Global Health) and all samples were co-stimulated with anti-CD28/49d antibody (Becton Dickinson, USA) for 6 hours at 37°C. At 2 hours, intra-cellular cytokine release was inhibited with 1µl of BD GolgiStop™ (Becton Dickinson, USA) at 10^6 cells/mL. Cells from 200µl of blood sample were lysed with 2ml of 1X FACs lysing solution then permeabilised with 500µl

of 1X permeabilising solution (Becton Dickinson, USA). Cells were washed with PBS/0.5% BSA buffer (Sigma Aldrich, USA), and stained with 3µl each of the surface antibodies: CD3-PercP, CD4-APC and 5µl of the intra-cellular cytokine antibody: IFN-γ-PE (all Becton Dickinson, USA). Cells were fixed and events acquired and analysed using FlowJo as described in section 2.1.7.8 (Figure 2.11).

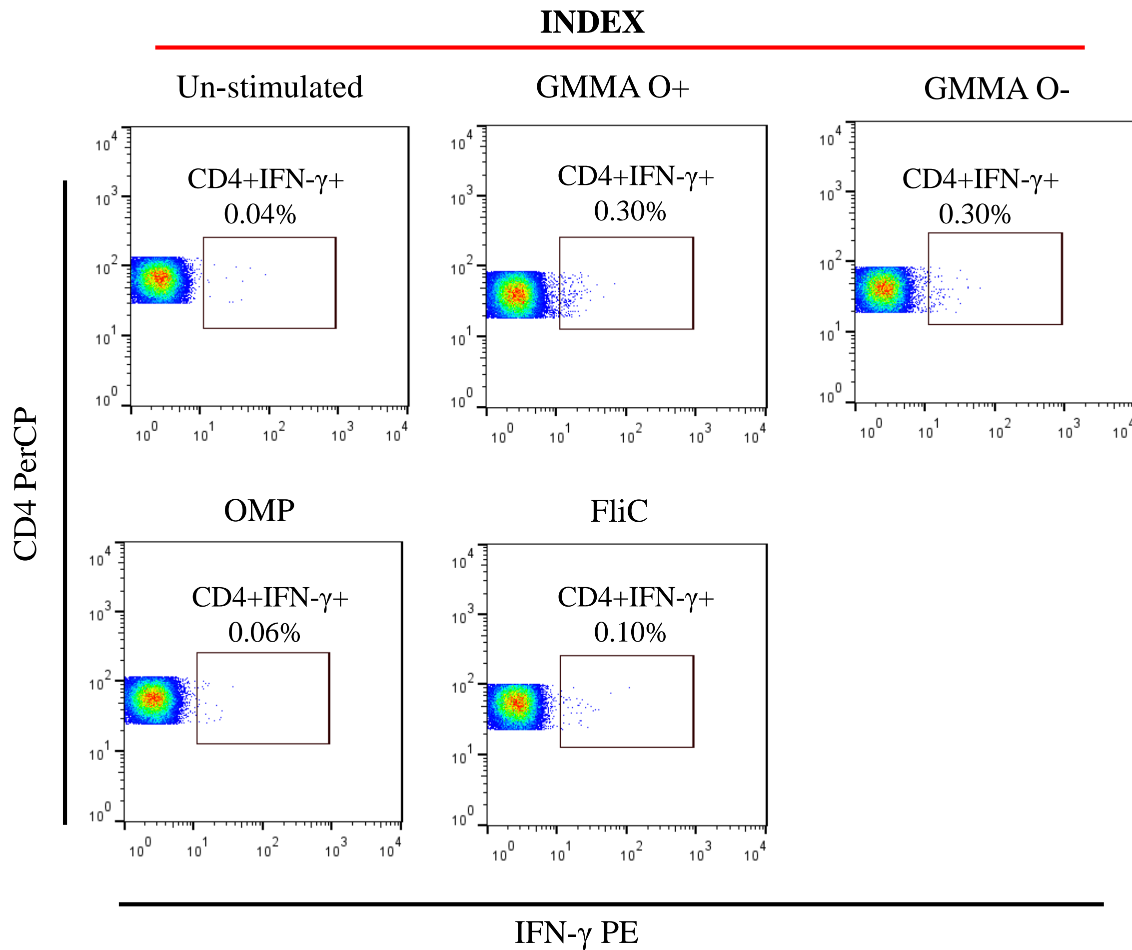

Figure 2. 11: Detection of T cells producing cytokines

A representative figure of antigen-specific CD4+ T cells producing IFN-γ. T cells were stimulated by various *Salmonella* antigens as indicated.

Representative plots of CD4+ T cells producing IFN-γ in unstimulated condition or stimulated with GMMA O+, GMMA O-, OMP, and FliC antigens. Index T cell responses at the point of diagnosis are shown (Figure 2.11).

2.3.6.4 B-cell ELISPOT for detection of *Salmonella* specific ASC

B cell immunity to specific pathogens including *Salmonella* are commonly evaluated using antigen specific ELISA in serum or plasma (Carlsson, *et al.*, 1975, Beasley, *et al.*, 1981, Strid, *et al.*, 2007). However, evaluation of B cell immunity using ELISA alone does not provide the whole picture of B cell immunity as this excludes the examination of memory B cells pool. It has therefore being argued that evaluation of B cell immunity by using both ELISA and B cell ELISpot give the complete picture of B cell immunity (Jahnmatz, *et al.*, 2013). B cell ELISpot is used to detect antigen specific secreting cells (ASC) and memory B cells. Active plasma blast (ASC) stimulated in vivo by either natural infection or vaccination can be examined directly by B cell ELISpot without *ex vivo* stimulation as is the case with memory B cells which require *ex vivo* stimulation to reach detectable levels (Jahnmatz, *et al.*, 2013). In this study IgG or IgA ASC were quantified by B cell ELISpot (without re-stimulation) as previously described (Kantele, *et al.*, 2012) with minor modifications. Millipore multiscreen 96-well ELISpot plates (EMD Millipore, USA) with a 0.45µm surfactant free mixed with cellulose ester membrane were coated with *Salmonella* O:4,5-LPS (ALEXIS Biochemicals), *Salmonella* O:9-LPS (ALEXIS Biochemicals), GMMA+ (*Salmonella enterica* Generalized Modules for Membrane Antigens expressing O antigen), GMMA- (*Salmonella enterica* Generalized

Modules for Membrane Antigens not expressing O antigen), *S*. Tymphimurim -FliC (S. Typhimurium flagellin protein) and *S*. Tymphimurim -OMP (S. Typhimurium outer membrane protein) [all adjusted to 5µg/ml] diluted in carbonate-bicarbonate buffer (Sigma Aldrich, USA) pH 9.6 (filtered through a 0.2µm syringe filter). Plates layout is shown in Figure 2.12. Coated plates were incubated at 4°C overnight. On day 2, plates were washed 6 times with 200µl/well of PBS then soaked in 200µl/well of PBS for 5 minutes. Plates were blocked with R10 medium (RPMI plus 10% Gibco® newborn calf serum (NBCS) [Life Technologies, USA]) (200µl/well) and incubated at 37°C in 5% CO2 for 1 hour. PBMCs were isolated from heparinized blood and processed within 4 hours after collection. PBMC pellets were re-suspended in R10 medium and cells counted and adjusted to $2.5x10^{6}$cells/ml. A cell suspension of 100µl was added to the blocked ELISpot plate to achieve $2.5x10^{5}$ cells/well and incubated at 37°C in 5% CO2 for 24 hours. Cells and supernatants were discarded and plates washed 5 times with PBS-Tween then soaked for 5 minutes. Alkaline-phosphatase goat anti-Human IgG and IgA antibodies were diluted 1:5000 in 0.2µm filtered PBS/10% NBBS and added 100µl/well to the appropriate wells and incubated at RT for 4 hours. Plates were washed five times with PBS-Tween, and four times with 200µl/well of distilled water. Alkaline phosphatase (AP) colour development buffer concentrate (Bio Rad, Switzerland) was diluted 1:25 with distilled water. 1:100 5-Bromo-4-chloro-3-indolyl-phosphate solution/nitro blue tetrazolium (BCIP/NBT) were diluted with 1:25 colour development butter. 200µl of newly prepared alkaline phosphates solution (BCIP/NBT) was added to appropriate well and incubated until spots were developed. Further colour development was stopped with

distilled water (200µ/well) without allowing background to become too dark. Lastly plates were washed twice with 200µl/well distilled water. Plates were air dried and read using AID ELISpot Reader version 4.0 (AID GmbH, Germany) (Figure 2.13).

		1	2	3	4	5	6	7	8	9	10	11	12
		$2.5x10^5$ cells/well				$2.5x10^5$ cells/well				$2.5x10^5$ cells/well			
Index	IgG	04 LPS	04 LPS	04 LPS NC	04 LPS NC	09 LPS	09 LPS	09 LPS NC	09 LPS NC	FliC	FliC	FliC NC	FliC NC
Index	IgA	04 LPS	04 LPS	04 LPS NC	04 LPS NC	09 LPS	09 LPS	09 LPS NC	09 LPS NC	FliC	FliC	FliC NC	FliC NC
Index	IgG	OMP	OMP	OMP NC	OMP NC	GMMA+	GMMA+	GMMA+ NC	GMMA+ NC	GMMA-	GMMA-	GMMA- NC	GMMA- NC
Index	IgA	OMP	OMP	OMP NC	OMP NC	GMMA+	GMMA+	GMMA+ NC	GMMA+ NC	GMMA-	GMMA-	GMMA- NC	GMMA- NC

Figure 2. 12: IgG and IgA antibody secreting cells ELISPOT plate layout

A representative plate lay out for detection of IgG and IgA ASC using ELISPOT

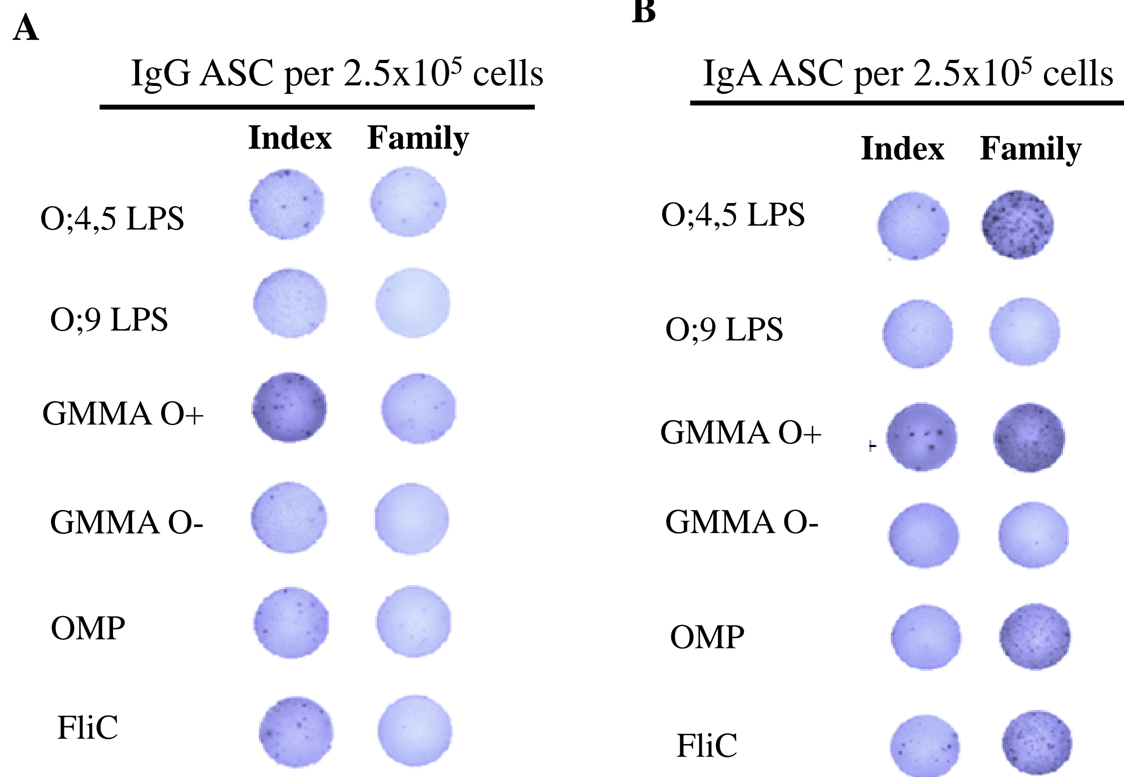

Figure 2. 13: IgG and IgA ASC per 2.5X10^{5}cells/ml

Representative images showing antigen-specific IgG antibody secreting cells (ASC) (Figure 2.13A) and IgA ASC (Figure 2.13B). Antigen-specific IgG and IgA ASC to 04-LPS, 09-LPS, GMMA O+, GMMA O-, OMP and FliC for both index and family member are indicated (Figure 2.13A, B).

2.4 Ty21a ORAL TYPHOID VACCINE INDUCED IMMUNITY IN THE PERIPHERAL BLOOD AND GUT MUCOSA OF HEALTHY ADULTS

2.4.1 Study design and participants

An interventional study was conducted to model natural *Salmonella* immunizing events in the gut mucosa and peripheral blood in healthy adults from the UK vaccinated with Ty21a Oral Typhoid vaccine. Participants were given three oral doses of the vaccine. One capsule was taken on each of days 0, 2 and 4, approximately one hour before a meal, with a cold or lukewarm drink, as recommended by the manufacturer and as licensed for use in the UK (Figure 2.14).

2.4.2 Sample size

This study aimed at recruiting 10 healthy adults in the vaccine group and 10 healthy adults in the control group in Liverpool. This was a pilot study and so power calculations were not done.

2.4.3 Inclusion and exclusion criteria

Healthy adults aged between 18 and 60 years, fluent in spoken English and those capable of giving informed consent were included in the study. Participants that were pregnant, had chronic illness or immune-compromised, anaemia, platelet count below 30,000 and those that had previous GI surgery were excluded. Individuals who had received the

parenteral typhoid vaccine within the past three years or the oral typhoid vaccine within the past twelve months, those who had previously travelled in countries endemic for typhoid (outside Europe, Australia or North America) and those with previous adverse reaction to vaccination were also excluded.

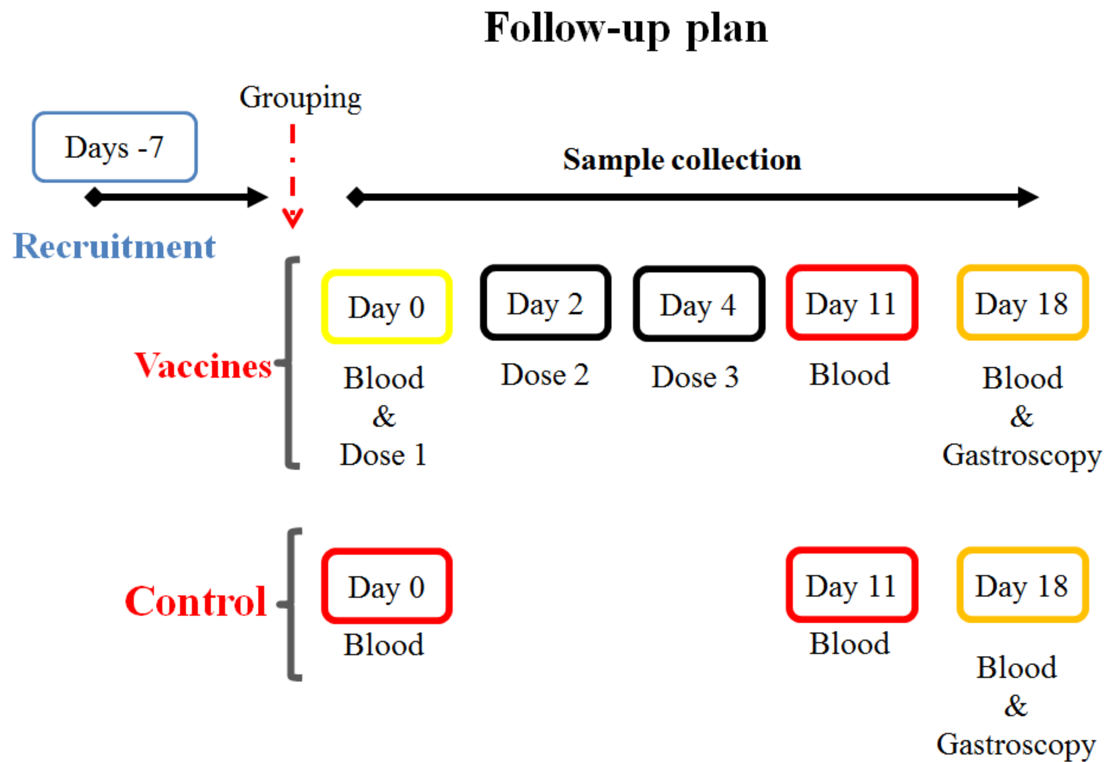

Figure 2. 14: Ty21a oral typhoid vaccine induced immunity in the peripheral blood and gut mucosa

A total of 9 vaccines and 8 controls (healthy adults age 18-65) were recruited in this intervention study to compare immune responses induced by oral Ty21 a Typhoid vaccine within the gastrointestinal tract (GIT) and peripheral blood. Three doses of oral

Ty21a vaccine were administered in the vaccine group only, and blood and gastroscopy specimens were collected as indicated (Figure 2.14).

2.4.4 Study location

This study was conducted in Liverpool and was sponsored by the Royal Liverpool University Hospital and the Liverpool School of Tropical Medicine (LSTM). The clinical work was carried out at the Royal Liverpool University Hospital and blood samples investigations were performed at LSTM.

2.4.5 Ethical consideration

Ethical approval for this study was obtained from the National Research Ethics Service (10/H1005/20). Informed consent was obtained from all study participants.

2.4.6 Laboratory methods

2.4.6.1 Collection of peripheral blood sample

A total of 30ml venous blood in 5ml BD sodium heparin tube (totalling 20ml) and 10ml BD serum tube (all Becton Dickinson, USA) was collected from both study groups on day 0 (pre-immunization sample), day 11 and day 18 follow up (Figure 2.15). Blood samples were collected by research study nurse.

2.4.6.2 Isolation of peripheral blood mononuclear cells for ICS and IPT

At total of 20ml of whole blood (WB) was collected in BD sodium heparin tubes (Becton Dickinson, USA) and was diluted with an equal volume of sterile PBS (Invitrogen, USA). Mixed blood/PBS was layered over half the volume of Lymphoprep™ (Axis-Shield, Scotland) and centrifuged at 800g for 20 minutes, with the brakes off at room temperature (RT). PBMCs were aspirated using a sterile Pasteur pipette into a separate 50ml BD Falcon™ Conical Centrifuge Tubes (Becton Dickinson, USA) and washed with 40ml sterile PBS (Invitrogen, USA) at 500g for 10 minutes. Cell pellets were next washed with 10ml of complete medium (50ml FBS [Invitrogen, USA] and 10ml 200mM L-glutamine [Sigma Aldrich, USA] were added to a 500ml bottle of RPMI-1640 [Sigma Aldrich, USA]) at 500g for 5 minutes. Pelleted cells were suspended in 2ml of complete medium and counted using a Neubauer Haemocytometer (Hausser Scientific, USA). Over 100 cells were counted if possible from 4 large squares to obtain a cell count per ml which was computed as follows: the product of cell count multiplied by 10^4 and divided by the number of large squares. Each sample cell concentration was adjusted to $5x10^6$ per ml and used for immuno-phenotyping and intra-cellular cytokine staining as described in section 2.4.6.5 and 2.4.6.6.

2.4.6.3 Collection of duodenum biopsy

Duodenum biopsy collection was performed by a Consultant Gastroenterologist. D2-D3 duodenal pinch biopsies were collected on day 18 from vaccinees and controls (Figure 2.15). Study participants fasted at home for 8 hours from midnight prior to the

endoscopic procedure. Sedation was optional; study participants were given up to 5mg maximum of midazolam intravenously. Nasal oxygen was administered during the procedure, and pulse oximetry monitoring was conducted throughout the procedure. Between 12 and 15, D2-D3 duodenal single-bite cold biopsies at 20-25cm insertion were collected using Boston Scientific large capacity ‘jumbo’ forceps (Boston Scientific, USA) which passed through a standard 2.8mm endoscopic biopsy channel. Biopsies were placed in MR15 medium (Mixture of 50ml Fetal Bovine Serum [Sigma Aldrich, USA], 10ml at 200mM L-glutamine [Sigma Aldrich, USA], 1ml Tazocin [piperacillin at 250mg/ml, tazobactam at 31.25mg/ml] [Wyeth Pharmaceuticals, USA] and 2.5ml amphotericin B at 250µg/ml (Sigma Aldrich, USA) and 500ml RPMI-1640 [Sigma Aldrich, USA)]), and transported on ice to the laboratory for processing. Study participants were observed for two hours post-procedure, given a drink and snack before being discharged home.

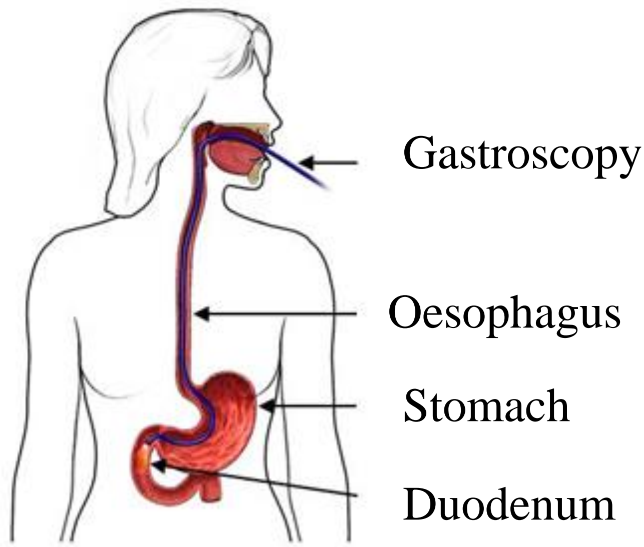

Figure 2. 15: Gastroscopy of the duodenum

2.4.6.4 Isolation of gut mucosal mononuclear cells

Gut mononuclear cells were isolated using a modification of the method previously described by Kaltsidis *et al* (Kaltsidis, et al., 2011). Biopsies were placed in 10ml MR15 medium on ice and were transported to the laboratory within 1 hour of collection. Samples were centrifuged at 400g for 10 minutes at room temperature, with the brake disabled. Biopsy pellets were re-suspended in 12.5ml CII-S medium (50mg Collagenase II-S [Sigma Aldrich, USA] plus 100ml MR15) and incubated at a 45° angle in a 37˚C shaking incubator at 220 rpm for 30 minutes. The tissue suspension was transferred into an open 50ml BD Falcon™ Conical Centrifuge Tubes (Becton Dickinson, USA). The tissue suspension was introduced by force through the syringe attached to a 16-gauge blunt-ended needle (Miltenyi Biotec, Germany) and then plunged and aspirated 4-5

times. Large aggregates were removed by passing the suspension through a 70µm cell strainer (Becton Dickinson, USA). Tissue fragments and clumps left were transferred from the cell strainer into the original tube by rinsing the cell strainer with 12.5ml of CII-S medium, ensuring that all remaining CII-S medium also entered the original tube. The original tube was placed back into the shaking incubator and the digestion and disruption procedure was repeated twice. Free cells from all three passages were pooled and centrifuged for 7 minutes at 500g. Pellets were re-suspended in 30ml MR15, and then centrifuged at 500g for 7 minutes. Pellets were re-suspended in 1ml MR15. Mononuclear cells were then adjusted to 1.0 $x10^6$/ml and seeded in Greiner CELLSTAR® 24-well plates (Greiner bio one, USA) at $1.0x10^6$/ml and rested overnight by incubating the plate at 37°C, 5% CO2. Cells were harvested, re-counted and adjusted to $5.0x10^6$/ml. Mononuclear cells were then used for intracellular cytokine producing T cells, as described in section 2.4.6.6.

2.4.6.5 Immuno-phenotyping for quantification of homing markers

This study aimed at characterizing homing properties of T cell subsets. The β7 integrin forms heterodimers with both the α4 and αE integrins (Stefanich*, et al.*, 2011). Since commercial anti-α4β7 antibody conjugated to a flourochrome of interest was not available, alternatively employed anti-β7 PE Cy7 antibody for T cell immunophenotying.

PBMCs were isolated from whole blood (WB) as described in section 2.4.6.2 and adjusted to $5.0x10^6$/ml. Test tubes were labelled as: fluorochrome Minus One (FMO) and

test for T cell surface phenotyping. A cell suspension of 200µl at 1.0 x10^6/ml was transferred to appropriate tubes, pelleted at 500g for 5 minutes and 100µl supernatant was removed. PBMCs were stained for T cell FMO condition with a pre-determined cocktail of surface staining antibodies: 5µl CD3-APC, CD4-APC H7, 2µl CD8-PE Cy7, 5µl CD19-PB (all Becton Dickinson, USA), and 5µl PBS (Invitrogen, USA). PBMCs were stained for the T cell test condition with a pre-determined cocktail of surface staining antibodies: 5µl CD3-APC, 5µl CD4-APC H7, 2µl CD8-PE Cy7, 2µl Integrin β7-PE Cy5 (all Becton Dickinson, USA), 2µl CCR9-PerCP Cy5.5 (BioLegend, USA), 3µl CD62L-AF700 (BioLegend, USA), 10µl CD45RA-FITC (Beckon Dickinson, USA), 5µl CCR7-PB (BioLegend, USA), and 5µl PBS (Invitrogen, USA). Stained PBMCs were incubated at 4°C in the dark for 20 minutes. Stained PBMCs were washed with 1ml PBS (Invitrogen, USA), centrifuged at 500g for 5 minutes. PBMCs were fixed in 1:10 diluted 300µl FIX solution (Becton Dickinson, USA) then acquired on BD FACSDIVA version 8 (Becton Dickinson, USA) within 24 hours. Memory T cells were gated and analysed using Flow Jo™ version 7.6.5 software (Tree star, USA).

2.4.6.6 Intracellular cytokine staining for detection of T cell producing cytokines

PBMCs isolated from whole blood (WB) and MMCs isolated from gut mucosal tissue were adjusted to 5x10^6/ml for antigen stimulation experiments. These antigen stimulation conditions were prepared: un-stimulated fluorochrome Minus One (US-FMO), un-stimulated stained (USS), Ty21a, influenza, and Staphylococcal enterotoxin B (SEB). 200µl PBMC or MMCs at 1.0 x10^6/ml were transferred appropriate micro-well (Greiner

bio one, USA). PBMCs or MMCs were stimulated as follows: US-FMO (no antigen stimulation), USS (no antigen stimulation), heat-inactivated Ty21a at 0.0632µg/ml (Berna Biotech, Switzerland), influenza (Influenza virus vaccine) at 0.225µg/ml (Solvay Biologicals B.V., Netherlands) and SEB at 100µg/ml (Sigma Aldrich, USA). Stimulated PBMCs or MMCs were incubated at 37 °C for 2 hours and treated with 1µl of BD Golgi-Plug (Becton Dickson, USA). Stimulated PBMCs or MMCs were incubated at 37˚C for a further 16 hours before harvesting. Non-adherent MMCs were removed by adding 200µl of RPMI-1640 medium and pipetting up and down. Harvested PBMCs or MMCs were pelleted at 500g for 5 minutes at 4˚C and the supernatant poured off. PBMCs or MMCs were re-suspended in 1ml PBS (Invitrogen, USA) containing 1µl ViViD (violet fluorescent reactive dye) (Invitrogen, USA) and incubated at 4˚C in the dark for 20 minutes. PBMCs or MMCs were suspended in 1ml PBS (Invitrogen, USA) and centrifuged at 500g at 4°C for 5 minutes. Supernatants were poured off and cells were stained with pre-determined cocktails of surface staining antibodies, as follows: Pre-determined cocktail of T cell FMO antibodies: 5µl CD3-APC, 5µl CD4-APC H7, 2µl CD8-PE Cy7, and 5µl CD19-Pacific Blue (all Becton Dickinson, USA). Pre-determined cocktail of surface antibodies for T cells: 5µl CD3-APC, 5µl CD4-APC H7, 2µl CD8-PE Cy7, 5µl CD19-Pacific Blue, 2µl β7-PE Cy5 (all Becton Dickinson, USA), 2µl CCR9-PerCP Cy5.5 (BioLegend, USA), and 5µl PBS (Invitrogen, USA). Stained cells were incubated at 4°C in the dark for 15 minutes and washed with 1ml PBS (Invitrogen, USA) for 5 minutes at 500g. Cells were treated with 250µl of Cytofix/Cytoperm (Becton Dickinson, USA), incubated at 4°C in the dark for 20 minutes. Cells were then washed

with 1ml Perm/Wash (Becton Dickinson, USA) centrifuged at 500g for 5 minutes. Permeabilised cells were stained with pre-determined cocktail of intracellular cytokine staining antibodies: 1µl IFNγ-AF700, 2.5µl TNFα-AF488, 10µl IL2-PE (all Becton Dickinson, USA), and 1.5µl Perm/Wash. These were incubated at 4°C in the dark for 30 minutes and washed with 1ml Perm/Wash for 5 minutes at 500g. Finally were fixed in 1:10 diluted 300µl FIX solution (Becton Dickinson, USA) then acquired on BD™ LSR II flow cytometer (Becton Dickinson, USA). CD3+CD4+ T cells producing IFN-γ, TNF-α and IL-2 were analysed for polyfunctional CD4+ T cells producing single, double and triple cytokines were further analysed by Boolean gates using Flow Jo™ version 7.6.5 software (Tree star, USA).

CHAPTER 3: DEVELOPMENT OF ADAPTIVE IMMUNITY TO NONTYPHOIDAL *SALMONELLA* IN MALAWIAN CHILDREN

Some of the work in this chapter has been published in the Journal of Infectious Diseases. The full manuscript is included in the appendix.

3.1 INTRODUCTION

NTS bacteraemia remains an important health problem in sub Saharan Africa particularly in young children and HIV infected individuals (Reddy*, et al.*, 2010, Feasey*, et al.*, 2012). To effectively control NTS bacteraemia in humans, public health interventions, such as vaccination alongside improvements in sanitation and hygiene in endemic regions, are required. Well characterized immune components that constitute immunity to NTS will help to inform the design of an effective vaccine for *Salmonella.* Immunity to *Salmonella* has been extensively studied in animal models compared to humans. For instance, mouse models of invasive *Salmonella* disease implicate innate immune cells (phagocytes), T cell and antibody-mediated immunity (Mastroeni*, et al.*, 1993, Tam*, et al.*, 2008). It is known that immunity to pathogens in mouse models is not always replicated in humans. Therefore, it is important to understand the similarities and differences between the immune response to *Salmonella* in animals and humans.

As discussed in chapter 1 (introduction chapter), NTS bacteraemia is particularly common among African children under two years of age suggesting that immaturity of the immune response may contribute towards susceptibility to invasive *Salmonella*

infection in this group. Immunity to *Salmonella* is complex and involves both humoral and cell-mediated mechanisms (Mastroeni, 2002, Mastroeni, 2003). Most Malawian children acquire anti-*Salmonella* IgG and IgM antibodies and bactericidal activity against NTS by the age of 2 years (MacLennan*, et al.*, 2008). Antibodies targeting NTS can effect bacterial killing through activation of complement cascade and assembly of the membrane attack complex (MacLennan*, et al.*, 2008). Antibodies opsonize NTS and, together with C3b deposition, facilitate internalization by phagocytes and subsequent killing of NTS through oxidative burst (Gondwe*, et al.*, 2010). These immune processes are thought to be critical for preventing extracellular growth and dissemination of NTS (MacLennan*, et al.*, 2008). However, salmonellae are facultative intracellular bacteria and individuals with primary immune-deficiencies affecting the IL12/23-IFN-γ axis (T helper 1 pathway) are particularly susceptible to NTS (Jouanguy*, et al.*, 1999, MacLennan*, et al.*, 2004), indicating an important role for cellular immune mechanisms and T cells, in particular, in immunity to *Salmonella*. This is supported by the close association between HIV/AIDS and NTS bacteraemia among adults in Africa, Europe and the USA (Gilks*, et al.*, 1990, Gordon*, et al.*, 2002), particularly those with CD4+ T cell counts of less than 200 cells/ul (Gordon*, et al.*, 2002). Prior to commencing ART, recrudescence of NTS bacteraemia is a common finding in this group, again indicating an important role for T cell immunity in clearing NTS infection and preventing chronic intracellular infection (Gordon*, et al.*, 2002). Adoptive transfer studies in mouse model of salmonellosis indicate that a combination of *Salmonella*-specific T cell and antibody is required for optimal protection against *Salmonella* infection (Mastroeni*, et al.*, 1993,

McSorley & Jenkins, 2000). T cells are particularly necessary to clear *Salmonella* from mice and prevent the development of chronic carrier states that could lead to relapse of infection (Mastroeni, 2003). Athymic mice and $CD28^{-/-}$ mice both show increased susceptibility to *Salmonella* infection suggesting that in the mouse model, clearance of *Salmonella* requires CD28-dependent activation of T cells (O'Brien & Metcalf, 1982, Mittrucker*, et al.*, 1999). Mice studies indicate that the T helper cell response to *Salmonella* is broad and is directed to protein antigens such as flagellin, porins and pilin (Mastroeni, 2002). Although it is known that CD4+ T cells orchestrate macrophage effector functions through IFN-γ and TNFα (Janssen*, et al.*, 2002, Gordon*, et al.*, 2005) and HIV-infected individuals with low CD4 counts are particularly susceptible to NTS bacteraemia (Gordon*, et al.*, 2010), the contribution of CD4+ T cell-mediated control of NTS in humans has not been well studied.

Class-switched (IgG isotype) and non-switched (IgM isotype) *Salmonella*-specific antibody levels have been shown to increase with age in young African children (MacLennan*, et al.*, 2008). The study hypothesis was that in the first two years of life, CD4+ T cell immunity to *S*. Typhimurium develop in parallel with the development of *S.* Typhimurium antibody immunity. Eighty healthy Malawian children aged 0-60 months were recruited in a cross-sectional study. *S*. Typhimurium-specific CD4+ T cells producing IFN-γ, TNFα and IL2 were quantified using intra-cellular cytokine staining. Antibodies to *S.* Typhimurium were measured by serum bactericidal activity assay (SBA), and anti- *S.* Typhimurium IgG antibodies by ELISA.

Hypothesis: In the first two years of life, CD4+ T cell immune responses to *S.* Typhimurium develop in parallel with the development of anti-*S.* Typhimurium antibodies.

Study objectives:

a) Characterize how T cell and antibody immune responses to *S.* Typhimurium develop in the first five years of life.
b) Examine the development of T cells and antibodies to *S.* Typhimurium in relation to *S.* Typhimurium bacteraemia epidemiology in children from Blantyre.

3.2 PARTICIPANTS RECRUITMENT AND METHODS

The methods for this chapter have been described in Chapter 2: study designs, materials, and methods, section 2.1.

In brief, 80 healthy Malawian children aged 0 to 60 months attending vaccination clinic, health check clinics and newborns in the maternity ward at Ndirande Health Centre (NHC) were recruited. A total of 20 adult participants were also recruited. NHC provide primary health care within the Blantyre city. According to Blantyre district health office (DHO) and QECH 2010/2011 annual report, it was projected that NHC to have catchment population (those receiving service from the health facility) of about 221,217 in the year 2012 (MCI, 2013). Child and adult participants were sampled blood at NHC. To characterise the acquisition of T cell immunity to NTS whole blood was quantified for

memory T cells and *Salmonella* specific-CD4+ T cells producing cytokines; IFN-γ, TNF-α and IL-2 by immuno-phenotyping and intracellular cytokine staining respectively. To characterise the acquisition of antibody mediated immunity to NTS, serum was quantified for complement fixing antibody by serum bactericidal assay and ELISA. These T cell and antibody responses to NTS were then examined in relation to age distribution of *S.* Typhimurium bacteraemia in under-five children.

3.3 STATISTICAL ANALYSES

GraphPad Prism version 5.0 (GraphPad Software, Inc, USA) was used to generate graphs and analyse the data. End-point response variables (antigen specific T cells producing cytokines, serum bactericidal activity and antibody titres) were examined for normality of distribution. The median was used as the measure of central tendency for non-normal distributed end points. Phases of the immune response were distinguished so as to further understand the evolution of immunity. Nonlinear regression models were used to fit to data relating *S.* Typhimurium-specific T cells and SBA responses with age. The inflection points of the resultant curves were taken to represent the boundaries of qualitatively different phases of immune response. The first period before the boundary was termed the early response and the subsequent period was termed the late response. The immune responses within these early and late periods were then modelled by linear regression. P-value of <0.05 was considered significant.

3.4 RESULTS

3.4.1 Healthy study participants

A total of 80 healthy children (Table 3-A), in 8 predefined age categories ranging from 0 to 60 months, were prospectively recruited at a large community health centre in Blantyre, Malawi, from March 2009 to January 2011. Children with malaria parasitemia, a positive HIV antibody test, severe anaemia (haemoglobin <7 g/dL), malnutrition (weight-for-height z score ≤ 2), or any other chronic illness were excluded from the study.

Table 3- A: General Characteristics, Nutritional and Haematological Profile

	Gender		
Parameter	Female	Male	All
Median participants (%)	35 (43.7)	45 (56.3)	80 (100)
Median age in months (range)	13.2 (0-52.5)	10 (0-47)	10.2 (0-52.5)
Median weight in kgs (range)	9.5(3.5-17)[a]	10(6-16.9)[b]	ND
Median height cm (range)	73.5(48-97)[a]	74(52-95)[b]	ND
Median weight for height z-score (range)	0.89(-1.9-4.6)[a]	1.4(-2-4)[b]	ND
Median lymphs x10^3/µl (range)	6.3(2.9-13.46)	5.3(2.2-10.4)	5.4(2.2-13.6)
Median Hgb in g/dl (range)	11.5(7.6-18.1)	11.2(8.0-17.7)	11.4(7.6-18.1)

Abbreviations: ND, Not done

[a] Twenty-five children aged 1-60 months were included.

[b] Thirty-five children aged 1-60 months were included.

3.4.2 Age distribution of *S.* Typhimurium bacteraemia in under-five children in Malawi

Between January 2006 and December 2011 *S.* Typhimurium bacteraemia was detected in 449 children <5 years of age presenting to QECH, of whom 359/449 (80%) were <2 years. The median age of *S.* Typhimurium bacteraemia was 13 months (range 0-60) (Figure 3.1). It was noted that age distribution for *S.* Typhimurium bacteraemia had not changed as previously reported (MacLennan, *et al.*, 2008).

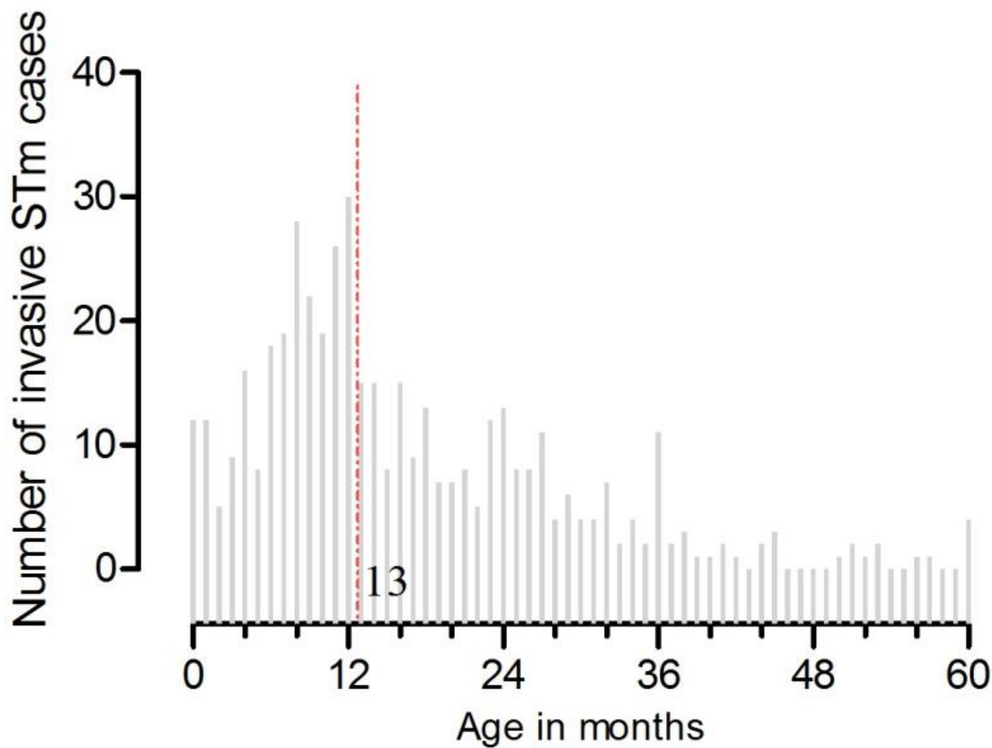

Figure 3. 1: Age distribution of *S.* Typhimurium bacteraemia in the first 5 years of life

Number of *S.* Typhimurium blood culture isolates was plotted against age in months. N= 449, the dashed line represents the median age = 13 months.

3.4.3 Development of memory CD4+ T cell subsets in children under-five years

To provide a context for the subsequent assessment of functional T cell memory, overall development of T cell subsets in Malawian children was assessed. Newborns are pathogen inexperienced (Ygberg & Nilsson, 2012) and therefore CD4+ T cells develop memory with age, enabling them to mount rapid immune responses to previously encountered pathogens. Naïve, effector and central memory CD4+ T cells can be differentiated by their extra-cellular expression of CD45RO and CCR7 (Mackay*, et al.*, 1990, Bunce & Bell, 1997). CD4+ T cell subsets were gated as described in Chapter 2: section 2.1.7.9. As expected (Chipeta*, et al.*, 1998), the proportion of CD4+CD45RO-CCR7+ naïve T cells decreased with age (r^2=0.246, slope -0.58, 95% CI [-0.83, -0.34] p=<0.01) (Figure 3.2A). The proportion of CD4+CD45RO+CCR7- EM T cells (r^2=0.119, slope 0.035, 95% CI [0.012, 0.057] p=<0.01) and CD4+CD45RO+CCR7+ CM T cells increased with age (r^2=0.455, slope 0.43, 95% CI [0.32, 0.55] p=<0.01) (Figure 3.2B and 3.2C). As expected the proportion of CD4+CD45RO-CCR7+ naïve T cells were lower in adults compared to children under-five (Figure 3.2A and 3.2D), while the proportion of CD4+CD45RO+CCR7- EM T cells and CD4+CD45RO+CCR7+ CM T cells were higher in adults compared to children under-five (Figure 3.2B-D).

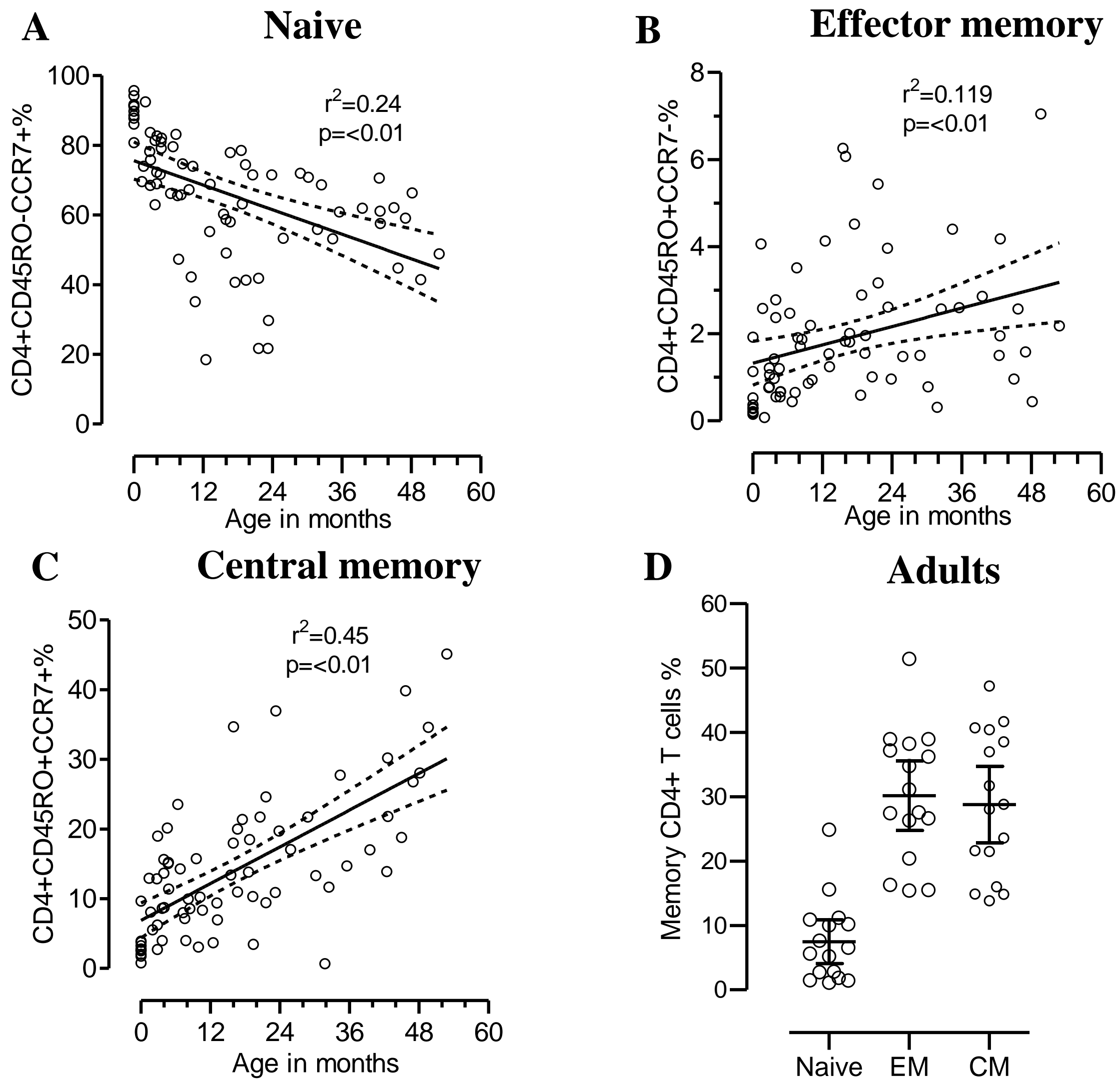

Figure 3. 2: Development of memory CD4+ T cell subsets in the first 5 years of life

Percentage of naïve CD4+ T cells: CD4+CD45RO-CCR7- (Figure 3.2A, n=73), effector memory CD4+ T cells: CD4+CD45RO+CCR7- (Figure 3.2B, n=73) and central memory CD4+ T cells: CD4+CD45RO+CCR7+) (Figure 3.2C, n=73) were plotted against age. Memory CD4+ T cells were determined by linear regression, represented by solid central line, and 95% CI represented by

dashed lines. Percentage of CD4+ T cell subsets in adults (Figure 3.2D, n=16). The mean, and 95% CI represented are shown.

3.4.4 Development of *S.* Typhimurium-specific intracellular cytokine staining assay

To determine the optimum concentration for stimulating whole blood (WB) for the detection of CD4+ and CD8+ T cells producing IFN-γ, WB was stimulated with varying concentrations of *S.* Typhimurium strain D23580 homogenate. Whole blood (WB) stimulation with *S.* Typhimurium homogenate at 100 µg/ml resulted in lowest magnitude of CD4+ T cells producing IFN-γ while stimulation at 1 µg/ml triggered the highest magnitude of CD4+ T cells producing IFN-γ (Figure 3.3A). WB stimulation with *S.* Typhimurium homogenate at 0.1µg/ml triggered lowest magnitude of CD8+ T cells producing IFN-γ. Similar to CD4+ T cells responses; WB stimulation at 1 µg/ml triggered the highest magnitude of CD8+ T cells producing IFN-γ (Figure 3.3B). It was resolved to use *S.* Typhimurium homogenate at 1µg/ml for WB stimulation in successive experiments. Optimum blood stimulation time, for the detection of cytokine producing T cells was investigated. WB stimulated with PMA/ION stimulated at varying time intervals was examined for CD4+ and CD8 T cells producing IFN-γ. Highest magnitude of CD4+ cells producing IFN-γ was detected at 8 hours of stimulation while for CD8+ T cells producing IFN-γ, it was detected at 6 hours of stimulation (Figure 3.3C-D). It was observed that the magnitude T cell responses (producing IFN-γ) increased progressively

at 2 to 6 hours (Figure 3.3C-D). Although, WB stimulation with PMA/ION was highest at 8 hours in CD4+ T cells, 6 hours stimulation was chosen in successive experiments.

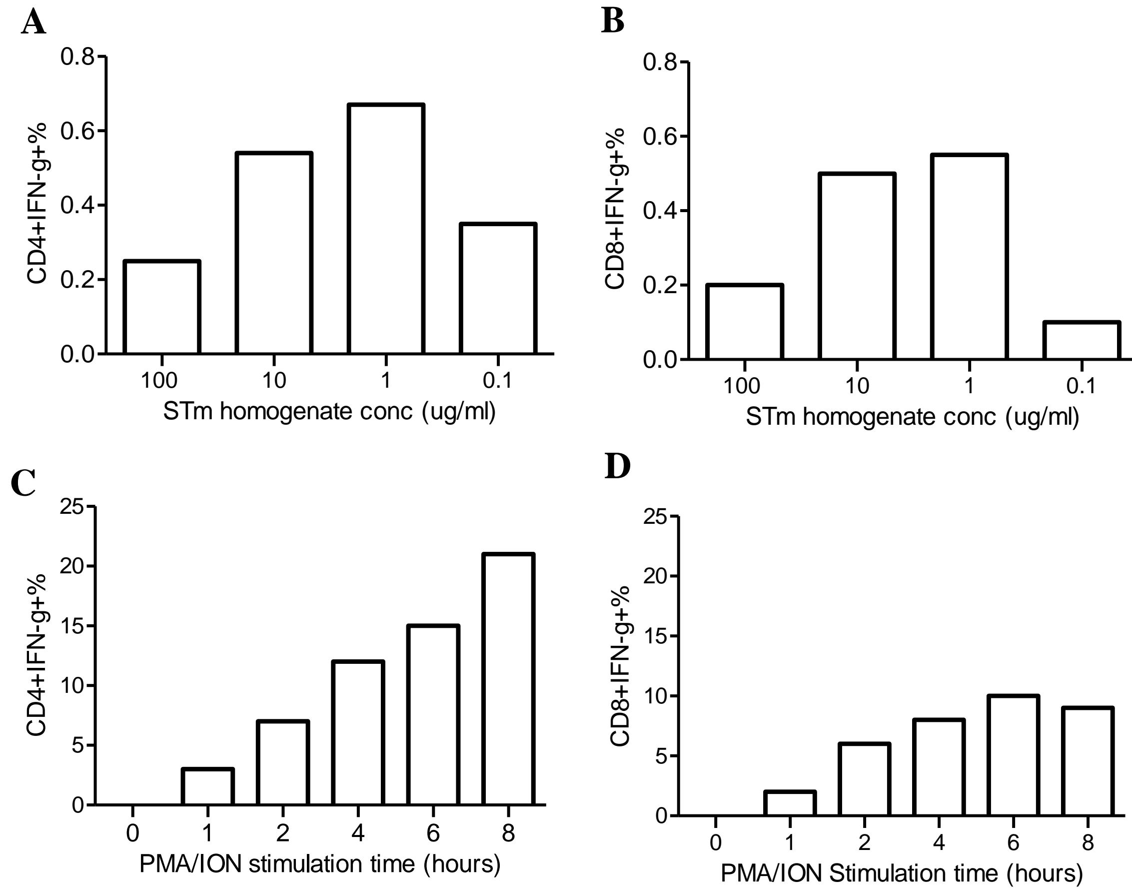

Figure 3. 3: Optimization of *S.* Typhimurium-specific intracellular cytokine staining assay

WB was stimulated *S.* Typhimurium homogenate at varying concentrations as indicated (Figure 3.3A-B). Percentage of CD4+ and CD8+ T cells producing IFN-γ are shown (Figure 3.3A-B). WB was stimulated with at varying time intervals as indicated (Figure

3.3C-D). Percentage of CD4+ and CD8+ T cells producing IFN-γ are shown (Figure 3.3A-B). For all experiments n=1

3.4.5 Early acquisition of *S*. Typhimurium-specific CD4+ T cell immune responses

To explore the hypothesis that CD4+ T cell immune responses to *S.* Typhimurium develop in parallel with acquisition of antibody-mediated immunity. CD4+ T cells were defined as described in Chapter 2, section 2.1.7.7 and 2.1.7.8. Contrary to this study hypothesis, *S.* Typhimurium-specific CD4+ T cells producing cytokine were detected early in life, peaked at 14 months and then decreased with age (Figure 3.4A).

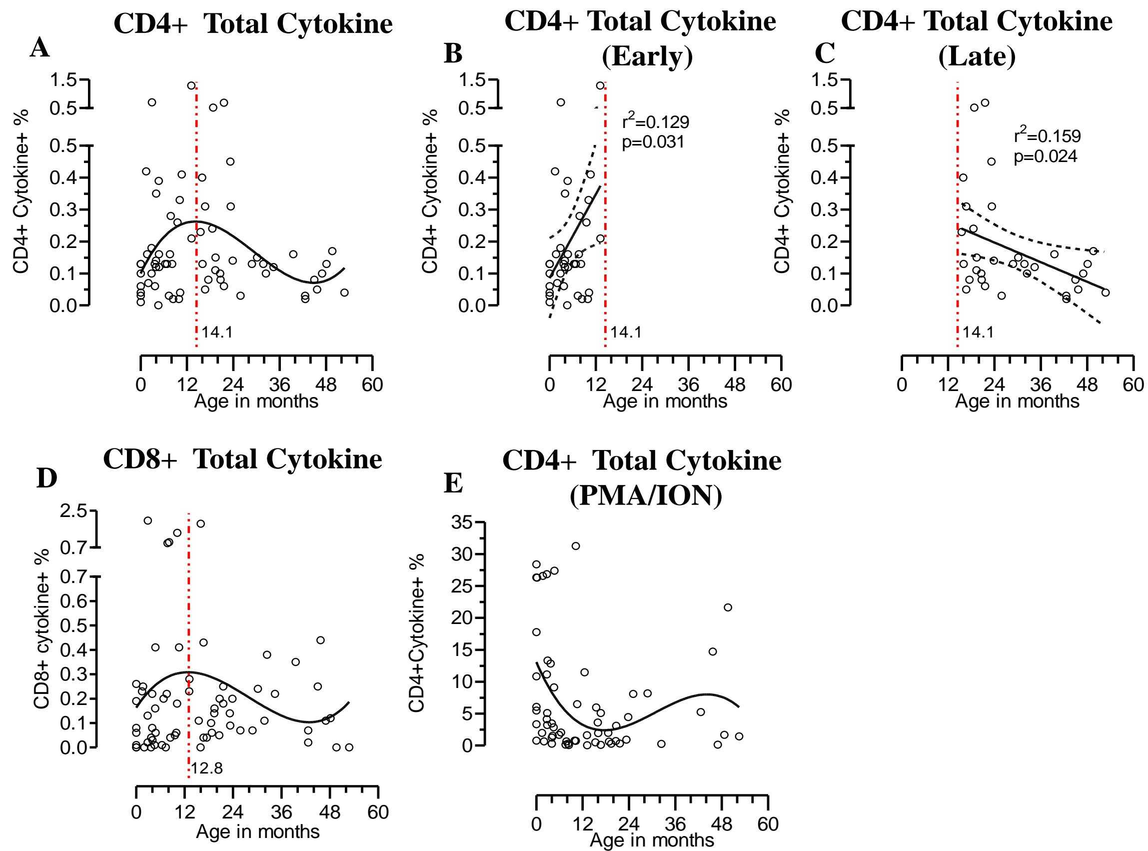

Figure 3. 4: Early acquisition of *S.* Typhimurium-specific CD4+T cell immune responses

Percentage of *S.* Typhimurium-specific CD4+ T cells producing total cytokine (Figure 3.4A, n=68) early (Figure 3.4B, n=36), late (Figure 3.4C, n=32), PMA/ION stimulated CD4+ T cells producing total cytokine (Figure 3.4E, n=62), Percentage of *S.* Typhimurium-specific CD8+ T cell producing cytokine (Figure 3.4D, n=62). Nonlinear polynomial regression models of third order were fit to data relating specific T cell cytokine response with age. *S.* Typhimurium-specific T cells response within early and late periods was determined by linear regression, represented by solid central line, and 95% CI represented by dashed lines.

This was further analysed by using the non-linear model peak points as defining early and late *S.* Typhimurium -specific CD4+ T cells. This showed early acquisition of *S.* Typhimurium -specific CD4+ T cell immunity (r^2=0.129, slope 0.021, 95% CI [0.002, 0.041], p=0.031), followed by a decrease in older children (r^2=0.157, slope -0.005, 95% CI [-0.009, -0.0006], p=0.024) (Figure 3.4A and 3.4C). These changes in intra-cellular cytokine profiles mirrored changes in IFN-γ- and TNF-α-, rather than IL-2-secreting cells (Figure 3.5). Unexpectedly, *S.* Typhimurium-specific CD4+ T cell responses were generally lower in adults compared to children under-five (Figure 3.5A-D).

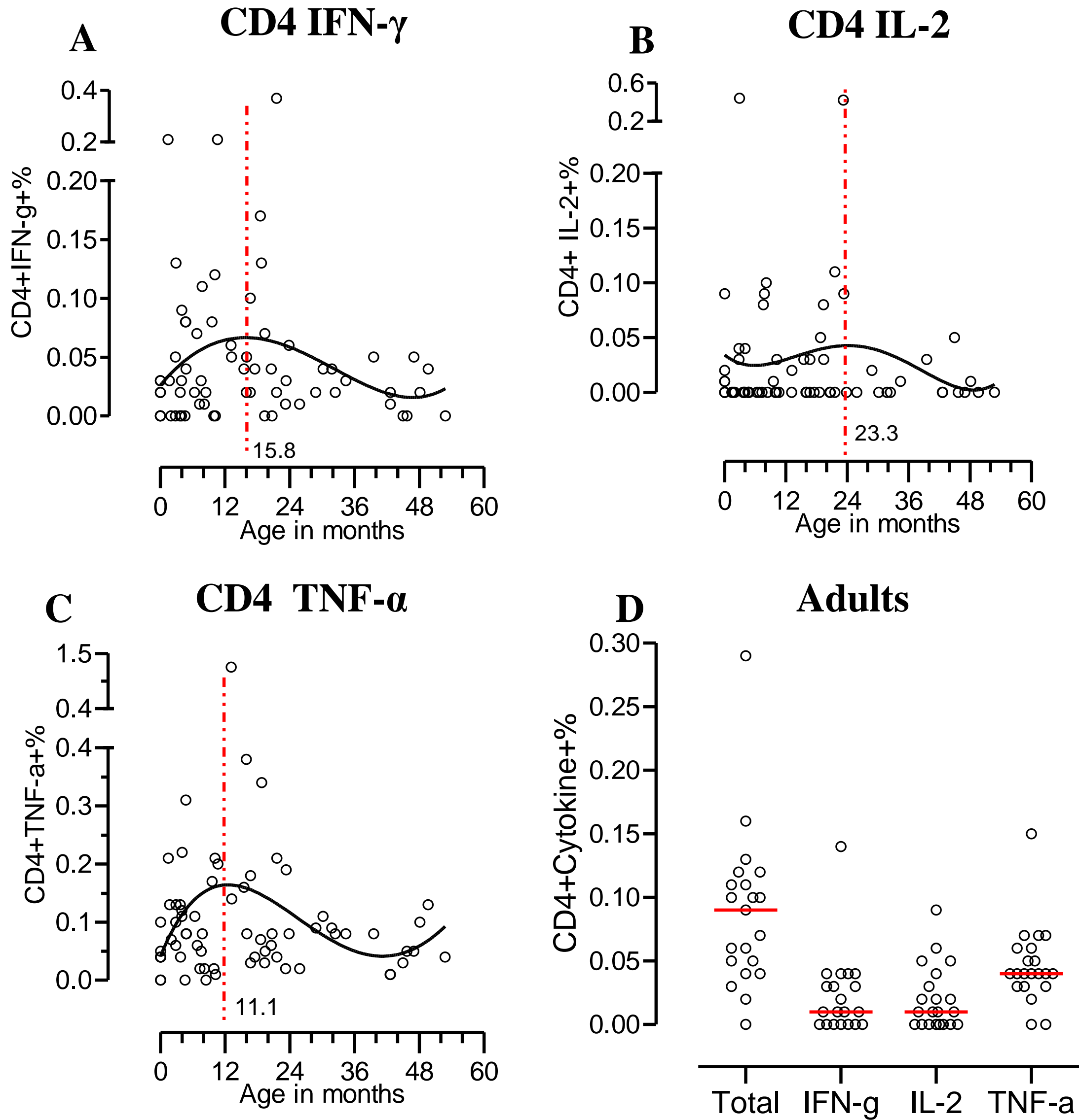

Figure 3. 5: Early acquisition of *S*. Typhimurium-specific CD4+T cell immune responses

Blood samples were analysed for *S*. Typhimurium-specific CD4+ T cells producing cytokines using ICS assay. Percentage of *S*. Typhimurium-specific CD4+ T cells producing IFN-γ (Figure 3.5A, n=68), IL-2 (Figure 3.5B, n=68), TNF-α (Figure 3.5C, n=67). The immune response with age was determined by nonlinear regression

polynomial models represented by solid line. The immune responses in adults (Figure 3.5D, n=20). Bars represent median.

S. Typhimurium-specific CD4+ cytokine responses did not correlate with PMA/ION stimulated CD4+ T cell cytokine responses ($r=0.109$, 95% CI[-0.128, 0.371] $p=0.426$) (Table 3-B), indicating that these responses to *S.* Typhimurium antigens were not simply due to a general maturation of the immune system (Figure 3.4E and Figure 3.6). Interestingly, the trend of *S.* Typhimurium -specific CD4+ cytokine responses were similar to *S.* Typhimurium-specific CD8 cytokine responses (Figure 3.4A and 3.4D). Similar trend of specific CD4+ and CD8+ responses re-affirms the pattern of *S.* Typhimurium-specific T cell immune responses.

Table 3- B: Association between immune variables

Parameter (s)	XY Pairs	Spearman r	95% Cl	P value
NTS vs PMA CD4+Cytokine+	55	0.109	-0.128, 0.371	0.426
SBA vs anti- *S*. Typhimurium -LPS IgG antibody titers	55	0.329	0.552, 0.062	0.01
SBA vs anti- *S*. Typhimurium -OMP IgG antibody titers	57	0.044	-0.226, 0.308	0.741
SBA vs anti- *S*. Typhimurium -FliC IgG antibody titers	58	-0.001	-0.266, 0.264	0.992
SBA vs anti-*E. coli*-LPS IgG antibody titers	50	0.031	-0.257, 0.314	0.830
CD4+ Cytokine+ vs anti- *S*. Typhimurium -OMP IgG antibody titers	65	0.137	-0.117, 0.375	0.275
CD4+ Cytokine+ vs anti- *S*. Typhimurium -FliC IgG antibody titers	67	0.174	-0.075, 0.404	0.157
CD4+ Cytokine+ vs anti- *S*. Typhimurium -OMP IgG antibody titers (early)[a]	39	0.405	0.088, 0.647	0.01
CD4+ Cytokine+ vs anti- *S*. Typhimurium -FliC IgG antibody titers (early)[a]	38	0.394	0.080, 0.637	0.01
CD4+ Cytokine+ vs anti- *S*. Typhimurium -LPS IgG antibody titers (early)[a]	36	-0.257	-0.547, 0.087	0.129

Abbreviations: Cl, confidence interval

[a] Early refers to parameters of participants aged less than 14 months

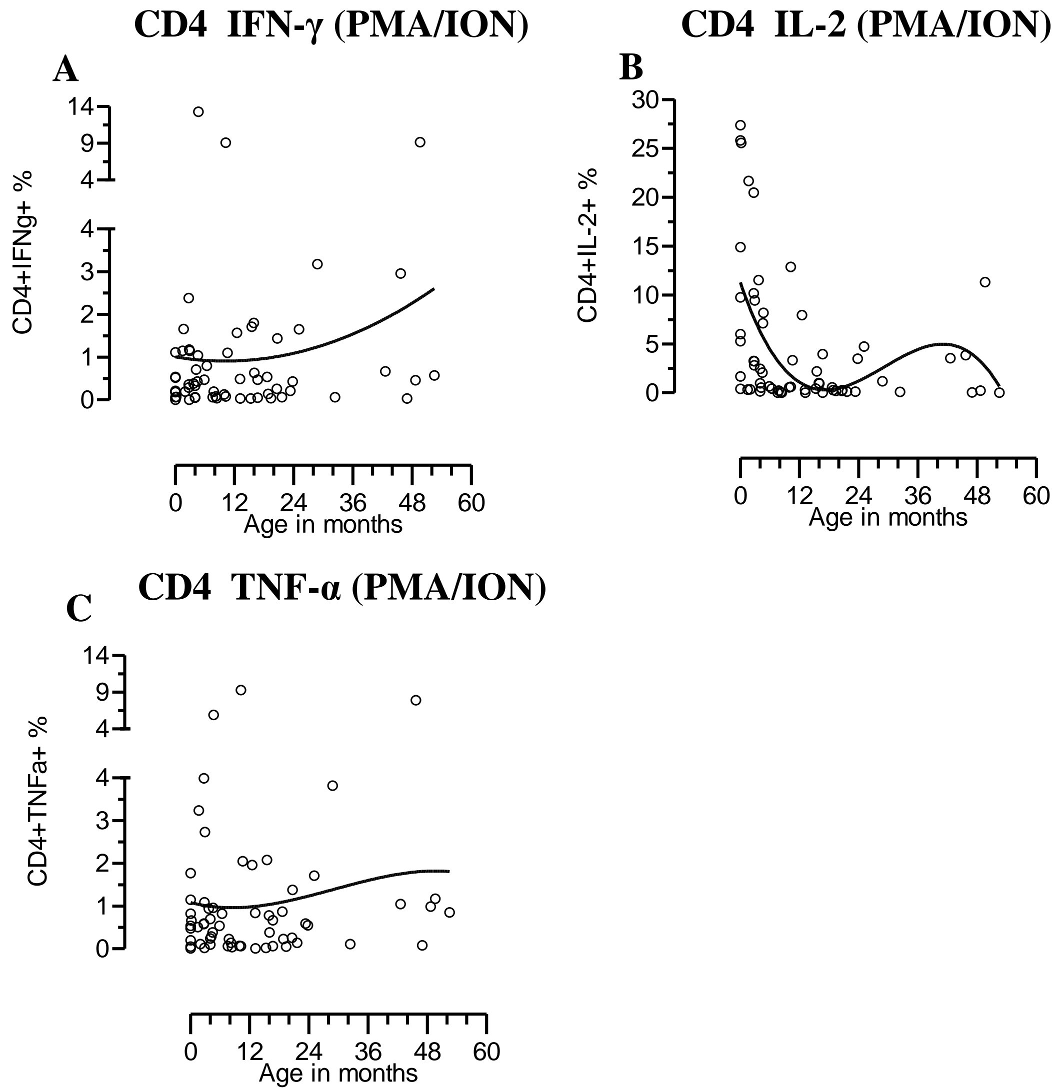

Figure 3. 6: Acquisition of PMA/ION-specific CD4+T cell immune responses

Blood samples were analysed for PMA/ION specific CD4+ T cells producing cytokines using ICS assay. Percentage of PMA/ION-specific CD4+ T cells IFN-γ (Figure 3.6A, n=62), IL-2 (Figure 3.6B, n=62), TNF-α (Figure 3.6C, n=62). The immune response with age was determined by nonlinear regression polynomial models represented by solid line.

Generation of antigen-specific multiple cytokine-producing cells is widely thought to indicate maturation of antigen-specific CD4+ T cell responses (Kannanganat, *et al.*, 2007). Maturation of *S.* Typhimurium -specific T cell responses in these healthy children (either double or triple cytokine producers) peaked mostly between 13-24 months and subsequently declined, while for IL2+TNFα+CD4 T cells, the response was sustained (Figure 3.7).

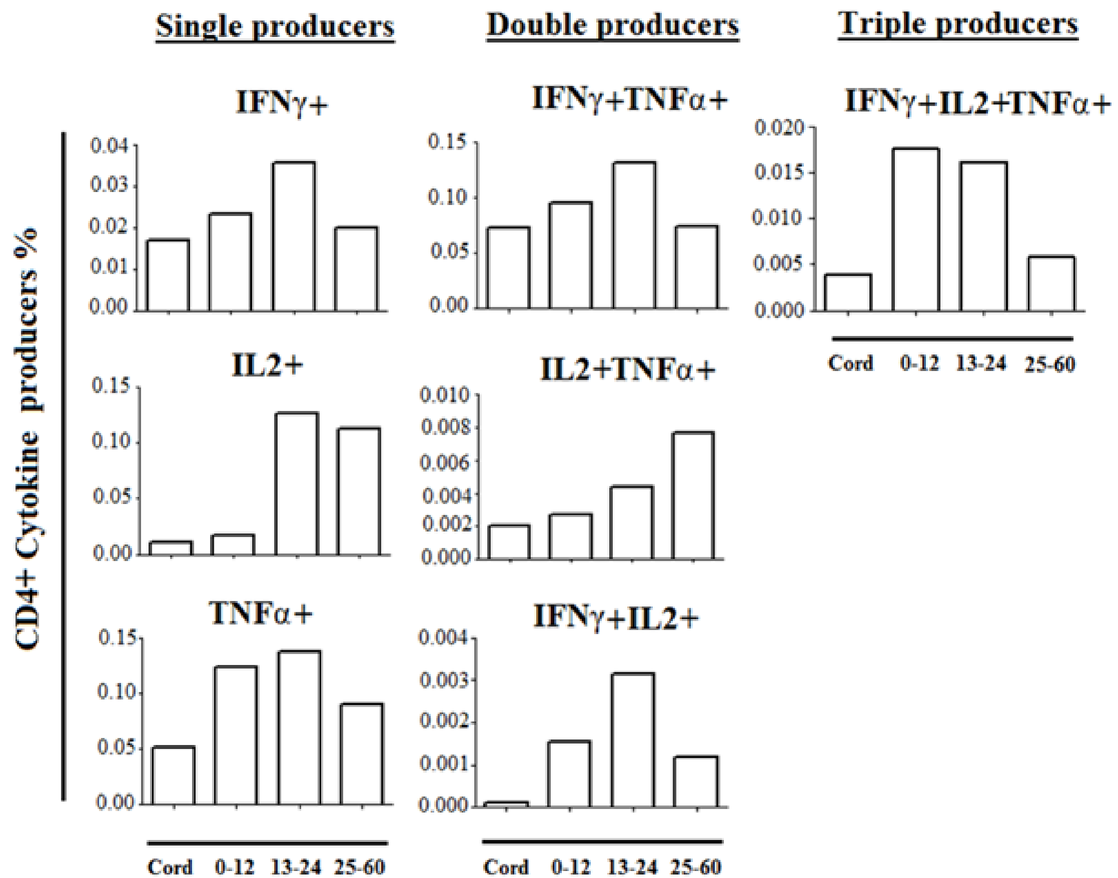

Figure 3. 7: *S*. Typhimurium-specific CD4+ T cell cytokine profiles in children

S. Typhimurium-specific CD4+ T cells producing IFN-γ, TNF-α and IL-2 were detected using ICS assay. CD4+ T cells producing multiple cytokines were analysed using FlowJo version 7.6.5 and graphs generated by Prism version 5.0. *S*. Typhimurium -specific CD4+ T cells producing single cytokine, double cytokines and triple cytokines were shown with age (Figure 3.7).

3.4.6 Delayed acquisition of *S.* Typhimurium -specific SBA

To confirm previous observations made in Blantyre by MacLennan *et al*, the same SBA assay and clinical *S.* Typhimurium strain D23580 (MacLennan*, et al.*, 2008) were used and described in Chapter 2, section 2.17.10. In line with the previous findings, *S.* Typhimurium-specific SBA declined in the first 8 months of life and then increased to a peak at 35 months (Figure 3.8A).

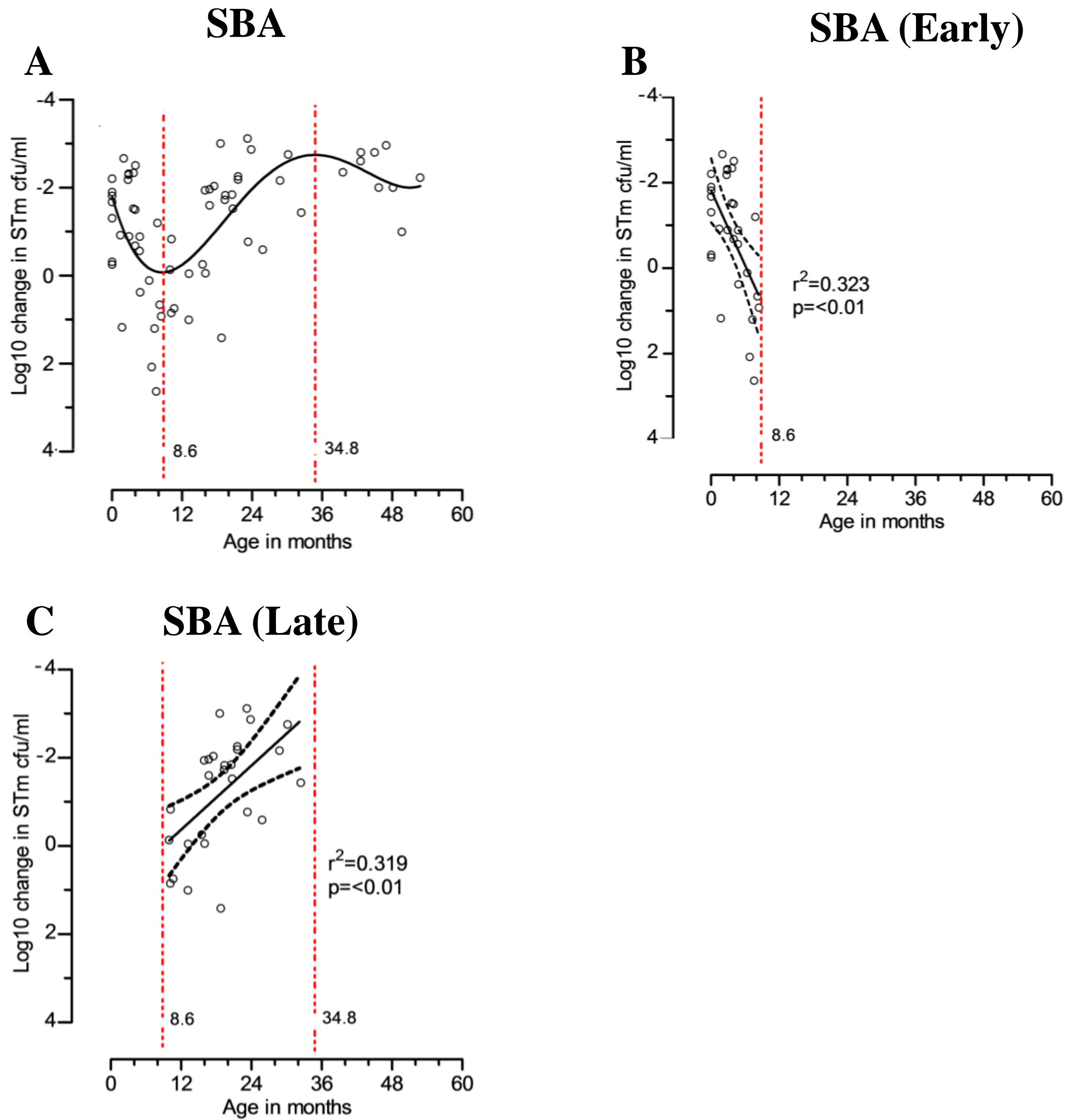

Figure 3. 8: Acquisition of *S.* Typhimurium-specific serum bactericidal activity among children

Log 10 change in *S.* Typhimurium cfu/ml from the control condition was plotted against age. The y-axis was inverted. Nonlinear regression polynomial model was represented by solid line (Figure 3.8A, n=65). SBA responses within early (Figure 3.8B, n=29), and late

periods (Figure 3.8C, n=27) was determined by linear regression, represented by solid central line and 95% CI represented by dashed lines.

To further analyse these trends in *S*. Typhimurium-specific SBA, periods were divided into early and late phases according to peak and nadir points, as before. *S.* Typhimurium -specific SBA declined in the first 8 months of life (r^2=0.323, slope 0.292, 95% CI [0.125, 0.459], p=<0.01), and then increased between 8-35 months (r^2=0.319, slope -0.121, 95% CI [-0.193, -0.048], p=<0.01), (Figure 3.8A and 3.8C). This *S.* Typhimurium-specific increase in SBA occurred later than that seen in T cell immunity to *S*. Typhimurium (Figure 3.4 and 3.8).

3.4.7 *S*. Typhimurium -specific SBA correlates with presence of antibodies targeting *S*. Typhimurium -LPS

Previous work in HIV-infected Malawian adults showed that excess anti-LPS IgG antibodies can inhibit complement-mediated killing of NTS *in vivo* while antibodies to outer membrane proteins (OMP) can mediate bactericidal activity (MacLennan*, et al.*, 2010). To clarify the antigenic targets of the *S.* Typhimurium-specific antibody in children, serum antibodies to *S.* Typhimurium LPS, OMP and FliC and *E. coli* 0127:B8 LPS were measured as described in Chapter 2, section 2.1.7.11. Anti- *S.* Typhimurium-LPS IgG antibody titers mirrored the pattern seen with SBA assay (Figure 3.8A and Figure 3.9A).

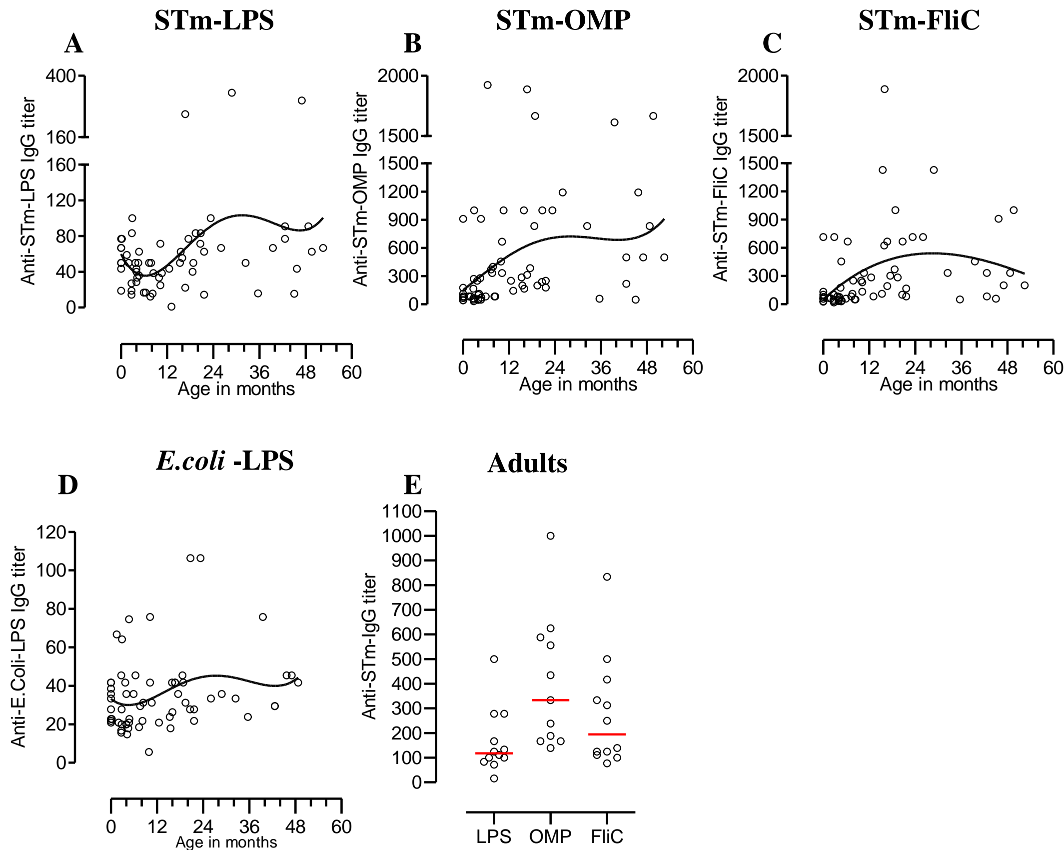

Figure 3. 9: Age dependent acquisition of specific anti- *S.* Typhimurium-IgG antibodies

Serum from children was tested for anti-*S*. Typhimurium-specific IgG antibodies by ELISA. Anti-*S*. Typhimurium-LPS IgG (Figure 3.9A, n=63), anti- *S*. Typhimurium OMP IgG (Figure 3.9B, n=66), anti-*S*. Typhimurium-FliC IgG (Figure 3.9C, n=67) and anti-*E.coli*-LPS IgG antibody titers (Figure 3.9D, n=63) were plotted against age. SBA with age was determined by nonlinear regression polynomial models represented by solid line. The immune responses in adults (Figure 3.9E, n=12). Bars represent median.

Anti- *S.* Typhimurium -OMP, were lowest at birth increasing with age while anti- *S.* Typhimurium -FliC IgG and anti-*E. coli*-LPS IgG antibody titers showed no particular trend with age (Figure 3.9B-D). The correlation between SBA and anti- *S.* Typhimurium-LPS IgG titers (r=0.329, 95% CI [0.552, 0.062] p=0.01) and the lack of correlation between SBA and anti-*E. coli*-LPS titers suggest that SBA is due to *S.* Typhimurium -specific rather than non-specific LPS antibodies (Table 3-B). A lack of a correlation with anti- *S.* Typhimurium -OMP and anti- *S.* Typhimurium-FliC suggest that these targets do not substantially contribute to SBA in these children.

3.4.8 *S.* Typhimurium -specific CD4+ T cell immune responses in early childhood are associated with generation of anti- *S.* Typhimurium protein antibodies

Having shown that *S.* Typhimurium -specific CD4+ T cells peak in early life (Figure 3.10), whether this immune memory was linked to the generation of anti- *S.* Typhimurium -OMP and anti- *S.* Typhimurium -FliC IgG antibodies was then investigated.

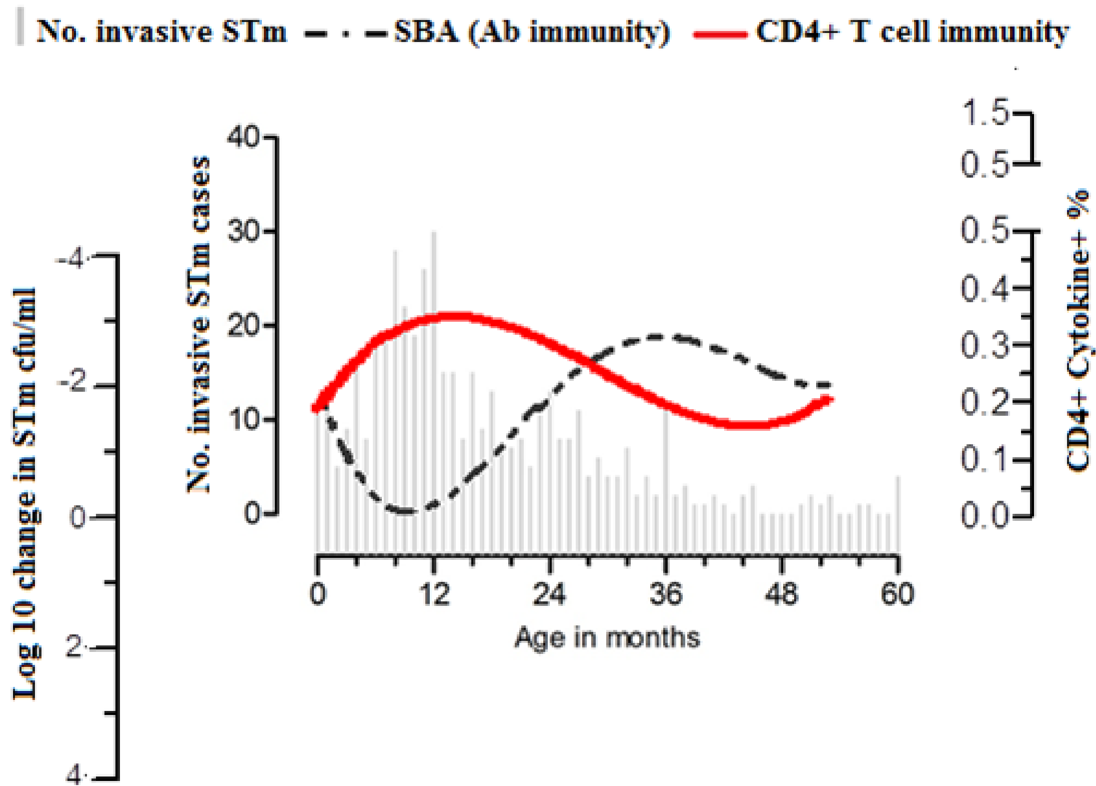

Figure 3. 10: Sequential acquisition of T cells and antibodies to *S*. Typhimurium in children

Age distribution of *S.* Typhimurium BSI in under-five children at QECH Blantyre, Malawi 2006-2011 was superimposed with kinetics of *S.* Typhimurium -specific CD4+T cell immune responses and *S.* Typhimurium -specific serum bactericidal activity (y-axis was inverted) in children (0-60 months age).

S. Typhimurium -specific CD4+ T cell immune responses correlate with anti- *S.* Typhimurium -OMP and anti- *S.* Typhimurium -FliC IgG antibodies in early childhood (r=0.405, 95% CI[0.088, 0.647] p=0.01 and r=0.394, 95% CI[0.080, 0.637] p=0.01 respectively) and not anti- *S.* Typhimurium -LPS IgG antibodies (r=-0.257, 95% CI[-

0.547, 0.087] p=0.129) (Table 3-B). This contemporaneous development of antibodies to *S.* Typhimurium OMP and T-cell immunity is in line with the conventional paradigm of the T cell dependent immune response to a protein antigen (Sinha*, et al.*, 1997, Mohr*, et al.*, 2010).

3.5 DISCUSSION

NTS bacteraemia remains an important cause of death among children in SSA. This study extend previous observations that complement-fixing antibodies to NTS are considered to be ‘protective’ and develop by 24 months of age (MacLennan*, et al.*, 2008). *S.* Typhimurium specific CD4+ immunity peaked in early life (14 months of age), suggesting exposure to *S.* Typhimurium or to cross reactive antigens. It is likely that this exposure occurs within the gut associated lymphoid tissues (GALTs) and primed T cells traffic into the peripheral circulation (Dougan*, et al.*, 2011). Surprisingly, in older children (>14 months) specific T cells declined in the peripheral blood, probably due to a decline in *S*. Typhimurium specific exposure within the GALT. Together these findings suggest unique phases of *S*. Typhimurium exposure and T cell priming (early phase) and this perhaps permits antigen-specific to CD4+ T cells spilling into the peripheral blood. It is possible that the late phase is characterized by limited *S*. Typhimurium exposure and pathogen clearance by the CM T cells. Some confirmation of these findings is noted in the correlation of *S*. Typhimurium -specific T cell immune responses in early life and the antibody titers to *S.* Typhimurium protein antigens and not *S.* Typhimurium LPS. Furthermore the trends of *S*. Typhimurium - specific CD4+ and CD8+ cytokine producers

were similar suggesting the maturation of both CD4+ and CD8+ T cells following *Salmonella* exposure.

Distribution of NTS within the gastrointestinal tract (GIT) by age in this population is not known. As previous demonstrated, this study has shown that *S.* Typhimurium bacteraemia peaks at 13 months of age and 80% of cases occur in children under-two among under-five children (MacLennan*, et al.*, 2008). Whether this age distribution in *S.* Typhimurium bacteraemia indicates asymptomatic *S*. Typhimurium colonization within the gut is not clear. Contracting *Salmonella* infection occurs through ingestion of contaminated food or fomites (Kariuki*, et al.*, 2006). In this population exclusive breast feeding is practiced mainly in the first 3 months of age and thereafter mixed feeding in commonly practiced (Kalanda*, et al.*, 2006, Kerr*, et al.*, 2007). It appears that the introduction of supplementary feeding coincides with the age group of *S.* Typhimurium bacteraemia. Risk factors of *S.* Typhimurium bacteraemia include malnutrition and HIV. The leaky gut mucosa in malnourished and HIV infected children (Reynolds*, et al.*, 1996, Papasavvas*, et al.*, 2011) might contribute to spread of *S.* Typhimurium normally localized in the GIT into the peripheral blood. The emergence of MDR and more virulent *S.* Typhimurium strain D23580 (driven by genetic degradation) (Kingsley*, et al.*, 2009), might explain changes in life style of an organism that causes localized gastroenteritis worldwide but predominately causes invasive disease in otherwise healthy children in SSA.

Serum killing of an invasive *S.* Typhimurium strain D23580 that is predominant in the SSA region requires IgM or IgM *S.* Typhimurium specific-antibodies (MacLennan*, et al.*, 2008). In the absence of the *S*. Typhimurium-specific IgG antibody, the SBA is abrogated (Heffernan*, et al.*, 1992, MacLennan*, et al.*, 2008). As previously demonstrated (MacLennan*, et al.*, 2008), this study demonstrates that passively acquired *S.* Typhimurium specific SBA from the mother gradually declines from birth to about 8 months of age. This is consistent with the observation that transfer of both immune serum and immune cells were necessary for protection to mouse-virulent strain *S.* Typhimurium C5 in innately susceptible BALB/c (ltys) mice (Mastroeni*, et al.*, 1993). After 8 months of age, presumably following exposure to *Salmonella*, antigen-specific SBA develops with age and peaks at 36 months. Interestingly, this study shows that this specific SBA correlates with *S.* Typhimurium specific antibodies targeting LPS and not OMP and FliC. MacLennan *et al* previously demonstrated that in HIV infected Malawian adults *S.* Typhimurium-specific antibodies targeting LPS were SBA inhibitory and not *S.* Typhimurium specific antibodies targeting OMP. This was due to the high concentration of *S.* Typhimurium-specific IgG antibodies targeting LPS, which is not the case in healthy Malawian children examined in this study. High concentration of *S.* Typhimurium specific IgG antibodies in HIV infected individuals are likely due to leaky gut that might permits transfer of LPS molecules into the peripheral blood (Papasavvas, *et al.*, 2011).

Taking into account age related acquisition of *S.* Typhimurium-specific T cell immune responses, it appears that acquisition of SBA is delayed. Perhaps not surprising, naturally acquired antibody responses to pneumococcal specific protein antigens appear early compared to antibodies to pneumococcal-specific-polysaccharide antigens in children (Rapola*, et al.*, 2000, Soininen*, et al.*, 2001). Furthermore, the process of generation of highly efficient isotype-switched IgG antibodies is dependent on CD4+ T cells and B cell cross talk (Cunningham*, et al.*, 2007), suggesting that mature complement-fixing antibodies to *S.* Typhimurium are expected to follow the emergence of specific T cells.

Immunity to *Salmonella* is considered complex due *Salmonella* facultative intracellular lifestyle and its strategies to escape even the competent host immune system. It has been hypothesized previously that high level of resistance to *Salmonella* is established in a step-wise fashion; innate immunity (innate cells and humoral immunity) preceding the adaptive immunity (beginning with the CD4+ T cell followed by the B cells and CD8+ T cells) (Mastroeni, 2002). In this study, the kinetics of *S.* Typhimurium -specific immunity (antibodies and T cells) and age distribution of *S.* Typhimurium bacteraemia were explored. Importantly cases of *S.* Typhimurium bacteraemia declines in parallel with decline in specific-CD4+ T cell immune responses and increase in SBA. Furthermore, cases of *S.* Typhimurium bacteraemia were more at the peak of CD4+ T cells responses, suggesting that CD4+ cells alone are not sufficient to clear *S.* Typhimurium infection and sequential acquisition of specific-SBA results in establishment of high level of resistance and decline in cases of *S.* Typhimurium bacteraemia.

Since salmonellae are facultative intracellular organism, complement-fixing antibodies are implicated in killing *S.* Typhimurium in the extracellular space and supports intracellular killing (Gondwe*, et al.*, 2010). CD4+ T cells helps macrophages intracellular killing of *S*. Typhimurium and importantly clears reside S. Typhimurium that can potentially cause relapse of *S.* Typhimurium bacteraemia (Mastroeni, 2003). Taken together these findings are in agreement with the hypothesis that *S.* Typhimurium immunity is acquired in a step-wise fashion and high level of resistant is only established when both the T cell and antibody immunity are fully mature.

Given the burden of iNTS in SSA (Reddy*, et al.*, 2010), a vaccine is urgently required. *S.* Typhimurium LPS O-antigen has considerable potential as a vaccine target and there are currently several groups developing conjugate vaccines for this purpose to overcome the short-lived T-independent antibody response generated by polysaccharide alone (MacLennan, 2013). Immunization with *S.* Typhimurium -OMP and *S.* Typhimurium-FliC induce both T cells response and production of antibodies in animal models, and these *Salmonella* derived proteins are therefore also being investigated as vaccine candidates, either separately (Gil-Cruz*, et al.*, 2009) or covalently linked to O-antigen as glycoconjugates (Simon & Levine, 2012).

Summary

In Malawian children T cell and antibody immunity to *S.* Typhimurium are acquired sequentially. Acquisition of both T cell mediated immunity and antibodies are required

for robust resistance to invasive *Salmonella* disease. Children below 2 years of age are more vulnerable to invasive *Salmonella* disease and public health intervention inform of vaccination is required. A vaccine that elicits both T cell and antibody immunity and also provide cross protection to common *Salmonella* serovars is required particularly in young children.

CHAPTER 4: *SALMONELLA* EXPOSURE AND DEVELOPMENT OF SPECIFIC IMMUNITY IN MALAWIAN CHILDREN

4.1 INTRODUCTION

As reviewed previously (chapter 1), Nontyphoidal *Salmonella* (NTS), principally *S.* Typhimurium and *S*. Enteritidis are among the commonest causes of bacteraemia in sub-Saharan Africa (SSA)(Gordon*, et al.*, 2008, Morpeth*, et al.*, 2009). NTS infections are most common in children below 3 years of age and HIV-infected individuals (Gordon*, et al.*, 2008, MacLennan*, et al.*, 2008, Graham & English, 2009). Understanding what constitutes naturally acquired protective immunity to *Salmonella* and defining the age group when this acquisition occurs is important for understanding the pathogenesis of the disease and for the development of an effective vaccine. Opsonic IgG antibodies to *Salmonella* facilitate efficient killing of *Salmonella* in the extracellular space, primarily the blood, by fixing-complement (MacLennan*, et al.*, 2008) and intracellular space of phagocytes by respiratory burst (Gondwe*, et al.*, 2010). Acquisition of serum bactericidal activity (SBA) to *S.* Typhimurium has been described in Chapter 3, as peaking at 36 months of age, which coincides with the decline in incidence of *S.* Typhimurium bacteraemia. Furthermore, Chapter 3 describes that CD4+ and CD8+ T cell immunity to *S*. Typhimurium are acquired early in life in Malawian children, peaking at 14 months of age, suggesting that the gut associated lymphoid tissues (GALTs) are exposed to *S.* Typhimurium or cross-reactive bacteria during this period.

Age distribution of *Salmonella* bacteraemia in Malawian children has been described in Chapter 3 and previously by MacLennan *et al* (MacLennan*, et al.*, 2008), as peaking at 13 months of age in under-five children but the age distribution of *Salmonella* exposure within the gastrointestinal tract (GIT) in this population is not known. *Salmonella* infection is thought to result from ingestion of contaminated food, water or contact with contaminated fomites (Kariuki*, et al.*, 2006). A cohort study of Mexican children from birth to 12 months of age, demonstrated that 40% of children were initially exposed to *Salmonella* (detected by culture) within the GIT (Cravioto*, et al.*, 1990). Under-five children have been shown to shed *Salmonella* in their stools for as long as 7 weeks (Buchwald & Blaser, 1984). In Malawi, in a study of children presenting to hospital with NTS bacteraemia, 40% (66/167) had *Salmonella* also isolated from the oropharynx, and 52% (73/141) had *Salmonella* also isolated from stool at the point of diagnosis (Msefula, 2009). *Salmonella* exposure within the oropharynx and/ or GIT might help to explain the natural immunizing events in children from SSA, and neither the acquisition of humoral immunity, following gut or oropharyngeal mucosal exposure, have previously been studied longitudinally in Malawian children. It is uncertain whether this *Salmonella* exposure within the GIT is asymptomatic or accompanied by an episode of diarrhoeal disease, which could be mild and transient or severe (Cravioto*, et al.*, 1990). This study therefore aimed to explore the hypothesis that in the first 18 months of life, the gastrointestinal tract is exposed to *Salmonella* serovars or other cross-reactive enteric pathogens with minimal symptoms which induce the development *Salmonella*-specific

serum immunity. This immunity might have the potential to protect against subsequent *Salmonella* infection and particularly invasive NTS disease.

Maternal breast milk antibodies (principally IgA) and trans-placental maternal IgG are thought to have a role in defence against pathogens in infancy. The protective role of breast milk against enteric pathogens has been recognized for a long time (France*, et al.*, 1980, Cravioto, 1990, Thomas*, et al.*, 2004, Shapiro*, et al.*, 2007); and has been shown to be critically important for Malawian infants (Kafulafula*, et al.*, 2010). Chapter 3 shows that NTS bacteraemia is most frequent as maternally acquired (trans-placental) serum antibody wanes and following discontinuation of breast feeding or following introduction of supplementary food. This suggests that either or both trans-placental IgG and breast milk IgA might play a role in preventing *Salmonella* colonisation of the gastrointestinal tract in the early months of life.

From this background, a prospective longitudinal cohort study of 60 healthy infants at Zingwangwa health centre (ZHC) in Blantyre, Malawi was conducted. These children and their mothers were recruited at 6 months of age and followed up until 18 months of age, to determine the association of *Salmonella* exposure within the GIT and oropharynx (examined at monthly intervals using stool and oropharyngeal samples, analysed by culture and real-time PCR) and the development of serum immunity to *Salmonella* (examined at 3 month intervals using serum killing assay). Furthermore, maternal breast milk bactericidal activities were quantified to explore their role in preventing *Salmonella* colonisation of the GIT.

Hypothesis: In the first 18 months of life, the gastrointestinal tract and / or oropharynx are exposed to *Salmonella* serovars which induce the development of *Salmonella*-specific serum immunity.

Study objectives:

c) To determine the pattern of *Salmonella* exposure events within the GIT and oropharynx in Malawian children over a period of 1 year from 6 to 18 months of age.
d) To determine whether *Salmonella* exposure events in children are associated with acquisition of *Salmonella*-specific serum killing.
e) To determine whether maternal breast milk kills *Salmonella ex vivo*.

4.2 PARTICIPANT RECRUITMENT AND METHODS

The participant recruitment and methods for this chapter have been described in Chapter 2 section 2.2. In brief, 60 healthy children and their mothers were recruited from Zingwangwa Health Centre (ZHC) at 6 months of age and followed for 1 year. ZHC provides primary health care to persons living in Soche Ward (SW) of Blantyre city. SW is one of the densely populated wards in Blantyre, with the population of 60,000 (MCI, 2012). Child participants were sampled stool and oropharynx swab at monthly intervals and were also sampled serum at 3 months intervals. Mothers of each child participant were sampled serum at study entry only and also sampled breast milk at 6 months intervals. To determine the pattern of *Salmonella* exposure, stool and oropharynx swabs

were examined for *Salmonella* using standard culture or PCR. Serum from children exposed to *Salmonella* or not was quantitatively examined for serum bactericidal activity to *S*. Typhimurium strain D23580, *S*. Typhimurium 037v4 and *Salmonella* species 049v3. Maternal breast milk was also quantitatively examined for breast milk bactericidal activity to *S*. Typhimurium strain D23580. At monthly intervals children were clinically examined (MUAC, Temperature, and malaria rapid test) and clinical history (diarrhoea, cough, antimalarial and antibiotics usage) was taken by the research nurse. Research nurse also administered a questionnaire to mothers to explore child feeding practices including breast feeding, water source and water treatment. Global Positioning System (GPS) coordinates for each child place of residence were captured by the research field worker.

4.3 STATISTICAL ANALYSES

Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, USA) and Stata SE® version 12.1 (StataCorp, USA) statistical analysis packages. End-point response variables (SBA) were examined for normality of distribution. Normally distributed immune variables were analysed using paired t test to compared groups at different time points. Two-tailed nonparametric, Mann-Whitney tests were used to compare immune variables at different time points. Kaplan-Meier survival function test was used to demonstrate the trend of *Salmonella* exposure events and feeding practices with age. Odd ratios were used to describe the relationship between *Salmonella* exposure events and clinical presentation, *Salmonella* exposure events and

feeding practices and *Salmonella* exposure events and serum bactericidal activity. Global Positioning System (GPS) visualizer (www.gpsvisualizer.com) was used to draw maps for the study participant's places of residence. Logistic regression analyses were used to describe the relationship between location absolute altitudes and *Salmonella* exposure events. 95% confidence intervals or interquartile range (IQR) were reported and *p* value of <0.05 or confidence intervals not crossing 1 were considered statistically significant.

4.4 RESULTS

4.4.1 Incidence of first *Salmonella* exposure within the GIT in a cohort of healthy Malawian children

A total of 60 healthy children were recruited (25 were male [42%]) at 6 months of age between August 2013 and December 2013, and these were followed until December 2014 for study specific procedures described in Chapter 2 section 2.2. A total of 630 stool samples from 60 children were examined for *Salmonella* (stool culture or rt-PCR) in this cohort. Kaplan–Meier survival analysis was used to describe the pattern of *Salmonella* exposure events. A total of 630 stool samples were examined for *Salmonella* (stool culture or rt-PCR) in this cohort, there were 105 observations beginning on or after the first exposure event within the GIT (stool samples). There were 525 observations remaining, from 60 children and 22 children were positive for *Salmonella* at least once during the follow up period. Mean number of visits per child was 9 (ranging from visit 1 to 13) and by the 13th study visit (18 months of age) 21.6% (13/60) of children, were lost

to follow up. These children (13/60) voluntarily withdrew from the study because their parents (father) or guardians (uncle/grandparent) were not willing for their child to participate in the study. Over the period of observation from 6 months to 18 months of age, 46.8% (22/47) of children were exposed to *Salmonella* (detected using culture and RT-PCR) within the GIT on at least one occasion (Figure 4.1).

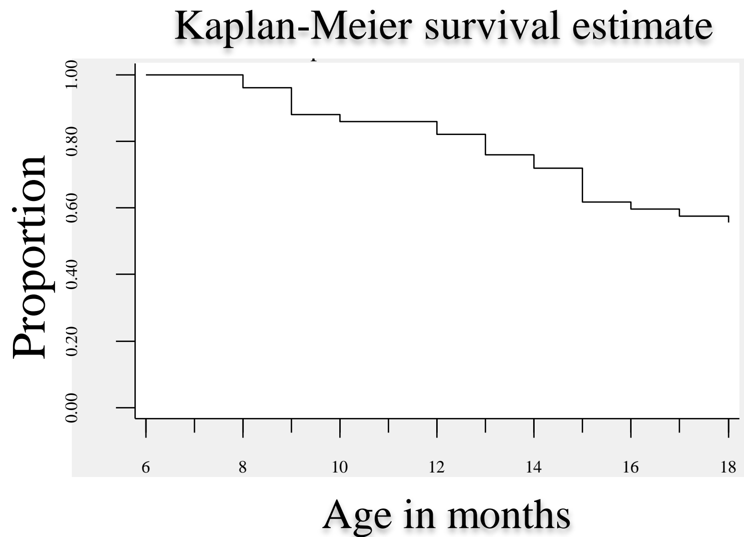

Figure 4. 1: Incidence of first *Salmonella* exposure within the GIT in a cohort of healthy Malawian children

Incidence of first *Salmonella* exposure within the GIT in a cohort of children recruited at 6 months of age, examined for *Salmonella* exposure at monthly intervals and exiting the study at 18 months. Using Kaplan-Meier survival function test, this figure shows the proportion of children remaining unexposed over time (Figure 4.1).

All stool samples (630) were examined by culture and from this total, 198 samples (these samples were of varying ages and were selected consecutively) were also examined by

real time PCR. Not all stool samples were tested using PCR because the test was developed when the study had already started and there was limited funding to test all samples. A total of 29 *Salmonella* exposure events were detected in stool by culture or real time PCR thus representing 4.6% (29/630) of the stool samples obtained and when *Salmonella* was detected in stool is shown (Figure 4.2A). *Salmonella* exposure events among female and male children were similar, 18/368 (4.8%) and 11/251 (4.2%) respectively. Out of these 29 exposure events, 6 were detected by rt-PCR only and none were detected by culture only. The remaining 23 were serotyped using standard procedures as described in Chapter 2 section 2.2.8.7. Among stool samples that tested culture or PCR positive, salmonellae isolates were principally *S*. Typhimurium 51.7% (15/29), followed by non-defined serovars 31% (9/29), *S*. Typhi 10.3% (3/29) and *S*. Enteritidis 7% (2/29) (Figure 4.2B). Among samples analysed by both culture and PCR, there were 7/29 (24%) children exposed to *Salmonella* at least twice during whole study period (5/7 [71%] were 1 month apart and 2/7 [28.5%] were 3 months apart) and none was exposed more than twice. Among children that were exposed twice to *Salmonella* within the GIT; 5/7 (71%) to *S*. Typhimurium, 1/7 (14.2%) to *S*. Typhimurium and *S*. Enteritidis and 1/7 (14.2%) to *S*. Typhi and non-defined *Salmonella* serovars. A total of 269 oropharynx swabs were tested for *Salmonella* by standard culture only and all samples tested negative for *Salmonella*.

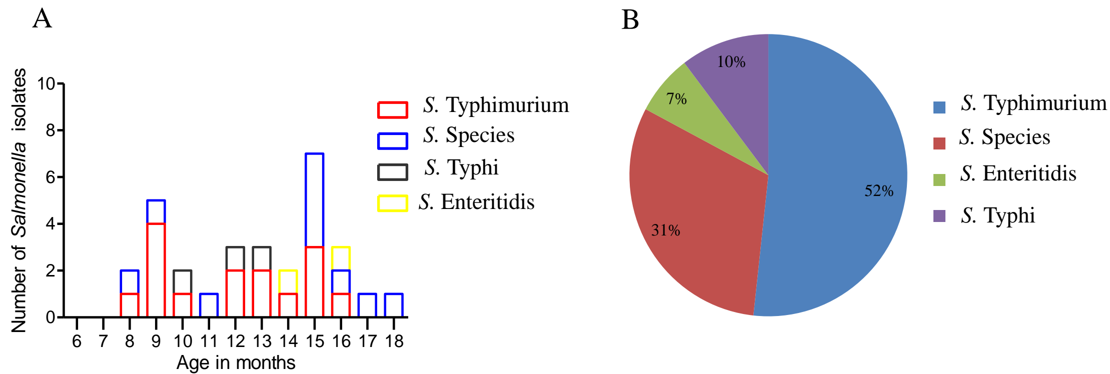

Figure 4. 2: Frequency of *Salmonella* exposure within the GIT in Malawian children

Salmonella detected in stool samples by either culture (n=630) or rt-PCR (n-198) were plotted against varying ages as indicated (Figure 4.2A). Distribution of *Salmonella* serovars detected by culture or rt-PCR are shown (n=29) (Figure 4.2.B).

4.4.2 Clinical features of *Salmonella* exposure

Young children may have a relatively high background rate of intercurrent gastrointestinal symptoms including diarrhoea, fever and vomiting (Uhnoo, *et al.*, 1986). To explore whether or not children exposed to *Salmonella* were more likely than unexposed children to be symptomatic, clinical history in relation to *Salmonella* exposure events were reviewed.

During the follow-up visits, there were a total of 54/563 (9.6%) current diarrhoea (defined as ≥ 3 loose stools per day) events recorded. There were 3/23 (13%) diarrhoea

events in children with *Salmonella* in their stool while 51/540 (9.4%) were found in stool negative children, OR 1.43 95% CI (0.41, 5.0) (Table 4-A). In the month prior to the follow-up visits, a total of 99/563 (17.6%) diarrhoea events were reported. 6/23 (26%) were reported in children that were exposed to *Salmonella* while 93/540 (17.2%) diarrhoea events were reported in non-exposed children, OR 1.69, 95% CI (0.65, 4.4) (Table 4-B). These findings showed that although there was a trend for children who had *Salmonella* detected in their stool to have diarrhoea or to have had a diarrhoea episode in the last month, this did not reach statistical significance. Rotavirus is important causative agent of diarrhoea in Malawi (Cunliffe*, et al.*, 2010). Recently the government of Malawi introduced rota virus vaccination (Madsen*, et al.*, 2014). Coverage of rota virus vaccination in this cohort was 100% (60/60) at 6 months of age (study entry). To what extent the frequency of diarrhoea in this cohort was influenced by the recently rolled-out rotavirus vaccination in Malawi is not clear, but since coverage was so high, there is unlikely to be a difference between those who did or did not experience exposure to *Salmonella*.

Table 4- A: Clinical presentation among children at current visit

	Total (%) (N=563)	Exposed (%) (N=23)	Non-exposed (%) (N=540)	Exposed vs non-exposed OR (95% CI)
Diarrhoea	54 (9.6)	3 (13)	51 (9.4)	1.43 (0.41, 5.0)
Vomit	21 (3.7)	2 (8.7)	19 (3.5)	2.6 (0.57, 11.9)
Cough	110 (19.5)	7 (30.4)	103 (19)	1.8 (0.74, 4.6)
Temp >37.8^{o}C	11(1.9)	2 (8.7)	9 (1.7)	5.6 (1.14, 27.6)
Malaria	5 (0.9)	0 (0)	5 (0.92)	ND
MUAC 11-12.5cm[a]	15 (2.6)	1 (4.3)	14 (2.6)	1.7 (0.21, 13.2)
MUAC 12.5-13.5 cm[b]	35 (6.2)	2 (8.6)	33 (6.1)	1.46(0.33, 6.4)
MUAC 11-13.5 cm[c]	50 (8.8)	3 (13)	47 (8.7)	1.57(0.45, 5.4)

ND refers to not done

[a] Moderate acute malnutrition (MAM)

[b] At risk of acute malnutrition

[c] Combination of MAM and those at risk of malnutrition

Table 4- B: Clinical history over the previous month among children

	Total (%) (N=563)	Exposed (%) (N=23)	Non-exposed (%) (N=540)	Exposed vs non-exposed OR (95% CI)
Diarrhoea	99 (17.6)	6 (26)	93 (17.2)	1.69 (0.65, 4.4)
Vomit	30 (5.3)	1 (4.3)	29 (5.4)	0.8 (0.104, 6.15)
Cough	127 (22.5)	5 (21.7)	122 (22.6)	0.95 (0.34, 2.6)
Antibiotic	139 (25.9)	7 (30.4)	132 (18.5)	1.35 (0.54, 3.35)
Antimalarial	8 (1.4)	1 (4.3)	7 (1.3)	3.4 (0.4, 29)

Malnutrition is commonly associated with NTS bacteraemia in children from SSA (Graham*, et al.*, 2000, Babirekere-Iriso*, et al.*, 2006). In this cohort, children Mid-Upper Arm Circumference (MUAC) ranging 11.5-19cm were recorded and the mean was 15cm.

MUAC measurements were grouped or defined as follows; <11cm severe acute malnutrition (SAM), 11-12.5cm moderate acute malnutrition (MAM), 12.5-13.5cm at risk of acute malnutrition and above 13.5cm well nourished. A total of 50/563 (8.8%) children had MUAC ranging 11-13.5cm and amongst these children 3/23 (13%) had *Salmonella* detected in theirs stool and 47/540 (8.7%) had no *Salmonella* detected in their stool (OR 1.57, 95% CI [0.45, 5.4]). Although not statistically significant, these findings suggested that malnutrition may be associated with a predisposition to *Salmonella* exposure in the gut. Similarly, there was a no significant difference for children to experience either cough (at current visit OR 1.8, 95% CI [0.74, 4.6]) or vomiting (at current visit OR, 2.6, 95% CI [0.57, 11.9] at the time when children stool was positive for *Salmonella* compared to when children stool was negative (Table 4-A).

A total of 11/563 (1.9%) fever events (Temp >37.8^{o}C) were recorded in this cohort at current visit, 2/23 (8.7%) were detected in children exposed to *Salmonella* while 9/540 (1.7%) were detected in non-exposed children, OR 5.6, 95% CI (1.14, 27.6) (Table 4-A). These findings suggest that children whose stool culture was positive for *Salmonella* were 5.6 times more likely to have a current fever than children without a positive stool culture for *Salmonella*. Clinical features including fever, cough, vomiting and diarrhoea in which stool culture was positive for *Salmonella* may have resulted in non-specific symptomatic febrile illness.

Recent or current malaria infections are an important comorbidity of NTS bacteraemia in children from SSA (Graham, *et al.*, 2000, Brent, *et al.*, 2006, Bronzan, *et al.*, 2007, Biggs, *et al.*, 2014). The burden of malaria is considerably high in Malawi, particularly, in under-five children (Mathanga, *et al.*, 2012), with asymptomatic parasitaemia rates ranging between 11.2-18.5% (Roca-Feltrer, *et al.*, 2012). In Blantyre, the *Plasmodium falciparum* parasite rate for children 2-<10 years is the lowest in Malawi (26%) (Bennett, *et al.*, 2013). It was therefore somewhat surprising that even for these very young urban dwelling children, at the current visit, only 0.9% (5/563) malaria events were recorded, none was among children that had *Salmonella* detected in their stool. Furthermore, during the previous month a total of 8/563 (1.4%) antimalarial usage events were recorded, 1/23 (4.3%) were recorded in children exposed to *Salmonella* and 7/540 (1.3%) were recorded in non-exposed children, OR 3.4, 95% CI (0.4, 29). This difference was not significant, but the trend would be in keeping with literature suggesting that recent (rather than current) malaria is a risk factor for *Salmonella* disease (Brent, *et al.*, 2006). It is also possible that antimalarial drugs were taken by children as syndromic management rather than a confirmed disease. These findings would need to be further explored in a much larger cohort.

During the previous month a total of 139/563 (25.9%) antibiotic usage events were reported, 30.4% (7/23) antibiotic usage events were reported in children who were exposed to *Salmonella* and 24.4% (132/540) were reported in non-exposed children, OR 1.35, 95% CI (0.54, 3.4). This difference was not statistically significant, but it is known

that administration of antibiotics may be a risk factor for more prolonged carriage of *Salmonella* (Miller*, et al.*, 1954), or may alternatively be a proxy marker for recent febrile illness caused by carriage or indeed malaria. Taken together these findings suggest that some *Salmonella* episodes (ranging 8.7% to 30.4%) detected were associated with non-specific symptomatic febrile illness. In keeping with the literature, recent malaria and prior antibiotic usage might be risk factors for *Salmonella* exposure in the GIT.

4.4.3 Child feeding practices and *Salmonella* exposure

Salmonella infection follows ingestion of contaminated food, water or contact with contaminated fomites (Kariuki*, et al.*, 2006). Most Malawian babies are exclusively breast fed and introduced to supplementary food after 3 months of age (Kalanda*, et al.*, 2006); milk formula feeding is rare, particularly in the demographic sampled. Chapter 3 demonstrates that T cell immunity to *S.* Typhimurium is detected early in life (peaking at 14 months of age) suggesting early exposure to *Salmonella* serovars or cross-reactive enteric bacteria. From this background, child feeding practices in this cohort were retrospectively explored. In this cohort, all children were being breast fed at 6 months (study entry). Supplementary feeding had been gradually introduced: 9% by the first 3 months of life; 29% by 4 months of age; 63% by 5 months of age and 91% by 6 months of age (Figure 4.3). At 18 months (study exit age), 8/47 (17%) of those children remaining in the study had discontinued breast feeding.

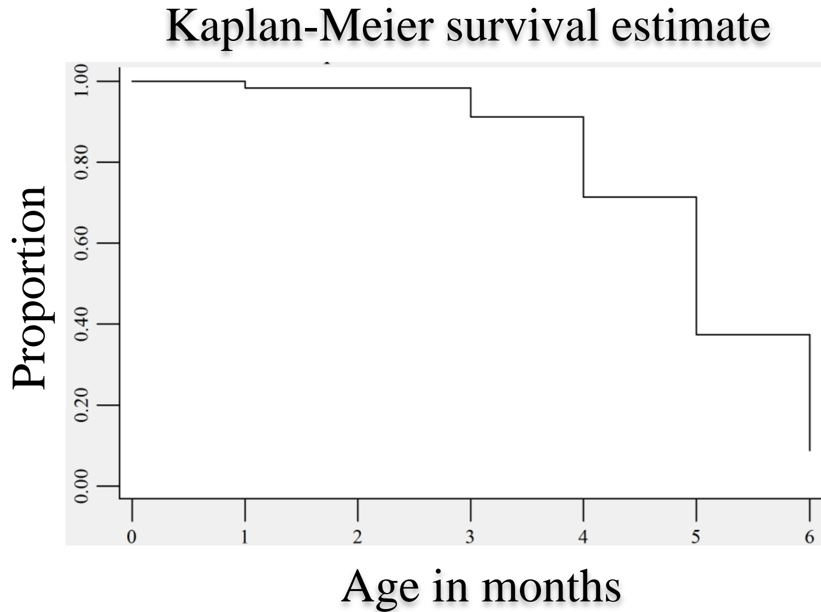

Figure 4. 3: Introduction of supplementary food in Malawian children

Mothers were asked, at the study entry point (6 months of age) when they introduced supplementary food to their babies. Using Kaplan-Meier survival function test, percentage of children exclusively breast fed during the first 6 months of life are shown (Figure 4.3).

Sources of water used by study participants at their homes were documented. A majority of families, 82.7% (466/563) were using tap water (Table 4-C). Among children that were exposed to *Salmonella*, 82.6% (19/23) were using tap water and among non-exposed children, 82.7% (447/540) were using tap water, OR 0.99, 95% CI (0.32, 2.97). Among children that were exposed to *Salmonella*, 26% (6/23) were using boiled water for drinking and among non-exposed children, 35.1% (190/540) were using boiled water,

OR 0.65, 95% CI (0.25, 1.67). This non-significant trend showed that boiling water for drinking might have reduced *Salmonella* exposure or transmission.

Table 4- C: Water source and *Salmonella* exposure

	Total (%) (N=563)	Exposed (%) (N=23)	Non-exposed (%) (N=540)	Exposed vs non-exposed OR (95% CI)
Tap	466 (82.7)	19 (82.6)	447 (82.7)	0.99 (0.32, 2.97)
Borehole	64 (11.3)	1 (4.3)	63 (11.6)	ND[a]
River	23 (4)	3(13)	20 (3.7)	3.9 (1.07, 14.2)
Bottle	10 (1.7)	0 (0)	10 (1.8)	ND[a]

[a]ND refers to not done

Furthermore, a total of 64/563 (11.3%) observations were made on use of borehole water. Among children that were exposed to *Salmonella*, 4.3% (1/23) were using borehole water and among the non-exposed children, 11.6% (63/540) were using borehole water. While using water from the borehole appeared to have reduced *Salmonella* transmission, this was not statistically significant. A total of only 23/563 (4%) children were using river water for drinking. Among *Salmonella* exposed children, 13% (3/23) were using river water and among non-exposed children, 3.7% (20/540) were using river water, and this finding was statistically significant, albeit with wide confidence intervals because of the small numbers; OR 3.9, 95% CI (1.07, 14.2). Together these findings suggest that *Salmonella* exposure occurred in these children following the introduction of supplementary food in nearly all children. Furthermore, using water from the river may be a risk factor for *Salmonella* exposure and although not statistically significant, use of

borehole or boiled water may be associated with reduced *Salmonella* transmission or exposure. A larger study would be needed to establish this definitively.

4.4.4 Living adjacent to a river or water stream may be a risk factor for *Salmonella* exposure in children

Both poor sanitary environment and food hygiene are important risk factors for the transmission of *Salmonella* (Crump & Mintz, 2010, Breiman, *et al.*, 2012). In this cohort, geographical locations of these children's homes (by protocol design coming from within 5 kilometres radius from ZHC) were explored. Using GPS co-ordinates study participant's homes locations were mapped by GPS visualizer (Figure 4.4A-B). Initially it appeared that children that were exposed to *Salmonella* appeared to reside in close proximity to the river or water streams. It is possible that families living nearer the river or water stream use contaminated water for household chores and also drinking. Children living at lower altitude relative to rivers might experience more "washout" surface water from contaminated sources or from contaminated standing groundwater near the river during rainy season when the water table is higher. However, when this was explored systematically using logistic regression analysis, the analysis did not show any statistical significant difference between the distribution of absolute altitude GPS locations of the homes of *Salmonella*-exposed and the non-exposed groups, OR 0.99, 95% CI (0.98, 1.0).

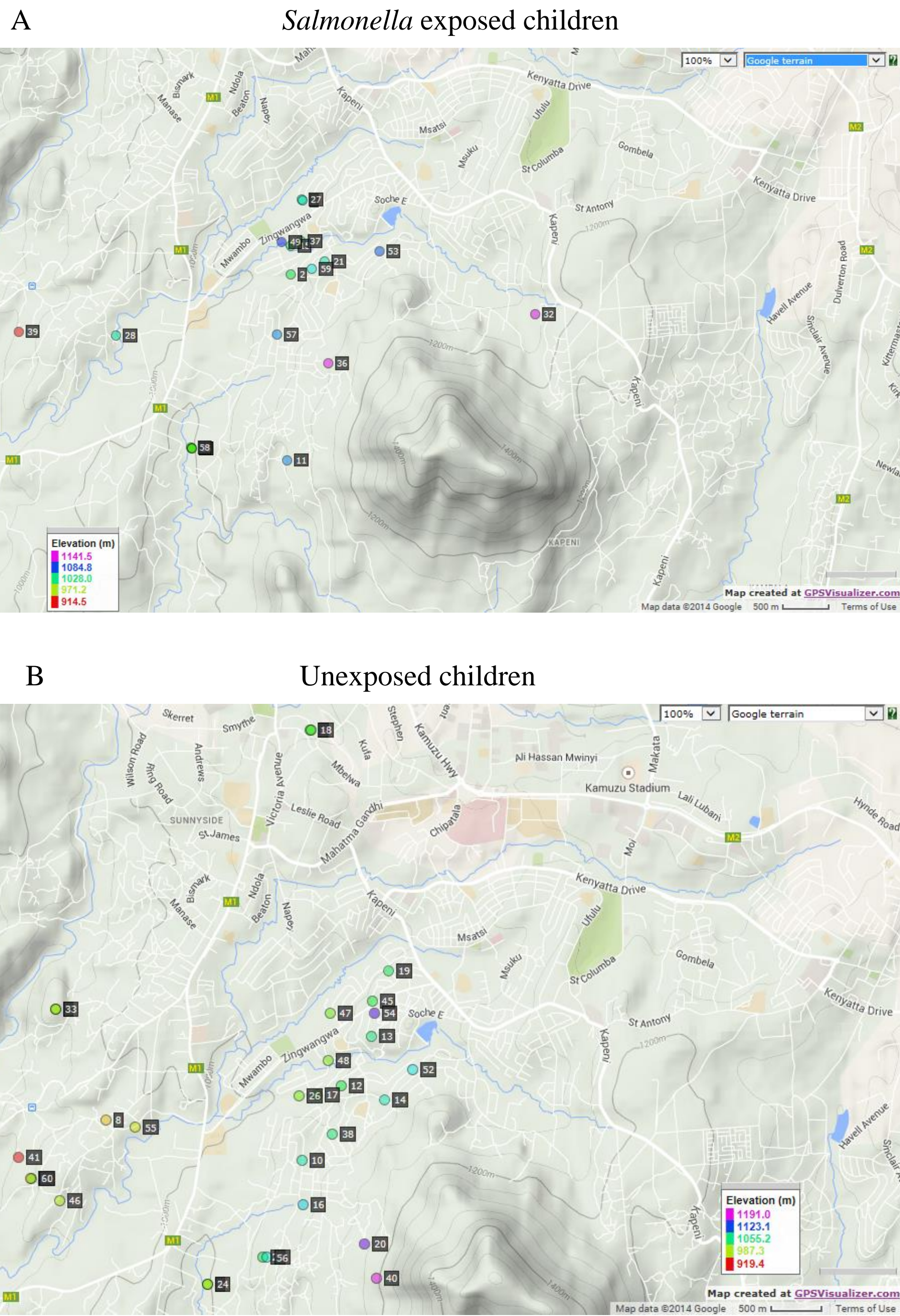

Figure 4. 4: GPS mapping of the homes of *Salmonella*-exposed and unexposed children

A Google terrain map showing the GPS location of homes of children that were *Salmonella*-exposed (Figure 4.4A) and homes of children that were never exposed to *Salmonella* (Figure 4.4.B)

Further more complex geospatial modelling analysis of absolute altitude, altitude above rivers, and distance from rivers is therefore planned, but is beyond the scope of this thesis, and further studies are required to understand the geospatial factors that are important in the transmission of *Salmonella* in this population.

4.4.5 Maternal breast milk lacks *Salmonella*-specific bactericidal activity

Maternal breast milk has a role in protecting children from a wide range of pathogens, including enteric pathogens (Hanson, *et al.*, 1990). Previously it was demonstrated that breast fed children had 5 fold reduced risk of *Salmonella* gastroenteritis infection compared to non-breast fed children (Borgnolo, *et al.*, 1996). Whether breast milk was able to kill or control the growth of *Salmonella* was investigated in this cohort. Chapter 2 section 2.2.8.5, describes the development of the milk bactericidal activity assay. Unexpectedly, breast milk did not inhibit the growth *S.* Typhimurium strain D23580 by a least -1 log10 change cfu/ml, while maternal serum from the same mothers used as a control, robustly inhibited growth of *S.* Typhimurium strain D23580 (p=<0.001, Mann-Whitney test) (Figure 4.5).

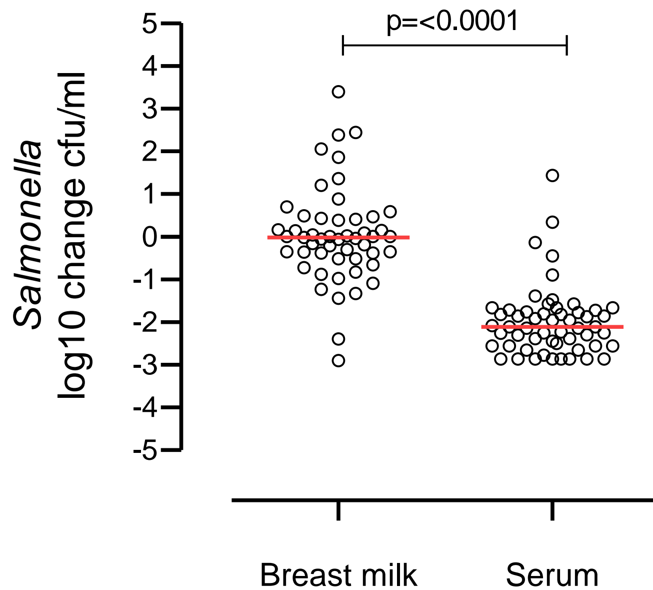

Figure 4. 5: Maternal breast milk lack invasive *Salmonella*-specific bactericidal activity

Log10 change in *S*. Typhimurium strain D23580 cfu/ml from the baseline condition in maternal serum and breast milk. PBS and heat inactivated milk were used as the baseline condition for maternal serum and breast milk respectively as describe in chapter 2 section 2.2.8.5. The red bars represents the mean (n=51).

These findings suggest that breast milk itself lacks bactericidal activity against *S.* Typhimurium strain D23580. However, it is possible that breast-feeding might limit *Salmonella* colonisation within the GIT by other mechanisms apart from direct bactericidal activity, such as inhibition of colonisation or mucosal adherence, or by altering the infant's gut microbiota (Weening, *et al.*, 2005, Roger, *et al.*, 2010, Mantis, *et al.*, 2011, Stecher & Hardt, 2011).

4.4.6 Relationship between *Salmonella* exposure and serum bactericidal activity in children

Chapter 3 describes that SBA to *S.* Typhimurium strain D23580 is acquired with age and peaks at 36 months of age in Malawian children, while T cell immunity to *S.* Typhimurium is acquired early in life, peaking at 14 months of age. Whether or not this antibody and T cell immunity development is facilitated by mucosal *Salmonella* exposure is a key question. In this cohort, two stool *Salmonella* isolates (*S.* Typhimurium ID 037v4 and *Salmonella* spp. ID 049v3 isolated from children 9 months and 8 months old respectively) were selected to be used in SBA experiments in parallel with *S.* Typhimurium strain D23580 (a sequenced and well-characterised blood isolate (Kingsley*, et al.*, 2009)). Using serum from 6 month-old children, D23580 and 037v4 resistance to serum killing was similar while 049v3 was significantly more sensitive to serum killing compared to D23580 and 037v4 (Mean; D23580 -0.09, 95% CI[-0.44, 0.24] vs 049v3 -2.52, 95% CI[-2.82, -2.22]), (Mean; 037v3 0.001, 95% CI[-0.40, 0.40] vs 049v3 -2.52, 95% CI[-2.82, -2.22]) (Figure 4.6A). Whether *S.* Typhimurium D23580 and *S.* Typhimurium 037v4 are genetically similar will be addressed by whole genome sequencing in future studies. In relation to SBA, there was positive correlation between SBA to D23580 and SBA to 037v4, using serum from children aged 6 to 18 months (r=0.64, 95% CI [0.56, 0.71], p=<0.0001) (Figure 4.6B). In our later SBA experiments D23580 and 037v4 were used in parallel because it was assumed that serum from older children (> 6months) would nearly all be competent to kill *Salmonella* 049v3.

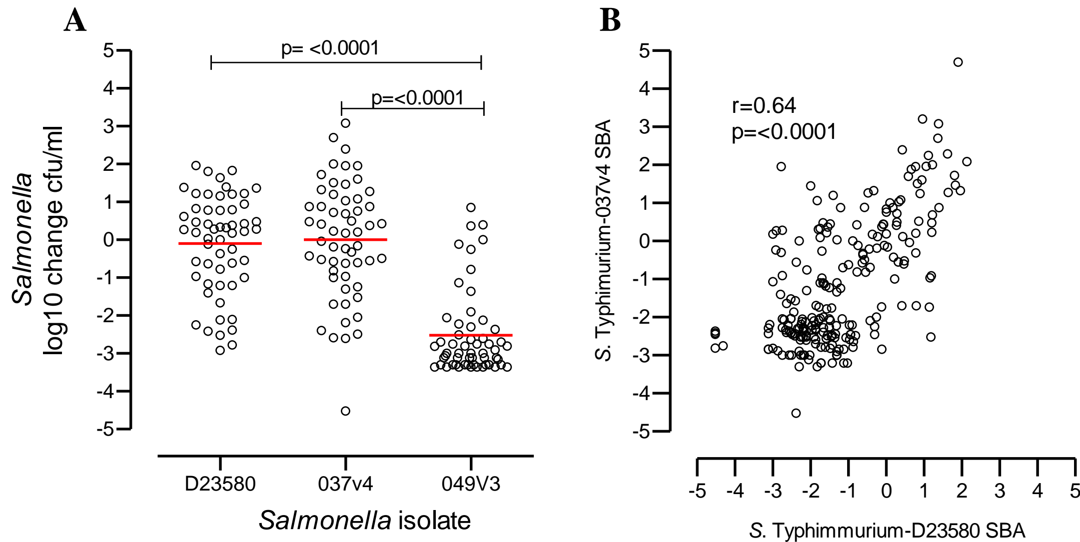

Figure 4. 6: Serum bactericidal activity to various *Salmonella* serovars

Log10 change in *Salmonella* serovars as indicated from baseline condition in serum from children at study entry (6 months) Figure 4.6A. The red bars represent the mean. Correlation of SBA to *S.* Typhimurium strain D23580 and 037v4 at 6 months to 18 months (Figure 4.6B). r represents Pearson correlation coefficient.

In keeping with previously presented data (chapter 3), serum bactericidal activity to both *S.* Typhimurium strain D2380 and *S.* Typhimurium ID 037v4 increased with age from 6 months to 18 months (Figure 4.7A-B). SBA to D23580 was significantly more inhibitory in children at 9 months, 12 months and 18 months compared to 6 months (Mean; 9 months -0.96, 95% CI[-1.43, -0.50] vs 6 months -0.09, 95% CI[-0.44, 0.24], p=0.003), (Mean; 12 months -2.02, 95% CI[-2.41, -1.62] vs 6 months -0.09, 95% CI[-0.44, 0.24], p=<0.0001), (Mean; 15 months -1.76, 95% CI[-2.04, -1.47] vs 6 months -0.09, 95% CI[-

0.44, 0.24], p=<0.0001), (Mean; 18 months -1.64, 95% CI[-1.86, -1.41] vs 6 months -0.09, 95% CI[-0.44, 0.24], p=<0.0001) (Figure 4.7A).

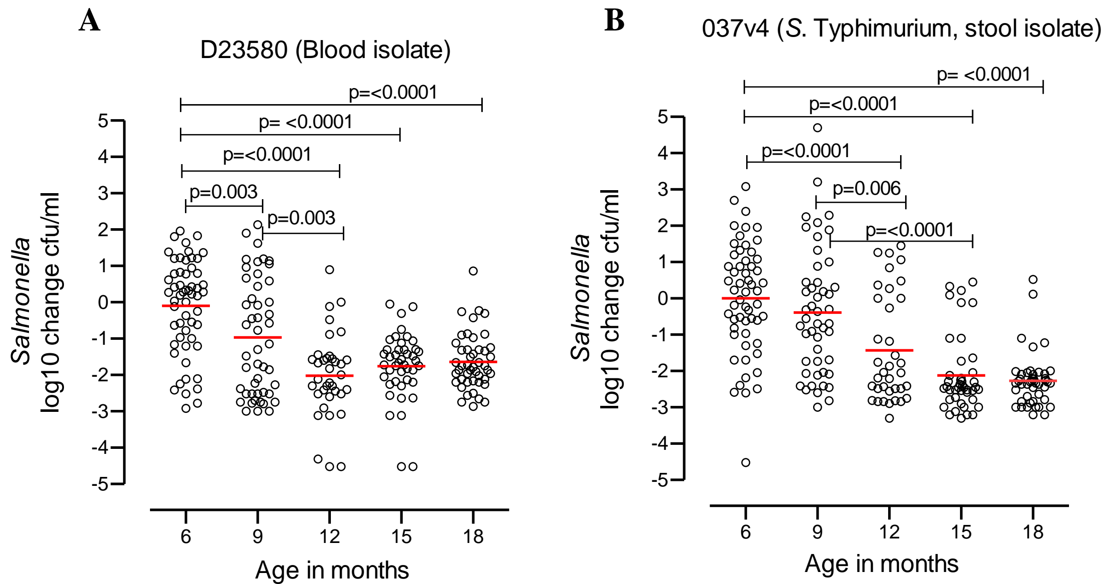

Figure 4. 7: Development of Serum bactericidal activity to *Salmonella* serovars

Log10 change in *S.* Typhimurium strain D23580 and 037v4 as indicated from baseline condition in serum from children at varying ages in months (Figure 4.7A-B). The red bars represent the mean.

SBA to D23580 was also significantly more inhibitory in children at 12 months compared to at 9 months (Mean; 12 months -2.02, 95% CI [-2.41, -1.62] vs 9 months -0.96, 95% CI[-1.43, -0.50], p=0.003). Similarly SBA to 037v4 was significantly more inhibitory in children at 12 months and 15 months compared to 6 months (Mean; 9 months -0.39, 95% CI[-0.89, 0.11] vs 6 months 0.001, 95% CI[-0.40, 0.40], p=0.41), (Mean; 12 months -1.42, 95% CI[-1.93, -0.92] vs 6 months 0.001, 95% CI[-0.40, 0.40],

p=0.006) and (Mean; 15 months -1.91, 95% CI[-2.2, -1.55] vs 6 months 0.001, 95% CI[-0.40, 0.40], p=<0.001) (Figure 4.7B). SBA to 037v4 was also significantly more inhibitory in children at 12, 15 and 18 months compared to 9 months (Mean; 12 months -1.42, 95% CI[-1.93, -0.92] vs 9 months -0.39, 95% CI[-0.89, 0.11] p=0.006), (Mean; 15 months -2.12, 95% CI[-2.4, -1.8] vs 9 months -0.39, 95% CI[-0.89, 0.11], p=<0.0001) and (Mean; 18 months -2.27, 95% CI[-2.49, -2.0] vs 9 months -0.39, 95% CI[-0.89, 0.11], p=<0.0001).

To explore whether SBA to *Salmonella* is facilitated by natural *Salmonella* exposure, SBA to D23580 and SBA to 037v4 in children exposed to *Salmonella* were quantitatively compared to non-exposed children at 9, 12, 15 and 18 months. SBA to D23580 among *Salmonella* exposed and non-exposed children were similar at 9 months (Median; exposed -0.61, IQR [-3- 1.28] vs non-exposed -1.3, IQR [-2.51- 0.43], p=0.839), 12 months (Median; exposed -1.96, IQR [-3.46- 1.1] vs non-exposed -2.1, IQR [-2.51- -1.52], p=0.81), 15 months of age (Median; exposed -1.57, IQR [-2.5- -1.3] vs non-exposed -1.69, IQR [-2.1- -1.3], p=0.61) and 18 months of age (Median; exposed -1.87, IQR [-2.15- -1.36] vs non-exposed -1.74, IQR [-2.2- -1.08], p= 0.61) (Figure 4.8A). Similarly, SBA to 037v4 among *Salmonella* exposed and non-exposed children was also similar at 9 months (Median; exposed 0.18, IQR [-1.71- -3.2] vs non-exposed -0.69, IQR [-2.07- -0.43], p=0.156), 12 months (Median; exposed -2.13, IQR [-2.8- 0.8] vs non-exposed -2.0, IQR [-2.5- 0], p=0.91), 15 months of age (Median; exposed -2.52, IQR [-3- -2.3] vs non-exposed -2.36, IQR [-2.5- -1.25], p=0.126) and 18 months of age (Median; exposed -2.3, IQR [-2.8- -2.2] vs non-exposed -2.31, IQR [-2.73- -2.04], p=0.66) (Figure

4.8B). SBA was observed to vary with age, as some children that had SBA of -1 log10 change in *Salmonella* cfu/ml at younger age had lower SBA at older age, suggesting intra-individual variability of this measurement over time (Figure 4.8C-D). This needs to be explored in future.

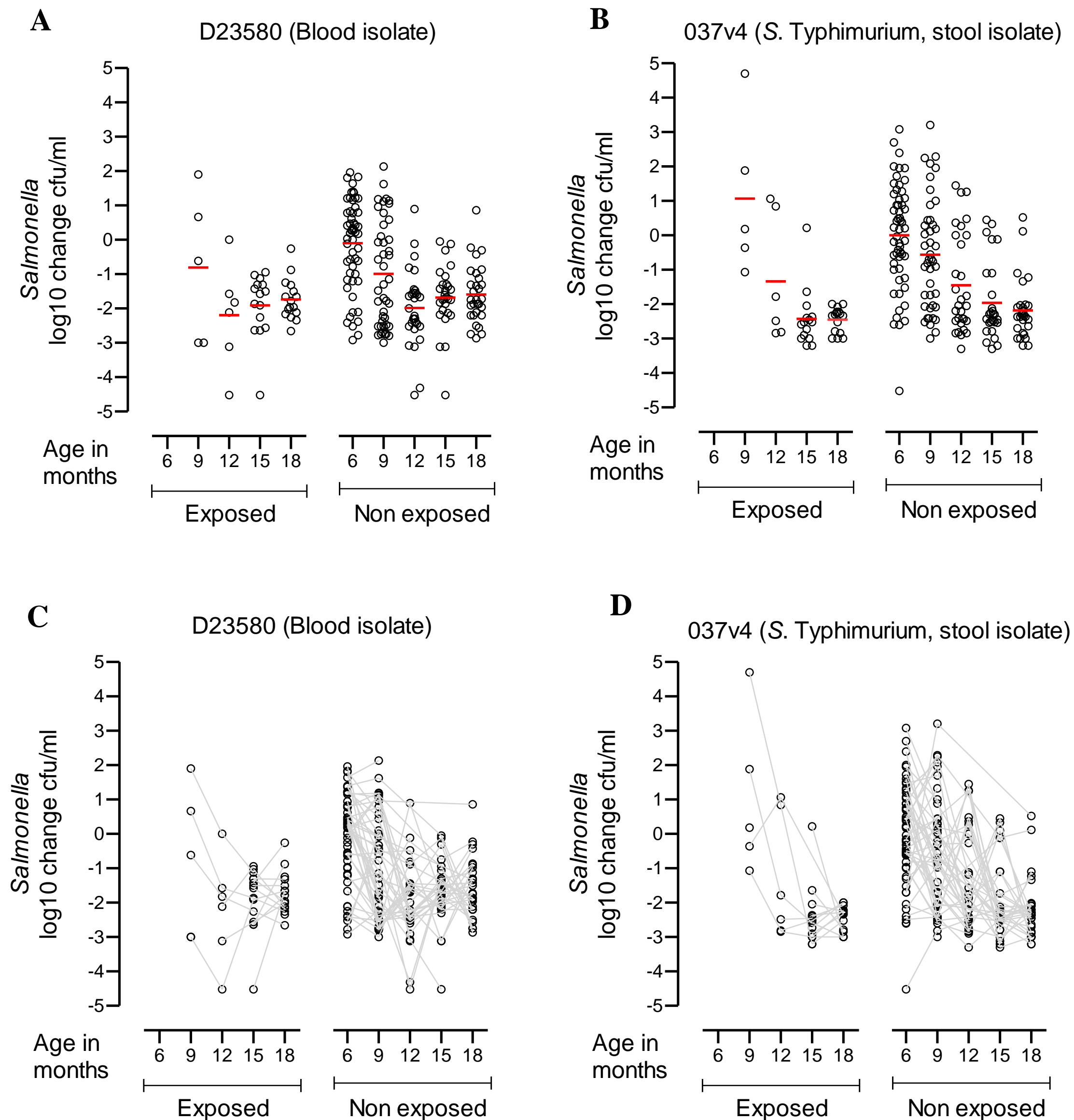

Figure 4. 8: Relationship between *Salmonella* exposure and serum bactericidal activity in children

Children serum killing capacity to *S.* Typhimurium strain D23580 or 037v4 are shown. Log10 change in *S*. Typhimurium strain D23580 or 037v4 cfu/ml from the control condition were plotted against varying ages as indicated (Figure 4.8A-D), grouped as exposed and non-exposed (Figure 4.8A-D), and individual SBA liked with age (Figure 4.6C-D). The bars represent the median. Child serum that attained of ≥ -1 Log10 change in *Salmonella* cfu/ml was considered 'protected'.

The relationship between SBA and *S.* Typhimurium and *Salmonella* exposure in children was further analysed by Chi squared analysis. SBA of ≥ -1 log10 change in *Salmonella* cfu/ml (considered as a surrogate of protective immunity) was used as a cut off. A total of 229 observations of SBA to *S*. Typhimurium D23580 or 037v4 were made (56 at 6 months, 48 at 9 months, 36 at 12 months, 43 at 15 months and 46 at 18 months) (Table 4-D and 4-E). Comparing SBA to both *S*. Typhimurium D2350 and 037v4 by specific age groups (between 6 and 18 months), there was a trend at 15 months that *Salmonella* exposure is associated with acquisition of potentially protective SBA but this did not reach statistical significance OR 3.04, 95% (0.32, 28.8) (Table 4-D and 4-E). Interestingly, acquisition of potentially protective SBA to *S.* Typhimurium D23580, when all observations over the period of 1 year (6-18 months) were considered, was 3.65 times more likely to occur in children who had *Salmonella* detected in stool than in children who had no *Salmonella* detected, OR 3.65, 95% CI(1.54, 8.65). Similarly, acquisition of potentially protective SBA to *S.* Typhimurium 037v4 for all observations over the period of 1 year was 4.25 times more likely to occur in children who had *Salmonella* detected

in stool than in children who had no *Salmonella* detected OR 4.25, 95% CI (1.79, 10). Taken together, these findings support the study hypothesis that *Salmonella* exposure within the GIT may facilitate the acquisition of potentially protective SBA to *Salmonella* in children.

Table 4- D: Relationship between SBA to *S.* Typhimurium strain D23580 and *Salmonella* exposure

Age		All SBA ≥ -1 log10 [%]	SBA ≥ -1 log10 Exposed [%]	SBA ≥ -1 log10 Non exposed [%]	Exposed vs non-exposed OR (95% CI)
6 months	Total N=56 (n/N)	14 (14/56) [25]	0 (0/0) [0]	14 (14/56) [25]	ND
9 months	Total N=48 (n/N)	25 (25/48) [52]	2 (2/5) [40]	23 (23/43) [53]	0.57 (0.87, 3.82)
12 months	Total 36 (n/N)	30 (30/36) [83]	5 (5/6) [83]	25 (25/30) [83]	ND[a]
15 months	Total 43 (n/N)	37 (37/43) [86]	14 (14/15) [93]	23 (23/28) [82]	3.04 (0.32, 28.8)
18 months	Total N=46 (n/N)	37 (37/46) [80]	14 (14/16) [87]	23 (23/30) [76]	2.13 (0.38, 11.7)
6-18 months	Total N=229 (n/N)	143 (143/229) [62.4]	35 (35/42) [83.3]	108 (108/187) [57.7]	3.65 (1.54, 8.65)

[a]ND refers to not done

Table 4- E: Relationship between SBA to *S.* Typhimurium strain 037v4 and *Salmonella* exposure

Age		All SBA ≥ -1 log10 [%]	SBA ≥ -1 log10 Exposed [%]	SBA ≥ -1 log10 Non exposed [%]	Exposed vs non-exposed OR (95% CI)
6 months	Total N=56 (n/N)	13 (13/56) [23]	0 (0/0) [0]	13 (13/56) [23]	ND[a]
9 months	Total N=48 (n/N)	17 (17/48) [35.4]	2 (2/5) [40]	15 (15/43) [34.8]	1.24 (1.24, 8.2)
12 months	Total N=36 (n/N)	25 (30/36) [69.4]	4 (4/6) [66.6]	12 (31/30) [70]	0.85 (0.13, 5.55)
15 months	Total 43 (n/N)	37 (37/43) [86]	14 (14/15) [93]	23 (23/28) [82]	3.04 (0.32, 28.8)
18 months	Total N=46 (n/N)	44 (44/46) [95.6]	16 (16/16) [100]	28 (28/30) [93]	ND[a]
6-18 months	Total N=229 (n/N)	136 (136/229) [59]	35 (35/42) [83.3]	101 (101/187) [54.7]	4.25 (1.79, 10)

[a]ND refers to not done

4.5 DISCUSSION

This cohort study extends the findings in Chapter 3 that SBA (peaking at 36 months) and T cell immunity (peaking at 14 months) to *S.* Typhimurium are naturally acquired in Malawian children. This cohort study has demonstrated that *Salmonella* exposure within the GIT occurs in the first 18 months of life, re-affirming our earlier hypothesis that the early acquisition of T cells immunity to *Salmonella* could be driven, at least in part, by exposure to *S.* Typhimurium. *Salmonella* exposure within the GIT was associated with acquisition of potentially protective SBA to *S.* Typhimurium in children aged between 6 and 18 months. However, there were many children who developed serum bactericidal

killing without detection of *Salmonella* exposure, and the time course of SBA acquisition in non-exposed children was not different from children who were exposed. It is anticipated that there were episodes of *Salmonella* exposure that were not detected, or that other exposures to cross-reacting bacteria also occurred which we did not detect.

In this cohort, 46.8% *Salmonella* exposure within the GIT of children aged ≤18 months was demonstrated, after children were prospectively followed for a period of 12 months (from 6 to 18 months of age). These findings are in keeping with a previous report from Mexican children that found that were 40% of children were exposed to *Salmonella* in the first year of life (Cravioto*, et al.*, 1990). Enteric pathogens exposure in that study was, however, examined at 2 weeks intervals. It is likely that higher than 46.8% *Salmonella* exposure might have been detected in our cohort if shorter time intervals to observe *Salmonella* exposure events were adopted because in some children shedding of *Salmonella* might be of shorter duration (< 4 weeks) than what was anticipated. It was, however, previously demonstrated that under-five year-old children, on average, shed *Salmonella* in their stools for as long as 7 weeks (Buchwald & Blaser, 1984). Cross-sectionally, *Salmonella* was detected in 4.6% (29/630) of stools tested in Malawian children aged 6-18 months. Importantly, among the *Salmonella* serovars isolated, *S.* Typhimurium was the predominant serovar (52%), followed by non-defined *Salmonella* serovars (31%), *S.* Typhi (10%) and *S.* Enteritidis (7%). A cross-sectional study in Blantyre, Malawi found asymptomatic *Salmonella* carriage in 2.4% (6/251) of children aged 0-2 years and 0.8% (1/131) of children aged 2-5 years from Ndirande community

(Msefula, 2009). This was lower than 4.6% detected in this cohort. The difference in magnitude of *Salmonella* detected in these studies might arise from differences in age distribution, from seasonal differences particularly during the wet season when the level of enteric pathogens including *Salmonella* has been reported to be high (Kariuki*, et al.*, 2006, Gordon*, et al.*, 2008, Morpeth*, et al.*, 2009) and also from methodological differences; in this study culture and PCR were used, while culture only was used in previous studies.

Salmonella infection occurs through the oral route following ingestion of contaminated food, water or contact with contaminated fomites (Kariuki*, et al.*, 2006) and it is possible that colonisation of the oropharynx could be immunizing. In this cohort throat swabs were examined for *Salmonella* to describe the pattern of *Salmonella* exposure within the oropharynx. Oropharynx swabs did not yield positive result for *Salmonella*, even in children that had stool culture *Salmonella* positive result. This was in keeping with a previous report that only detected *Salmonella* in throat swabs of children with acute bacteraemia and not asymptomatic children and adults from the communities (Msefula, 2009). These differences may arise from secondary infection in oral cavity in children with *Salmonella* bacteraemia (Sirsat, 2013) and which could be unlikely in children asymptomatically carrying *Salmonella* within the GIT. This study, however, did not use molecular methods to increase the sensitivity of detection from throat swabs. Further studies are required to investigate variations in colonization pattern of *Salmonella* in different tissues.

Although this cohort of *Salmonella* exposed children was intended to be a survey of asymptomatic children, at the point of the stool being positive for *Salmonella* (positive stool culture), children were more likely to have fever (>37.8º C), and there was a trend for them to have a range of non-specific symptoms such as diarrhoea, vomiting or cough, at the time of positive stool sample. As previously reported (Bar-Meir*, et al.*, 2005), it is possible that these febrile children may have had sub-clinical blood-stream infections, which were not detected. Incidence of bacteraemia among children with gastroenteritis caused by NTS may range from 3-41% (Meadow*, et al.*, 1985, Bar-Meir*, et al.*, 2005). This might suggest that transient or low-level blood stream infection with NTS may be common outside of hospital admissions, and this might be important in relation to the development immunity.

Among children that had *Salmonella* in their stool there was a non-statistically significant trend that diarrhoea was more likely occur than in children with no *Salmonella* in their stool. Diarrhoea episodes (13% and 26% at current visit and during previous month respectively) amongst *Salmonella* in this cohort may be explained by NTS strains ability to trigger pro-inflammatory diarrhoea (Zeng*, et al.*, 2003).

Current or recent malaria infection is commonly associated with *S.* Typhimurium bacteraemia in children from SSA (Graham*, et al.*, 2000, Bronzan*, et al.*, 2007, Biggs*, et al.*, 2014). Surprisingly, in this cohort only a few children were diagnosed with malaria infection (<1%). Among children that had *Salmonella* in their stool, there was a non-

statistically significant trend that they had taken anti-malarial drugs in the previous month. However, it is also possible that some children had taken antimalarial drugs as syndromic management rather than a confirmed disease. Malaria infection is thought to impair phagocyte effector functions and this favours *S*. Typhimurium to break mucosal host immunity and cause bacteraemia (MacLennan, 2012). World Health Organisation (WHO) already recommend the prescription of antibiotics, to treat possible non-diagnosed bacterial blood stream infections [BSI] in children presenting to hospital with severe malaria infection (Church & Maitland, 2014). Further studies into malaria and *Salmonella* co-infection are required to understand the biology and provide possible immunological or clinical management interventions.

Importantly, in this cohort usage of antibiotics was high (25.9%) compared usage of antimalarial drugs (1.4%). Antibiotics are thought to disturb the GIT microbial communities and potentially favour *Salmonella* colonisation as normal flora resistance is compromised (Lolekha, 1986, Pavia*, et al.*, 1990, Gradel*, et al.*, 2008, Endt*, et al.*, 2010, Kaiser*, et al.*, 2012). Although statistically non-significant, there was a trend that having taken recent antibiotics had a higher risk of having *Salmonella* in stool. Coughing was commonly reported clinical presentation in this cohort (22.5%), so it is possible that children took these antibiotics to treat cough, a common childhood symptom. Cough is known to be a nonspecific symptom of invasive NTS disease among children, and is also common in typhoid fever (Graham*, et al.*, 2000, Gordon*, et al.*, 2002, Feasey*, et al.*, 2012). Alternatively, antibiotic-usage may be an indirect proxy marker for fever from

previous malaria, which is also known to be a risk factor for *Salmonella* disease. Sadly, high usage of antibiotics is thought to drive antimicrobial resistance (Lolekha, 1986) and could have huge implications in management of bacterial infections using antibiotics, particularly in SSA where resources are also limited.

There have been few studies into factors (i.e. food and water intake, environmental) that contribute to *Salmonella* transmission in this population. As shown before (Kalanda*, et al.*, 2006), Malawian babies in this cohort were introduced to supplementary food gradually from around 9% at 3 months to 91% by 6 months. A minority of children were exclusively being breast fed at 6 months of age (9%). Un-expectedly, maternal breast milk lacked bactericidal effect to *S.* Typhimurium strain D23580 while maternal serum robustly inhibited growth of *S.* Typhimurium strain D23580. Previous reports recognised 'protective' role of breast milk in breast fed children to pathogenic infections including *Salmonella* gastroenteritis (France*, et al.*, 1980, Borgnolo*, et al.*, 1996). In this study, levels of antibodies in maternal milk were not quantified and the contribution of antibodies to maternal milk bactericidal activity was also not explored. The contribution of IgA to complement fixing has been controversial and it is generally agreed that it does not efficiently fix complement which is required for killing of invasive *S*. Typhimurium strain D23580 (Michetti*, et al.*, 1992, Roos*, et al.*, 2001, Woof & Kerr, 2006). The level of IgG and IgA antibodies specific for *Salmonella* in maternal milk is not known and this needs to be investigated in future. Human breast milk exhibit specific IgG and IgA antibodies to pathogens including *Haemophilus influenza, Campylobacter jejuni,*

Helicobacter pylori and Streptococcus pneumoniae (Shapiro*, et al.*, 2007). Human breast milk also contains complement (Ogundele, 2001) and has been demonstrated to opsonise serum sensitive *E.* coli strain (Ogundele, 1999, Ogundele, 2000), indicating that it could be activated *in vivo*. Together these findings indicates that breast milk may lack direct bactericidal activity against *S.* Typhimurium strain D23580 and suggest that breast-feeding might limit *Salmonella* colonization within the GIT by other mechanisms such as contribution to normal flora colonization resistance by promoting growth of bacteria that resist *Salmonella* invasion and prevention of *Salmonella* adherence to intestinal epithelial cells through opsonisation or blocking of *Salmonella* fimbriae or pili (Weening*, et al.*, 2005, Mantis*, et al.*, 2011, Stecher & Hardt, 2011).

Normally water intake begins very early after birth in Malawian children. In this cohort most families had access to tap-water (82.7%) but a majority of them were taking non-boiled water (64.8%). Importantly, among children that had *Salmonella* in their stool, there was a non-statistically significant trend that using non-boiled water contributed to being exposed to *Salmonella* while use of boiled water appeared to prevent *Salmonella* transmission and colonisation. A minority of families were using water from the river (4%) or a borehole (11.3%). Using water from the river had 3.9 times higher risk of being exposed to *Salmonella* among children. Borehole and boiled water appeared to reduce or prevent *Salmonella* transmission, but these were not statistically significant. Taken together these findings suggest that *Salmonella* exposure occurred in these children, following the introduction of supplementary food, and water sources including tap

water(particularly when used non-boiled) or river water might have an important role in transmission of *Salmonella* infection. In the developed world, typhoid cases have considerably reduced (Clark*, et al.*, 2010), while in sub-Saharan Africa, Asia and the Indian sub-continent typhoid fever remain endemic mainly due to limited access to clean water, poor sanitation and poor food hygiene (Crump & Mintz, 2010, Breiman*, et al.*, 2012) which hugely contribute to *Salmonella* transmission. Improvements in water, sanitation and hygiene could reduce transmission of *Salmonella* and other enteric pathogens these populations.

Observations made on geographical locations of exposed and non-exposed children residences seem to suggest that living in close proximity to the river or water stream contributed to *Salmonella* transmission. There was no evidence of statistical difference in absolute altitude position of the homes of those exposed to *Salmonella* and compared to those who were non-exposed. It is possible that families living near the river or water stream use this water for household chores or also drinking. It has previously been demonstrated that residents in lower grounds (low latitudes) are at risk of *Salmonella* infection (Baker*, et al.*, 2011). Waste disposal in Blantyre is fairly good in the commercial area and low density areas, but poor in high density areas and among low income earners (including Soche Ward) (Kumwenda*, et al.*, 2012, MCI, 2012). In Blantyre city, Soche Ward, water taps frequently run dry coupled with high water bills (MCI, 2012), some families resort to use of river and stream water. It is not clear whether living in close proximity (either by distance or altitude) to the river or water stream might

be a risk factor for contracting *Salmonella* infection. Further investigations including more complex, geospatial analyses studies are required, to generate hypotheses stimulating future studies aimed at understanding the role of environmental factors (including water sources) in the transmission of *Salmonella* infection in this population.

The ultimate objective of this project was to provide an answer as to whether or not *Salmonella* exposure within the GIT facilitates development of serum immunity. For the first time in this population, this cohort study demonstrated 46.8% overall *Salmonella* exposure in Malawian children that were prospectively examined at 1 month intervals from 6 to 18 months of age. In keeping with findings in a cross sectional study (chapter 3), SBA increased with age from 6 months to 18 months. In keeping with the study hypothesis, acquisition of potentially protective SBA to *S.* Typhimurium D23580 and 037v4 from 6-18 months was 3.65 and 4.25 times more likely to occur in children who had *Salmonella* detected in stool than in children who had no *Salmonella* detected suggesting that *Salmonella* exposure within the GIT facilitates the acquisition of potentially protective SBA to *Salmonella* in children. However, observations were made that children that had no *Salmonella* detected in their stool also developed SBA to *S.* Typhimurium D23580 and 037v4. This might have arised from suboptimal sensitivity to detect all *Salmonella* exposures, because other exposures to cross-reactive species also contribute to protection. Multiple *Salmonella* exposure events might be required to develop potentially protective SBA. This cohort described 24% (7/29) of children that were at least exposed twice to *Salmonella*. A majority of these were exposed twice to *S.*

Typhimurium 71% (5/7). Whether development of potentially protective SBA varies following single or multiple exposure events to the same serovar or different serovar is the key question to be explored in future. The effect of *Salmonella* exposure in this study was also hindered by the unanticipated and intra-individual variation in SBA at different time points and further studies might address this issue.

Summary

This cohort study revealed that the magnitude of *Salmonella* exposure within the GIT in Malawian children is high in early life (< 18 months of age) when the potentially protective SBA is not fully mature. True to this study hypothesis, *Salmonella* exposure within the GIT is associated with acquisition of SBA. Maternal breast milk lacks direct bactericidal effect to *S.* Typhimurium strain D23580. Non-specific clinical features including fever, and possibly diarrhoea, vomiting and cough are associated with *Salmonella* exposure events in a small proportion of episodes. Further studies are required to determine whether previous administration of antibiotic and recent malaria infection and feeding practices including taking river and non-boiled water are possible risk factors of *Salmonella* exposure. These findings underlie the need for a better understanding of the relationship between *Salmonella* exposure within the GIT and the development of protective immunity and also *Salmonella* exposure within the GIT and spread into systemic tissues. Public health interventions including improvements in sanitary environments, food hygiene and access to clean and safe water are required in addition to administration of a cross protective *Salmonella* vaccine in this population.

CHAPTER 5: DEVELOPMENT OF T CELL AND ANTIBODY MEDIATED IMMUNITY IN RESPONSE TO INVASIVE *SALMONELLA* INFECTION

5.1 INTRODUCTION

Nontyphoidal *Salmonella* (NTS), principally *S.* Typhimurium and *S.* Enteritidis, frequently causes bacteraemia in children under 2 years and HIV infected individuals in sub-Saharan Africa (SSA)(Graham*, et al.*, 2000, Graham*, et al.*, 2000, Bahwere*, et al.*, 2001, Berkley*, et al.*, 2005, Gordon & Graham, 2008, Bassat*, et al.*, 2009, Graham, 2010, Feasey*, et al.*, 2012). In contrast, *S*. Typhi typically causes bacteraemia in children older than 2 years and adults in Malawi and elsewhere in SSA (Feasey*, et al.*, 2010, Breiman*, et al.*, 2012, Lutterloh*, et al.*, 2012). As described in Chapter 1, both NTS and *S*. Typhi isolates from blood are frequently multi-drug resistant in SSA (Kariuki*, et al.*, 2010, Lutterloh*, et al.*, 2012). To effectively control *Salmonella* bacteraemia, a comprehensive approach is required, including vaccination and improvements in sanitation and food hygiene to prevent new infections and transmission. The rational design of protective vaccines requires better understanding of the targets and determinants of naturally acquired immunity.

Although its remains poorly defined, humoral immunity appears to constitute a key component of protective immunity to both NTS and *S.* Typhi bacteraemia in children from endemic countries (MacLennan*, et al.*, 2008, Pulickal*, et al.*, 2009). CD4+ T helper 1 (Th1) immunity constitutes another important component of protective immunity

against *Salmonella* (Mastroeni*, et al.*, 1993, MacLennan*, et al.*, 2004). In Chapter 3, we show that the development of both CD4+ T cell and SBA immunity to *Salmonella* in healthy children coincides with the decline in cases of NTS bacteraemia, suggesting establishment of high level of resistance.

As discussed in Chapter 1, currently they are a number of research groups working on the development of an effective vaccine for *Salmonella* bacteraemia. Pre-clinical work has demonstrated that both LPS-O;4,5 (O antigen specific for *S.* Typhimurium) (Grimont PA. D and Weill, Cited 2007) and LPS-O;9 (O antigens shared by *S.*Enteritidis and *S.* Typhi) (Grimont PA. D and Weill, Cited 2007) antigens have considerable potential as vaccine targets (Colwell*, et al.*, 1984). Pure polysaccharide vaccines induces the generation of antibodies which are short-lived (Simon*, et al.*, 2011, MacLennan, 2013), in contrast polysaccharide conjugated vaccines induce the generation of antibodies that are T cell-dependent and are long-lived (Simon*, et al.*, 2011, MacLennan, 2013). Pre-clinical work has also demonstrated that immunisation with outer membrane proteins (OMP) and flagellin protein FliC derived from *Salmonella* induces both T cell and antibody immune responses (Udhayakumar & Muthukkaruppan, 1987, Cunningham*, et al.*, 2004, Gil-Cruz*, et al.*, 2009, Bobat*, et al.*, 2011). Whether or not these protein vaccine candidates can confer superior protection separately (Gil-Cruz*, et al.*, 2009) or covalently linked to O-antigen as glyco-conjugates (Simon & Levine, 2012) is not clear and is currently being investigated. Recently, MacLennan and colleagues adopted a previous described Generalised Modules for Membrane Antigens (GMMA) platform for *Shigella* (Berlanda

Scorza*, et al.*, 2012), to generate GMMA from *Salmonella* (MacLennan, 2013). GMMA are outer membrane particles naturally released by *Salmonella* during growth, and contain high amounts of periplasmic and outer membrane protein (MacLennan, 2013). This is achieved through the disruption of the Tol-Pal system which leads to an increase in rate of GMMA natural release enabling exploitation of GMMA as vaccine candidate (Berlanda Scorza*, et al.*, 2012). Generation of GMMA with O antigen (GMMA O+) and lacking O antigen (GMMA O-) involves ultracentrifugation of culture supernatants from *tolR*- and *tolR-wabaP*-mutants of *Salmonella* serovar of choice (Berlanda Scorza*, et al.*, 2012). GMMA have been shown to be highly immunogenic in mice (MacLennan, 2013) but their importance in humans as vaccine candidate has not been evaluated.

It is thought that natural *Salmonella* bloodstream infection results in the acquisition of effector and memory B and T cells (Mittrucker & Kaufmann, 2000) and Chapter 4 demonstrates that *Salmonella* exposure within the GIT facilitate development of 'protective' serum bactericidal activity to invasive NTS strain D23580 in children (6 to 18 months). In this study, a cohort of children with *Salmonella* bacteraemia was evaluated for immune response to *Salmonella* specific antigens during the acute phase of infection and at 1 month in convalescence. In addition, healthy family members from the index cases' households were recruited for similar immunological investigations. Immunity was evaluated *ex vivo*, using immunological assays including the intracellular-cytokine staining assay (for detection of *Salmonella*-specific CD4+ T cells producing

IFN-γ) and the B cell ELISpot assay (for detection of *Salmonella*-specific IgG or IgA antibody secreting cells [ASC]).

Hypothesis: Invasive *Salmonella* infection induces the development of *Salmonella* specific B cell and CD4+ T cell immunity.

Study objectives:

1. To determine the immunological importance of *Salmonella* derived proteins; FliC, OMP, GMMA O+ and GMMA O- and polysaccharide antigens; LPS O; 4,5 and LPS O; 9 as inducers of B cell and T cell immunity.
2. To determine whether or not *Salmonella* bacteraemia primes the development of memory CD4+ T cell immunity.

End points:

1. Measure and compare *Salmonella*-specific CD4+IFN-γ+ T cells, induced by *ex vivo* stimulation of T cells with *Salmonella* derived proteins; FliC, OMP, GMMA O+ and GMMA O, in a cohort of children with *Salmonella* bacteraemia at acute phase, convalescent phase (at 1 month) and in healthy family members.
2. Measure and compare *Salmonella*-specific IgG and IgA-secreting cells, targeting *Salmonella* derived proteins; FliC, OMP, GMMA O+ and GMMA O- and

polysaccharide antigens; LPS O;4,5 and LPS O;9, in children with *Salmonella* bacteraemia at acute phase and in healthy family members.

5.2 PARTCIPANTS RECRUITMENT AND METHODS

The methods for this chapter have been described in chapter 2, section 2.4.

Study participants: Children admitted and diagnosed with *Salmonella* bacteraemia were termed index cases. This project aimed at recruiting equal numbers of children presenting with NTS and *S.* Typhi bacteraemia. Unexpectedly only children with *S.* Typhi bacteraemia were recruited as cases of NTS bacteraemia were rare during the recruitment period. Children (aged below 15 years) with *Salmonella* bacteraemia were recruited sequentially. These children were presenting to QECH with a Gram negative bacteraemia which was then confirmed as *Salmonella* by routine identification as described in Chapter 2, section 2.15. These index cases were recruited and blood samples drawn within 72 hours of diagnosis for immunological investigations, typically 1-2 days after treatment with recommended antibiotics (ceftriaxone and ciprofloxacin) had been commenced. From each index case, a convalescent blood sample was collected at 1 month. Healthy family members (both children and adults) living in the same household as index cases were also recruited into the study and blood samples were collected at entry. Healthy family members were not age matched with index cases.

Antigens used for immunological investigations: All antigens used in this study have considerable potential to be used a vaccines and are currently being investigated in pre-clinical studies. Outer membrane protein (OMP) was generated from *S*. Typhimurium as a rough preparation as previously described (Gil-Cruz*, et al.*, 2009). These OMP are thought to cross-react with *S.* Typhi due to conservation of the OmpC and F dominant proteins (Maclennan*, et al.*, 2014). Flagellin protein FliC was generated from *S.* Typhimurium. *S*. Typhimurium flagellin reacts to anti-Hi antibodies whiles *S*. Typhi reacts to anti-Hd antibodies according to the Kauffman-White scheme (Grimont PA. D and Weill, Cited 2007). To what extent *S*. Typhi and *S*. Typhimurium FliC cross-react is not clear. GMMA O- were generated from mutant *S.* Typhimurium strain D23580 (*wabaP*-mutants) while GMMA O+ was generated from wild type *S*. Typhimurium strain D23580 (MacLennan*, et al.*, 2008). GMMA proteins derived from *S.* Typhimurium cross-react with *S.* Typhi. LPS-O; 4,5 and LPS-O;9 were commercial *S*. Typhimurium LPS and *S*. Enteritidis LPS respectively (ALEXIS, Biochemicals). In addition *S*. Enteritidis LPS-O;9 cross-reacts with *S.* Typhi LPS as these share LPS O; 9 antigen (Grimont PA. D and Weill, Cited 2007).

5.3 STATISTICAL ANALYSES

Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, USA). Percentages of CD4+IFN-γ+ T cells and absolute counts of IgG or IgA ASCs were examined for normality of distribution using D'Agostino and Pearson omnibus normality test. Non-normally distributed immune responses (T cells and ASCs),

groups were compared using Wilcoxon matched paired t test. End point immune response for each group, or interquartile range (IQR) were reported, and *p* value of less than 0.05 was considered statistically significant.

5.4 RESULTS

5.4.1 Study participant's demographics and clinical presentation

In this cohort study, a total of 20 children were recruited, less than <15 years of age (Median; 8 years range [0.8-13.9]) presenting to QECH with *S.*Typhi bacteraemia. Among these index cases, 10/20 (50%) were female. A total of 21 healthy family members of index cases (Median; 30 years, range [12-40 years]) were also recruited into the study (Table 5-A). Among these healthy family members 20/21 (95.2%) were female. About one third (35%) of index cases had diarrhoea at the acute phase (Table 5-A). A majority of index cases were vomiting (75%) while coughing was rare (10%) at the acute phase. As expected, the family members to index cases were generally asymptomatic (Table 5-A).

Table 5- A: Study participant's demographic and clinical characteristics

Description	Index [a] (%) n=20	Family (%) n=21
Sex	Male 10 (50)	Male (4.7)
Median age in years (range)	8 (0.8-13.9)	30 (12-40)
Median PCV (range)	34 (21-45)	ND [c]
Diarrhoea	7/20 (35)	0/21 (0)
Vomit	15/20 (75)	2/21 (9.5)
Cough	2/20 (10)	1/21 (5)
HIV status	1/20 (5)	4/21 (19)
MUAC 1	15 (10-23)	ND
Currently breastfeeding	2(10)	ND
Median breast feeding discontinued (range)	24 (11-36)	ND

[a]All the 20 index cases recruited had blood culture confirmed *S*. Typhi result

ND refers to not done

5.4.2 High percentage of FliC- and OMP-specific-CD4+IFN-γ+ T cells in healthy family members

Both FliC and OMP have previously been demonstrated to induce protective immunity in mice (Udhayakumar & Muthukkaruppan, 1987, McSorley, *et al.*, 2000, Cunningham, *et al.*, 2004) but their importance in humans as markers of antigen-specific memory T cells has received limited attention.

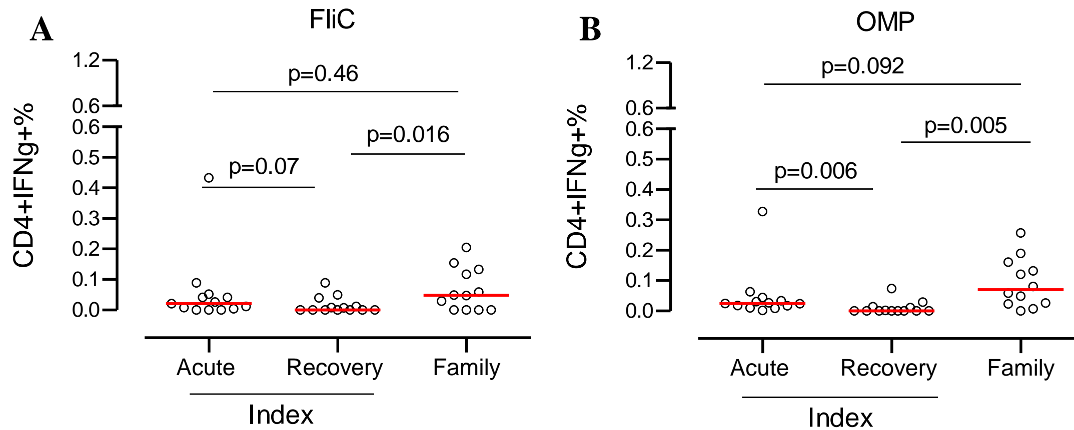

Figure 5. 1: High percentage of specific- CD4+IFN-γ+ T cells in healthy family members

Percentage of CD4+IFN-γ+ T cells in the peripheral blood among children with *Salmonella* bacteraemia; index cases at acute phase (n=12), at 1 month into recovery phase (n=12) and in healthy family members (n=12). Antigen-specific CD4+IFN-γ+ T cells were examined using ICS assay, stimulated with *Salmonella* protein antigens; FliC and OMP as indicated (Figure 5.1A-B) and described in Chapter 2. The bars represent the median. Groups were compared using Wilcoxon matched paired t test and considered significant with *p* value<0.05.

CD4+ T cells producing IFN-γ were quantified in index cases at the acute phase and 1 month in recovery phase and also in healthy family members. There was no statistical difference between FliC-specific CD4+IFN-γ+ T cells in index at acute phase cases and recovery phase (Median acute 0.021, IQR[0-0.046] vs recovery 0, IQR [0-0.026], p=0.078), (Figure 5.1A). OMP-specific CD4+IFN-γ+ T cells were significantly higher in

index cases at acute phase compared to recovery phase (Median 0.025, IQR [0.013-0.039] vs 0, IQR [0-0.012], p=0.006)(Figure 5.1B).

There was no statistical difference in percentage of both FliC-specific and OMP-specific CD4+IFN-γ+ T cells between healthy family members and index cases at acute phase; FliC (Median family 0.048, IQR [0-0.12] vs acute 0.021, IQR [0-0.046], p=0.46) (Figure 5.1A), OMP (Median family 0.07, IQR [0.023-0.15] vs acute 0.025, IQR [0.013-0.039], p=0.092) (Figure 5.1B). The percentage of both FliC-specific and OMP-specific CD4+IFN-γ+ T cells were significantly higher in healthy family members compared to index cases at recovery phase, FliC (Median family 0.048, IQR [0-0.12] vs recovery 0, IQR [0-0.026], p=0.016) (Figure 5.1A), OMP (Median family 0.07, IQR [0.023-0.15] vs recovery 0, IQR [0-0.012], p=0.005) (Figure 5.1B).

5.4.3 LPS-O antigen expression on GMMA elicits robust *Salmonella*-specific CD4+IFN-γ+ T cells

Generalised Modules for Membrane Antigens (GMMA) are thought to be highly immunogenic in mice (MacLennan, 2013). *Salmonella* derived GMMA antigens are generated with or without LPS O antigens. It is not known whether GMMAO+ and GMMA O- would elicit different antigen-specific memory T cell responses *ex vivo*. Firstly, GMMA O- specific CD4+IFN-γ+ T cells were quantified in cohort of index cases and healthy family members. The percentage of GMMA O- specific CD4+IFN-γ+ T cells

were higher during acute phase compared to recovery phase and healthy family members, but these did not reach statistical difference (Median acute 0.05, IQR [0.01-0.10] vs recovery 0.002, IQR [0-0.07], p=0.51), (Median acute 0.05, IQR [0.01-0.10] vs family 0.003, IQR [0-0.08], p=0.62) (Figure 5.2A).

Secondly, GMMA O+ specific CD4+IFN-γ+ T cells were quantified in the same cohort of index cases and healthy family members. GMMA O+ specific CD4+IFN-γ+ T cells were significantly higher in index cases during acute phase compared to recovery phase (Median acute 0.09, IQR [0.03-0.27] vs recovery 0.03, IQR [0-0.12], p=0.018) (Figure 5.2B). Furthermore, it was observed that healthy family members had higher percentage of CD4+IFN-γ+ T cells in GMMA O+ condition compared to GMMA O- condition (Figure 5.2A-B), suggesting that LPS O antigen augmented this T cell immune response. Consequently, there was no statistical difference in percentage of GMMA O+ specific CD4+IFN-γ+ T cells between index cases at acute phase and healthy family members (Median acute 0.09, IQR [0.03-0.27] vs family 0.14, IQR [0.03-0.31], p=0.7) (Figure 5.2B).

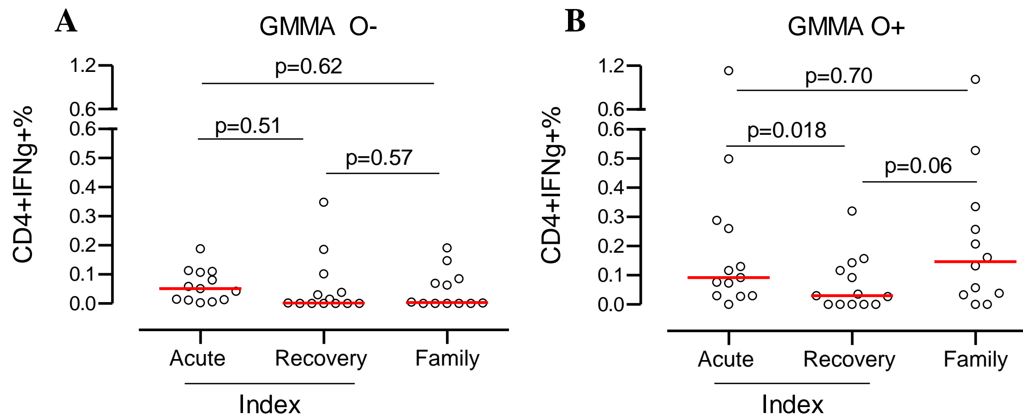

Figure 5. 2: LPS-O antigen expression on GMMA induces highest magnitude of antigen CD4+IFN-γ+ cells

Percentage of CD4+IFN-γ+ T cells in the peripheral blood among children with *Salmonella* bacteraemia; index cases at acute phase (n=12), at 1 month into recovery phase (n=12) and in healthy family members (n=12). Antigen-specific CD4+IFN-γ+ T cells were examined using ICS assay, stimulated with *Salmonella* protein antigens GMMA O- and GMMA O+ as indicated (Figure 5.2A-B) and described in Chapter 2. The bars represent the median. Groups were compared using Wilcoxon matched paired t test and considered significant with *p* value <0.05.

5.4.4 IgG and IgA ASC targeting *Salmonella* derived proteins and LPS O antigens are elevated during acute phase of *Salmonella* bacteraemia

Specific B cells are generated following *Salmonella* infection (Kantele, *et al.*, 2012). B cell immune responses to specific antigen are commonly evaluated using ELISpot assay

(which measures antibody secreting cells i.e. effector B cells [plasma blast] or memory B cells) and ELISA (measure antibodies secreted in plasma or serum) (House*, et al.*, 2008, Kantele*, et al.*, 2012). In this study, *Salmonella*-specific IgG and IgA antibody secreting cells (ASCs) were quantified in the peripheral blood circulation using a B cell ELISpot assay described in Chapter 2, section 2.3.6.4. This study, aimed at measuring transient effector B cells in the peripheral blood without *ex vivo* stimulation and IgA and IgG ASCs only at acute phase and in healthy family members were examined. There was no statistical difference in absolute counts of IgA-ASC targeting; FliC, OMP, GMMA O- and GMMA O+ among index cases were higher compared to healthy family members; FliC (Median index 44, IQR [7-62.5] vs family 13.5, IQR [1.87-37], p=0.62), OMP (Median index 36, IQR [8.5-38] vs family 14.7, IQR [1.62-35.2], p=0.43), GMMA O- (Median index 30.5, IQR [22-39] vs family 10, IQR [1.12-31.2], p=0.58), GMMA O+ (Median index 34.6, IQR [8-51.5] vs family 8.5, IQR [0.37-19.8], p=0.18) (Figure 5.3A). In contrast to IgA absolute counts, absolute counts of IgG-ASC targeting all proteins examined (FliC, OMP, GMMA O- and GMMA O+) were all significantly higher in index cases compared to healthy family members; FliC (Median index 51.5, IQR [24.3-105.8] vs family 10.5, IQR [2.68-17.3], p=0.007), OMP (Median index 32, IQR [14.6-59.1] vs family 8.1, IQR [1.37-20], p=0.015), GMMA O- (Median index 55, IQR [14.5-104.9] vs 5.5, IQR [1.87-26], p=0.015), and GMMA O+ (Median index 47.4, IQR [30.3-111] vs 10, IQR [2.75-17], p=0.007) (Figure 5.3B).

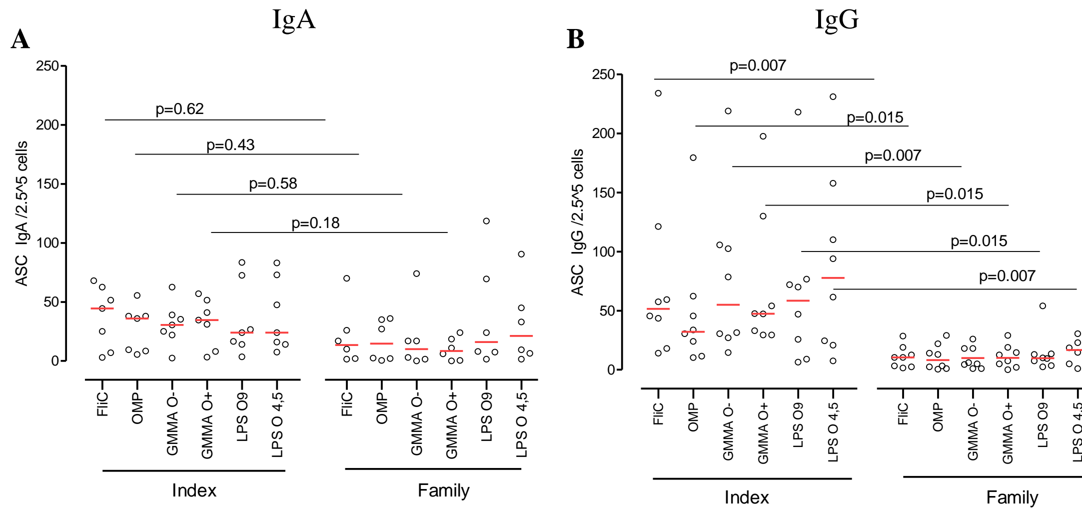

Figure 5. 3: IgG and IgA ASC targeting *Salmonella* derived proteins and LPS O antigens are elevated during acute phase of *Salmonella* bacteraemia

Number of antigen specific antibody secreting cells (ASC) secreting IgA and IgG among children with *Salmonella* bacteraemia (Index IgA [n=7], IgG [n=8]) and family members (IgA [n=6], IgG [n=8]) as indicated (Figure 5.3 A-B). B cell ELISpot plates were coated with *Salmonella* antigens as indicated and described in Chapter 2 (Figure 5.3A-B). The bars represent the median. Groups were compared using Wilcoxon matched paired t test and considered significant with *p* value <0.05.

There was no statistical difference in absolute counts of IgA-ASC targeting LPS O;9 and LPS O;4,5, detected in index cases compared to healthy family members; LPS O;9 (Median index 24, IQR [14-72.5] vs family 16, IQR [6-81.7], p=0.62), LPS O;4,5 (Median index 24, IQR [14-73] vs family 21.2, IQR [5.2-56.3], p=0.62) (Figure 5.3A). In contrast to IgA-ASC specific absolute counts, absolute counts of IgG-ASC targeting

LPS-O;4,5 and IgG-ASC targeting LPS O;9 were significantly higher in index cases at acute phase compared to healthy family members; LPS O;9 (Median index 58.5, IQR [13.2-75.5] vs family 9.8, IQR [4.5-13.3], p=0.015), LPS O; 4,5 (Median index 77.8, IQR [21.6-231] vs family 16.6, IQR [3.87-30.5], p=0.007) (Figure 5.3B).

In this project, only children with *S.* Typhi bacteraemia were recruited. Unexpectedly, the absolute counts of IgA-ASC and IgG-ASC targeting LPS-O;4,5 (derived from *S.* Typhimurium) and IgA and IgG-ASC targeting LPS-O; 9 (derived from *S*. Enteritidis, O9 shared with *S*. Typhi) were similar in index cases (Figure 5.3 A-B). To explore this further, absolute counts of both IgA and IgG ASC targeting LPS O; 9 and LPS O; 4,5 were linked (Figure 5.4A-B) and found that individual responses were similar (Figure 5.4A-B). Furthermore, these absolutes counts correlated strongly; IgA ASC targeting LPS O; 9 and O; 4, 5 (r=0.76, p=0.03) and IgG-ASC targeting LPS O; 9 and O; 4,5 (r=0.86, p=0.01). These findings suggest cross-reactivity occurs in antibody responses to *S*. Typhi and *S*. Typhimurium during infection.

Taken together, these findings show that IgG-ASC targeting *Salmonella* derived LPS O antigens and proteins are highly elevated during acute phase of *Salmonella* bacteraemia compared to IgA-ASC targeting *Salmonella* derived LPS O antigens and proteins. IgG-ASC targeting LPS O antigens specific for *S*. Typhi and *S*. Enteritidis appear to cross-react with *S.* Typhimurium LPS O antigen, suggesting these might have a broader role in controlling *Salmonella* infection caused by various *Salmonella* serovars.

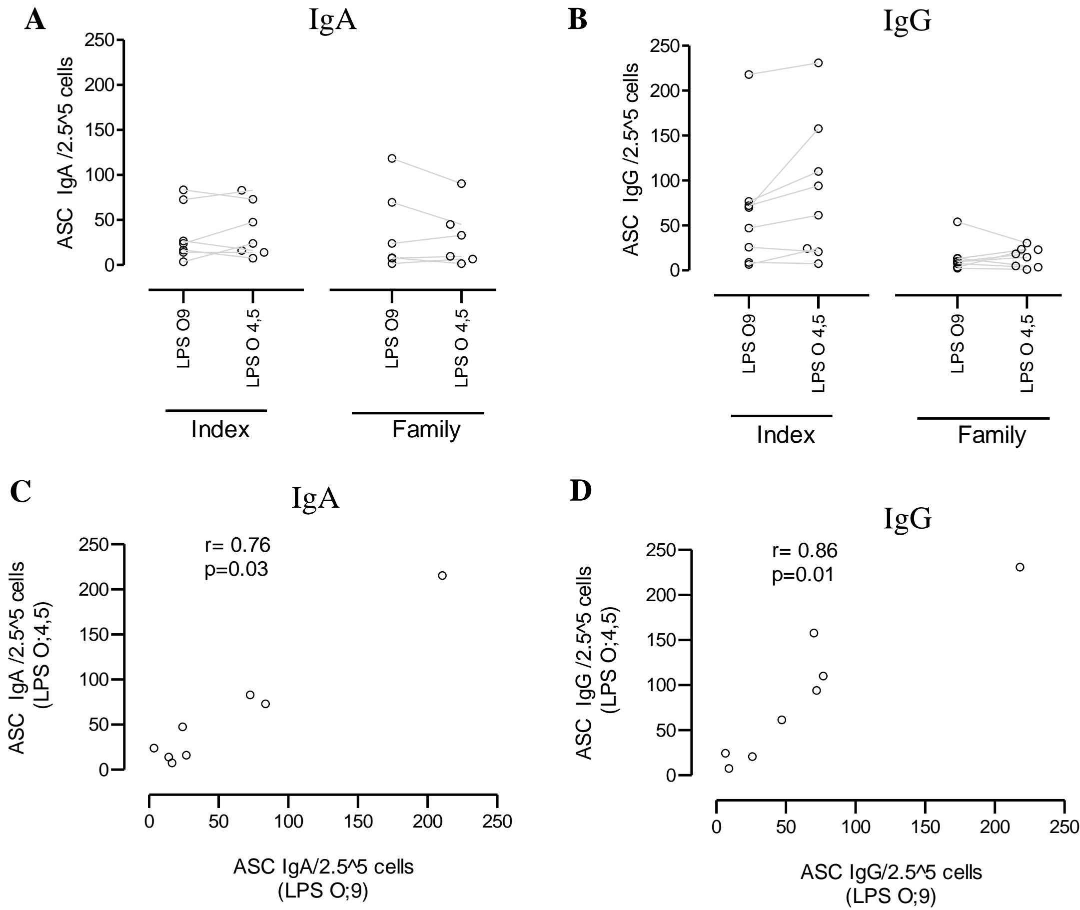

Figure 5. 4: LPS-O antigen-specific ASC in typhoid patient's cross-react with O4-LPS

Number of antigen specific antibody secreting cells (ASC) secreting IgA and IgG among children with *Salmonella* bacteraemia (Index) (Index IgA [n=7], IgG [n=8]) and healthy family members (IgA [n=6], IgG [n=8]). B cell ELISpot plates were coated with *Salmonella* antigens as indicated. Absolute counts were linked (Figure 5.4A-B). Correlation of IgA and IgG targeting LPS O;9 and LPS O; 4,5 are shown (Figure 5.4C-D). r value representing spearman correlation coefficient is shown.

5.5 DISCUSSION

The burden of *Salmonella* bacteraemia caused by NTS and *S*. Typhi is considerably high in SSA (Reddy*, et al.*, 2010, Agnandji*, et al.*, 2011, Breiman*, et al.*, 2012). This requires public health interventions including the development of an effective *Salmonella* vaccine**.** Previous studies in mice, have demonstrated that immunization with *Salmonella* derived proteins such as flagellin and outer membrane protein and polysaccharides such as *S*. Typhimurium LPS O antigen confers protection to virulent *Salmonella* infection (Udhayakumar & Muthukkaruppan, 1987, Simon*, et al.*, 2011).

This study extend these pre-clinical observations in mice by evaluating the importance of *Salmonella* derived FliC, OMP, GMMA O+ and GMMA O- and LPS O antigens as targets of effector B cell immune response and markers of memory CD4+ T cell immunity. This study evaluated the importance of these vaccine candidates *ex vivo* by quantifying *Salmonella*-specific IgG and IgA ASCs and CD4+IFN-γ+ T cells in a cohort of children with *S.* Typhi bacteraemia at acute phase and convalescent phase and in healthy family members. Serum IgG antibody immunity constitutes a key component that controls *Salmonella* bacteraemia in humans (MacLennan*, et al.*, 2008, Pulickal*, et al.*, 2009). Opsonic IgG antibodies targeting LPS O antigen fixes complement to effect serum bactericidal activity to NTS and *S.* Typhi (MacLennan*, et al.*, 2008, Pulickal*, et al.*, 2009). Although, IgG antibodies targeting LPS O antigen are considered to correlate with protection to invasive NTS and *S*. Typhi bacteraemia, correlates of invasive *Salmonella* protection and the targets of protective antibodies are poorly defined. This study, show

that absolute counts of IgG-ASC targeting FliC, OMP, GMMA O- and GMMA O+ were significantly higher in index cases compared to healthy family members. In contrast absolute counts of IgA-ASC targeting FliC, OMP, GMMA O- and GMMA O+ were higher in index cases compared to healthy family members, but this did not reach statistical difference. Previous studies in Bangladesh demonstrated that secretory IgA targeting crude membrane proteins (MP) are elevated at acute phase of *S.* Typhi infected patients compared to healthy controls (Sheikh*, et al.*, 2009, Khanam*, et al.*, 2013). It is possible that the results from this current study and the Bangladesh studies differ due to methods employed (Bangladesh studies measured MP-specific IgA antibodies secreted by B cell lymphocytes in supernatants using the ELISA as the readout while the current study measured IgA expressed by the ASC using direct ELISpot). Even though both the Bangladesh studies and this current study aimed at examining transient B cell responses and *ex vivo* stimulation was not required, it is possible that measuring antibodies in lymphocytes supernatants technique is more sensitive compared to direct ELISpot.

These findings may also reflect minimal contribution of IgA immunity in response to *S.* Typhi infection in the peripheral blood compared to IgG immunity. Lee and colleagues previously showed that immunized mice lacking IgA or the polymeric Ig receptor acquired robust protection against *Salmonella* infection, suggesting that systemic, rather than mucosal, B-cell responses are important for protective immunity to virulent *Salmonella* (Lee*, et al.*). This is also consistent with previous observations that IgA antibodies play a crucial role in primary defence (within the gut mucosa) against enteric

pathogens including *Salmonella* (Griffin & McSorley, 2011) while IgG antibodies are crucial for controlling *Salmonella* infection in systemic organs (MacLennan*, et al.*, 2008). Compartmentalisation of IgA ASCs and IgG ASCs following varying routes of immunisation including oral and systemic have been reported before (Quiding-Jarbrink*, et al.*, 1997), whether this might have important implications in vaccine design and administration (oral or parental vaccine) targeting *Salmonella* is the key question. It remains possible that IgA ASCs are important but not detectable peripherally. Furthermore, *Salmonella*-specific IgG are required to fix complement in immune serum, and this enables killing of complement resistant invasive *Salmonella* strains including *S*. Typhimurium strain D23580 (MacLennan*, et al.*, 2008), while IgA antibodies capacity to activate complement is poor and the role of IgA in complement mediated killing or opsonophagocytosis has been controversial (Michetti*, et al.*, 1992, Roos*, et al.*, 2001, Woof & Kerr, 2006).

Despite the fact that this study only examined children with *S.* Typhi bacteraemia, absolute counts of IgG ASC and IgA ASC targeting LPS-O;4,5 (specific for *S.* Typhimurium) and LPS-O; 9 (Shared by *S*. Enteritidis and *S*. Typhi) were similar in index cases. Furthermore, only absolute counts of IgG ASC targeting LPS-O;4,5 and LPS-O; 9 were significantly higher in index cases compared to healthy family members. Importantly, these findings suggest that secreted IgG antibodies targeting LPS O; 9 induced by *S*. Typhi infection, cross-reacts with *S*. Typhimurium LPS O: 4, 5 antigen *in vivo*. These findings are in agreement with a previous report (Kantele*, et al.*, 2012). They

demonstrated that Ty21a vaccinees and patients with enteric fever had a strong gut-directed cross-reactive plasma blast response against *Salmonella* serovars sharing the two O-antigens (LPS O; 9, 12) (shared by *S.* Typhi and *S*. Enteritidis). They also demonstrated a weaker gut-directed cross-reactive plasma blast response against *Salmonella* serovars sharing one (LPS O;12) with *S*. Typhi including Typhimurium (Kantele*, et al.*, 2012). Whether this cross-reactivity observed *ex vivo* in our currently and the previous study (Kantele*, et al.*, 2012), results in cross-protection to multiple *Salmonella* serovars in vaccinated or naturally infected individuals is the key question. For instance, is it possible that oral Ty21a vaccination for typhoid fever can confer protection to *S.* Typhimurium and *S*. Enteritidis infection? It has previously been shown that attenuated *S*. Typhi vaccines including Ty21a and CVD 909 induces opsonophagocytic functional antibodies in humans that cross-react with *S*. Paratyphi A and *S.* Paratyphi B (Wahid*, et al.*, 2014). These observations need further studies to determine whether or not already licenced vaccines such as oral Ty21a could confer protection to NTS and be used in endemic countries. In SSA, *Salmonella* serovars principally; *S.* Typhi, *S*. Typhimurium and *S*. Enteritidis frequently causes bacteraemia in children and adults (Reddy*, et al.*, 2010, Agnandji*, et al.*, 2011, Breiman*, et al.*, 2012). A vaccine that confers protection to multiple *Salmonella* serovars would be highly desirable.

In recent years protein array and proteomic technology, has been used to determine antibody targets and potential diagnostic markers during invasive *Salmonella* infections

in both mice and humans (Lee*, et al.*, Charles*, et al.*, 2010). Current diagnostic tools including blood culture and Widal test for *Salmonella* bacteraemia have had considerable setbacks (Khanam*, et al.*, 2013). The significant increase in absolute counts of IgG ASCs targeting *Salmonella* derived antigens; FliC, OMP, LPS O;4,5 and LPS O;9 in index cases compared to healthy family members suggests that secreted serum IgG antibodies targeting these antigens might be valuable as diagnostic marker for *S*. Typhi infection in endemic countries. But antigens examined in this current study lack specificity between NTS and *S.* Typhi (For instance antibodies targeting LPS O;9 cross-reacts with LPS O;4,5, crude preparation of OMP derive from *S*. Typhimurim cross-reacts with *S.* Typhimurium). Further studies using both protein array and proteomic technology in a natural infection study design, are required to determine unique antigens for exploration as diagnostic tools.

Both during the early and secondary phases of *Salmonella* infection, CD4+ T cells provide help to monocytes and macrophages to efficiently kill *Salmonella* through the generation of pro-inflammatory cytokines such as IFN-γ and TNF-α (Mastroeni*, et al.*, 1992, Mastroeni, 2002). A study on Vietnamese typhoid patients showed that *S.* Typhi infection induces a molecular signature that is mainly pro-inflammatory in nature during the acute phase (Thompson*, et al.*, 2009). A study on Bangladesh typhoid patients showed that *S.* Typhi specific-CD4+ T cell immune responses as evidenced by IFN-γ production (detected by ELISpot and ICS) and proliferation are increased at acute phase and convalescence phase (14-28 days)(Sheikh*, et al.*, 2011). In this cohort of index cases,

the proportion of *Salmonella*-specific CD4+IFN-γ+ T cells was higher during acute phase and declined at 1 month in convalescence. This decrease in *Salmonella*-specific CD4+IFN-γ+ T cells observed, contradicts previous reports in typhoid patients, which showed that CD4+ T cell immune responses specific *S*. Typhi proteins expressed in vivo during human infection, including StaF, StbB, CsgF, and CsgD, OppA, STY2195, and PagC were generally sustained at convalescent phase (14-28 days) (Sheikh*, et al.*, 2011, Bhuiyan*, et al.*, 2014). The important differences observed between this study and the two Bangladesh studies; protein antigens (Bangladesh study panel of purified proteins expressed during *S*. Typhi infection versus proteins express on the surface of *S*. Typhimurium) used to stimulate T cells and the timing for blood sampling particularly during the convalescence phase (Bangladesh study 14-28 days versus current study at 1 month). Whether this decline in specific CD4+ T cells observed a month into the recovery period reflects changes in memory CD4+ T cells in systemic circulation or lack of establishment of memory CD4+ T cell immunity is not clear.

Perhaps this reduction in specific CD4+ T cell immune responses might result from the decrease in effector memory T cells in the systemic circulation as they differentiate into central memory T cells and migrate to the secondary lymphoid tissues. In this study immunological tool that were used, essentially measures effector CD4+ T cells producing IFN-γ specific for *Salmonella.* A short-term (6 hours stimulation plus CD28/49d co-stimulation) intracellular cytokine staining assay was used. It may have been possible to detect sustained *Salmonella*-specific CD4+ T cells immune response in the recovery

period, if immunological tools that typically measures the central memory CD4+ T cells such as proliferations assay were employed (Lundin*, et al.*, 2002, Salerno-Goncalves*, et al.*, 2002). Several factors including taking antibiotics and *Salmonella* mediated immune modulation might have a role in the decline of specific CD4+ T cells at 1 month into recovery phase. Administration of antibiotics is thought to compromise the development of specific memory CD4+ T cell immunity (Griffin*, et al.*, 2009) through a mechanism that is not very clear. Through the SPI2 T3SS, *Salmonella* has the capacity to escape host defence by compromising the priming of naïve CD4+ T cells in natural infection. *Salmonella* is thought to exploit development of immune memory by reducing its antigen availability, avoiding phagosome-lysosome fusion and down-regulation of T cell receptor (Matsui, 1996, Tobar*, et al.*, 2006, Bueno*, et al.*, 2007, Srinivasan & McSorley, 2007).

It was observed that in healthy family members, the majority of whom were mothers, the percentage of *Salmonella* specific-CD4+IFN-γ+ T cells were generally higher (in *ex vivo* stimulated T cells with FliC, OMP, GMMA O+) compared to index cases at acute phase. These *Salmonella*-specific CD4+IFN-γ+ T cells reflect presence of memory CD4+ T cell mediated immunity in adults in this population. These memory T cells most likely are developed as a result of previous *Salmonella* exposure.

Generalised Modules for Membrane Antigens (GMMA) are thought to be highly immunogenic in mice (MacLennan, 2013, Koeberling*, et al.*, 2014). Interestingly, this study demonstrated that T cells stimulation with GMMA O- resulted in generation of

higher percentage of CD4+IFN-γ+ T cells in index cases during acute phase compared to healthy family members. Furthermore, in healthy family members, T cell stimulation with GMMA O- resulted in generation of lower percentage of CD4+IFN-γ+ T cells compared to T cells stimulation with GMMA O+. The lower percentage of CD4+IFN-γ+ T cells in GMMA O- condition among healthy family members can be attributed to lack of LPS O antigen. Natural LPS antigen has a profound effect on CD4+ T cell immune responses although this has been explored more intensively in mouse models than in humans (McAleer & Vella, 2008, Chilton, *et al.*, 2013). LPS stimulates CD4+ T cell immune responses through toll like receptor 4 (TLR4). This TLR4 signalling cause APCs to up-regulate antigen bearing MHC class II chains and co-stimulatory molecules, both of which push T cell clonal expansion and mount specific effector functions in mice (McAleer & Vella, 2008). How LPS O antigen in GMMA O+ augments generation of *Salmonella*-specific CD4+IFN-γ+ T cell immune responses needs to be explored in humans.

Limitations

The major limitation of this study was that only children with *S*. Typhi bloodstream infection were recruited although it was planned to recruit children presenting with *S*. Typhi and those presenting with NTS. Recruitment of one group of children suffering from NTS and another group presenting with *S*. Typhi bacteraemia would have offered us the opportunity to compare the nature of immunity induced by exposure to either one of

these invasive *Salmonella* serovars. Furthermore, healthy family members that were used as healthy controls were mainly adults as compared to the index cases who were children. It is very likely that immune responses to *Salmonella* differ in different age groups. A group of age-matched children as controls would have been ideal. All proteins antigens used in this study were derived from both wild type and *tolR-wabaP* mutant (lack LPS O antigen) *S*. Typhimurium strain D23580. All protein antigen preparations (FliC, OMP, GMMA O- and GMMA O+) were derived from *S*. Typhimurium. Some proteins antigens derived from *S*. Typhi would have allowed appropriate comparison.

Summary

In children with *S*. Typhi bacteraemia, IgG antibodies targeting *S*. Typhi LPS O; 9 antigens were high during acute infection and appeared to cross-react with *S*. Typhimurium LPS O; 4, 5, suggesting pre-existing *S*. Typhi IgG antibodies specific for LPS O;9 might have a wider role in controlling *S.* Typhimurium infections. Further studies are required to explore the potential of already licensed vaccines in providing protection against nontyphoidal *Salmonella* serovars including *S.* Typhimurium and *S.* Enteritidis. *Salmonella* specific effector memory CD4+ T cells were elevated during the acute phase which decreased one month in convalescence. This might have resulted from effector CD4+ T cell differentiation to central memory CD4+ T cell and subsequently migrate to secondary lymphoid tissues. In contrast to GMMA O-, LPS O antigen on expression GMMA O+ appears to act as an adjuvant and augments the generation of

Salmonella-specific CD4+IFN-γ T cells. Clinical studies are required to study the immunogenicity induced by *Salmonella* derived GMMA O+ and GMMA O- as vaccines.

CHAPTER 6: Ty21a ORAL TYPHOID VACCINE INDUCED IMMUNITY IN THE PERIPHERAL BLOOD AND GUT MUCOSA OF HEALTHY ADULTS

6.1 INTRODUCTION

About 21.7 million people worldwide contract typhoid fever annually (Crump, et al., 2004, Crump & Mintz, 2010). In the developed world, typhoid cases have considerably reduced (Clark, et al., 2010), while in sub-Saharan Africa, Asia and the Indian sub-continent typhoid fever remain endemic mainly due to limited access to clean water, poor sanitation and poor food hygiene (Crump & Mintz, 2010, Breiman, et al., 2012). Clinical management of typhoid fever is problematic in endemic countries, due to the emergence of multi drug resistant (MDR) *S*. Typhi (Rowe, et al., 1997, Crump & Mintz, 2010, Kariuki, et al., 2010, Aggarwal, et al., 2011, Zaki & Karande, 2011). Besides other public health interventions such as improvements in sanitation and food hygiene, vaccination for typhoid was recommended by world health organization (WHO) as a means to control *S*. Typhi infections since 2008 (WHO, 2008).

As described in Chapter 1, oral Ty21a is a vaccine for typhoid fever and was generated in the 1970s following chemical mutagenesis of the wild type strain *S.* Typhi Ty2 and does not express the Vi polysaccharide and *galE* gene (Germanier & Fuer, 1975, Guzman, et al., 2006). Ty21a (Vivotif) contains attenuated *S.* Typhi Ty2 in lyophised form, and each capsule contains no less than $2x10^9$ viable cells (Guzman, et al., 2006). Oral Ty21a vaccine is one of the two currently licensed vaccines for typhoid fever, the

other being the Vi polysaccharide (VCP) parenteral vaccine (Germanier & Fuer, 1975, Guzman, *et al.*, 2006, Khan, *et al.*, 2010). Both are used mainly by travellers from developed world when they visit endemic areas, but may also be used for disease control within endemic areas. As discussed in Chapter 1, clinical trials demonstrated that oral Ty21a vaccine is extremely safe and well tolerated (Olanratmanee, *et al.*, 1992, Guzman, *et al.*, 2006). A meta-analysis of randomised controlled trials demonstrated that Ty21a vaccination has three years cumulative protective efficacy of 51% (Fraser, *et al.*, 2007). Ty21a vaccine induces both antibody and T cell immune responses (Pasetti, *et al.*, 2011). However, the nature of oral Ty21a vaccine-induced antibody and T cell immune responses within the gut mucosa has not been directly examined and neither has this immune response within the gut mucosa been compared to peripheral blood immune responses.

Oral vaccines or enteric pathogens induce immune response within the gut associated lymphoid tissues (GALTs) (Pasetti, *et al.*, 2011). Naïve T cells are primed when they encounter peptide antigen through local dendritic cells (DC) and imprinted within the regional mesenteric lymphoid tissues to express gut mucosa specific trafficking molecules including α4β7 and CCR9. Vitamin A (retinol) metabolite retinoic acid (RA) has been implicated in imprinting gut mucosa homing molecules such as α4β7 and CCR9 on T cells (Johansson-Lindbom, *et al.*, 2003, Iwata, *et al.*, 2004). B cells and T cells expressing integrin α4β7 interacts with mucosal vascular address in cell adhesion molecule 1 (MAdCAM1), which is constitutively expressed on endothelial venules in

intestinal lamina propria (LP) and Peyer's patches (PP) (Berlin*, et al.*, 1993). B cells and T cells express chemokine receptor CCR9 and interact with the gut-associated chemokine ligand CCL25/TECK (Kunkel*, et al.*, 2000, Iwata*, et al.*, 2004). CCL25 has been implicated in trafficking blood lymphocytes into the mucosal epithelial tissues of the gut, respiratory and urogenital tracts (Kunkel*, et al.*, 2003). CCR9+ T cells are not found within the colon, perhaps due to lack of CCL25 expression in the colon. In contrast, CCL28 is expressed by colonic epithelial cells and interacts with CCR10 (Kunkel*, et al.*, 2003). It is therefore thought that CCR9 promotes B cells and T cells homing within the small bowel while CCR10 promotes these lymphocytes homing within the large bowel. CD62L is mainly expressed on naïve lymphocytes and facilitates trafficking into the secondary lymph nodes (SLN) via high endothelial venules (HEV). Homing to the SLN is directed by T cell expression of CD62L which interacts with Gly-CAM-1, CD34 and SGL-1 expressed by endothelial cells, and expression of CCR7, which interacts with CCL19, CCL21 expressed by the lymph nodes (Streeter*, et al.*, 1988). A majority of central memory (CM) T cells express high CD62L and this permits CM T cells to reside within the lymphoid tissues, in contrast effector memory (EM) T cells express low CD62L, and permits these cells to migrate to infected tissues for antigen-specific effector functions (Sallusto*, et al.*, 1999). It has previously been shown, that oral Ty21a vaccination induces a peripheral *S.* Typhi-specific CD4+ and CD8+ T cell immune responses and a majority of these peripheral cells express gut homing markers including α4β7 and CCR9 (Lundin*, et al.*, 2002, Salerno-Goncalves*, et al.*, 2002). However, what

happens at the site of immune induction (the gut mucosa) in humans has not previously been addressed.

This study aimed at modelling natural *Salmonella* infection induced immune responses using oral Ty21a vaccination. A model for natural *Salmonella* infection and immune responses occurring within the gut mucosa and peripheral blood will help to understand natural immunizing events that occurs in young children exposed to gut localised NTS and invasive NTS disease in SSA. Currently, there is no licenced vaccine for NTS, understanding the immunizing events that occur in this model will help to inform the design of future NTS vaccine candidates. This model will also help refine the design of immunological investigations and tools needed to investigate natural *Salmonella* infection and related immunizing events. Thus to model natural *Salmonella* infection induced immune responses, an interventional study was carried out in healthy adults comprising of vaccinated group (received 3 doses of oral Ty21a vaccine) and a control group (unvaccinated) and examined T cell immune responses within the peripheral blood (at 11 days and 18 days) and gut mucosa (at day 18 only) post-vaccine administration.

Hypothesis: *Salmonella* infection through the oral route induces the generation of specific immune responses within the gut mucosa and by using gut mucosa specific homing markers, antigen-specific T cells reflecting mucosal responses can effectively be examined non-invasively in the peripheral blood.

Study objectives:

1. To examine and compare oral Ty21a vaccine induced T cell immune responses in the peripheral blood and gut mucosa.
2. To examine the usefulness of gut mucosa homing markers in evaluating these antigen-specific immune responses.

Endpoints:

1. Measure Ty21a specific CD4+ and CD8+ T cells producing IFN-γ, TNF-α and IL-2 in peripheral blood and gut mucosa of vaccinated and unvaccinated subjects.
2. Measure Ty21a-specific CD4+ and CD8+ T cells co-expressing gut homing markers (β7 and CCR9) producing IFN-γ, TNF-α and IL-2 in peripheral blood of vaccinated and unvaccinated subjects.

6.2 **PARTICPANTS, MATERIALS AND METHODS**

The methods for this chapter have been described in Chapter 2: study designs, materials, and methods, section 2.4.

Briefly, in this prospective interventional study, a total of 17 healthy adults (Median age 21 years and range [18-30]) from the UK were recruited. The participants were randomly assigned to vaccine and control groups. There were a total of 9 vaccinated (female 55% [5/9]) and 8 unvaccinated participants (female 25% [2/8]). Vaccinees were given three oral doses of the vaccine. Ty21a (Vivotif) contains attenuated *S.* Typhi Ty2 in lyophised form, and each capsule contains no less than $2x10^9$ viable cells. One capsule was taken

on each of days 0, 2 and 4, approximately one hour before a meal, with a cold or lukewarm drink, as recommended by the manufacturer and as licensed for use in the UK. Blood samples were collected at day 0 (before vaccine administration), day 11 and day 18 while mucosal specimens were collected only at day 18, at gastroscopy. *Salmonella* specific peripheral and gut mucosal T cell cytokine responses; IFN-γ, IL-2, TNF-α were quantified by the intracellular cytokine staining assay. In these experiments peripheral blood mononuclear cells and gut mucosal mononuclear cells were stimulated with heat-inactivated Ty21a at 0.0632µg/ml (Berna Biotech, Switzerland) and influenza (Influenza virus vaccine) at 0.225µg/ml (Solvay Biologicals B.V., Netherlands) and SEB at 100µg/ml (Sigma Aldrich, USA) were used as control stimulants. Peripheral blood memory T cell subsets (naïve, central and effector) were quantified by immuno-phenotyping using CD45RA, and CCR7 markers and T cells homing were quantified by immuno-phenotyping using β7, CCR9, and CD62L markers.

6.3 **STATISTICAL ANALYSES**

Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, Inc., USA). Percentages of T cells producing cytokines were examined for normality of distribution using D'Agostino and Pearson omnibus normality test. In case of normally distributed immune responses, paired groups were compared using two-tailed parametric t test. While non-normally distributed immune responses, paired groups were compared using Wilcoxon signed ranked test, and non-paired groups were compared

using two tailed nonparametric Mann Whitney test. Mean or median and 95% confidence intervals (CI) or interquartile range (IQR) for each group immune response were reported. A p value of less than 0.05 was considered statistically significant.

6.4 RESULTS

6.4.1 Peripheral blood Ty21a-specific CD4+/CD8+Cytokine+ T cell responses remain un-changed following Ty21a vaccination at day 11 and 18

In this intervention study, *Salmonella*-specific CD4+ and CD8+ T cells producing a combination of cytokines (addition of cells producing single, double and triple combinations of cytokines IFN-γ, TNF-α and IL-2, here termed as Cytokine+) in the peripheral blood were quantified using the intracellular-cytokine staining (ICS) assay as described in Chapter 2. CD4+ T cells producing cytokines, for instance IFN-γ, were gated as shown in figure 6.1.

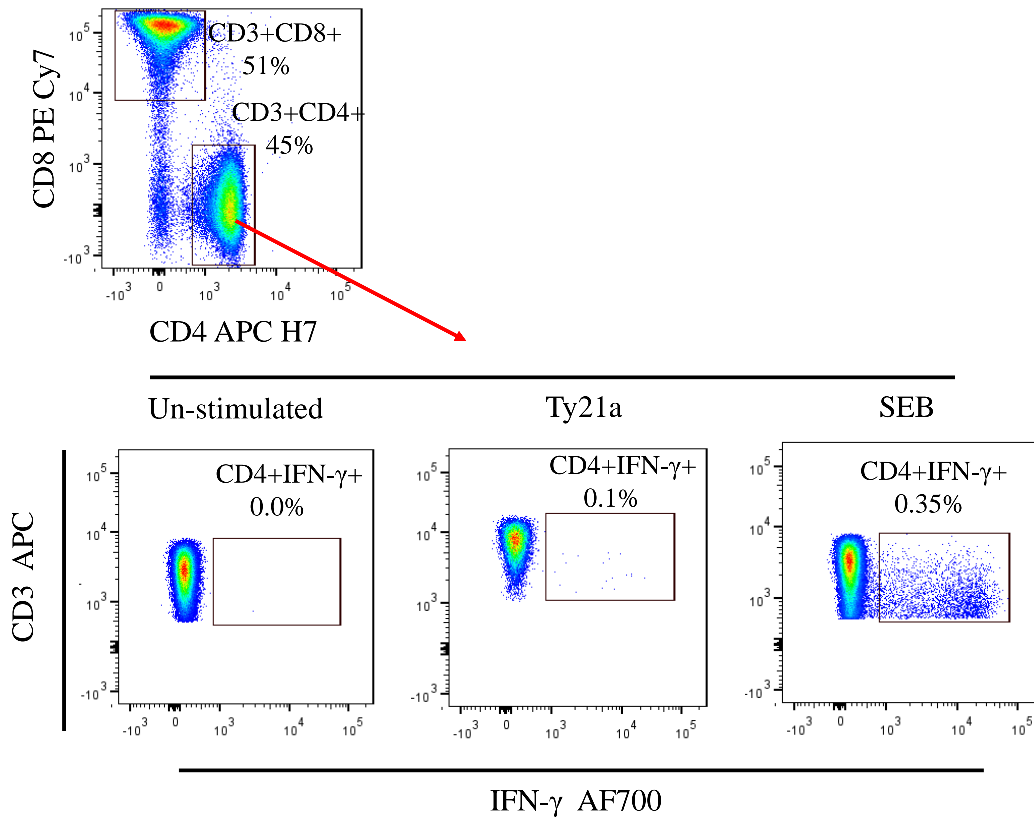

Figure 6. 1 Gating strategy for detection of CD4+ T cells producing cytokines

From the CD4+ T cells gate shown (Figure 6.1 top), CD4+ T cells producing IFN-γ were defined as CD4+IFN-γ+ (Figure 6.1 top and bottom plots). Representative plots of un-stimulated condition and Ty21a and SEB stimulated conditions are shown (Figure 6.1 bottom plots).

There was no evidence of statistical difference in the percentages of Ty21a-specific T cell immune responses at day 0 (baseline) comparing the vaccinated and unvaccinated subjects (CD4+Cytokine+ T cells, median; 0.062%, IQR [0.015-0.077] vs 0.02%, IQR

[0.016-0.029], p=0.31) and (CD8+Cytokine+ T cells, median; 0.05%, IQR [0.002-0.13] vs 0.04%, IQR [0-0.11], p=0.8) (Figure 6.2A and 6.3A).

There was no evidence of statistical difference in the percentages of Ty21a-specific CD4+Cytokine+ T cells and CD8+Cytokine+ T cells at day 11 and 18 in comparison with day 0 in vaccinated subjects (CD4+Cytokine+ T cells, median; 0.062%, IQR [0.015-0.077] at day 0 vs 0.023%, IQR [0.019-0.075] at day 11, p=0.87 and 0.02%, IQR [0.010-0.058] at day 18, p=0.68) and (CD8+Cytokine+ T cells, median; 0.05%, IQR [0.002-0.13] at day 0 vs 0.03%, IQR [0.004-0.065] at day 11, p=0.25 and 0.04%, IQR [0.01-0.098] at day 18, p=0.34) (Figure 6.2A and 6.3A).

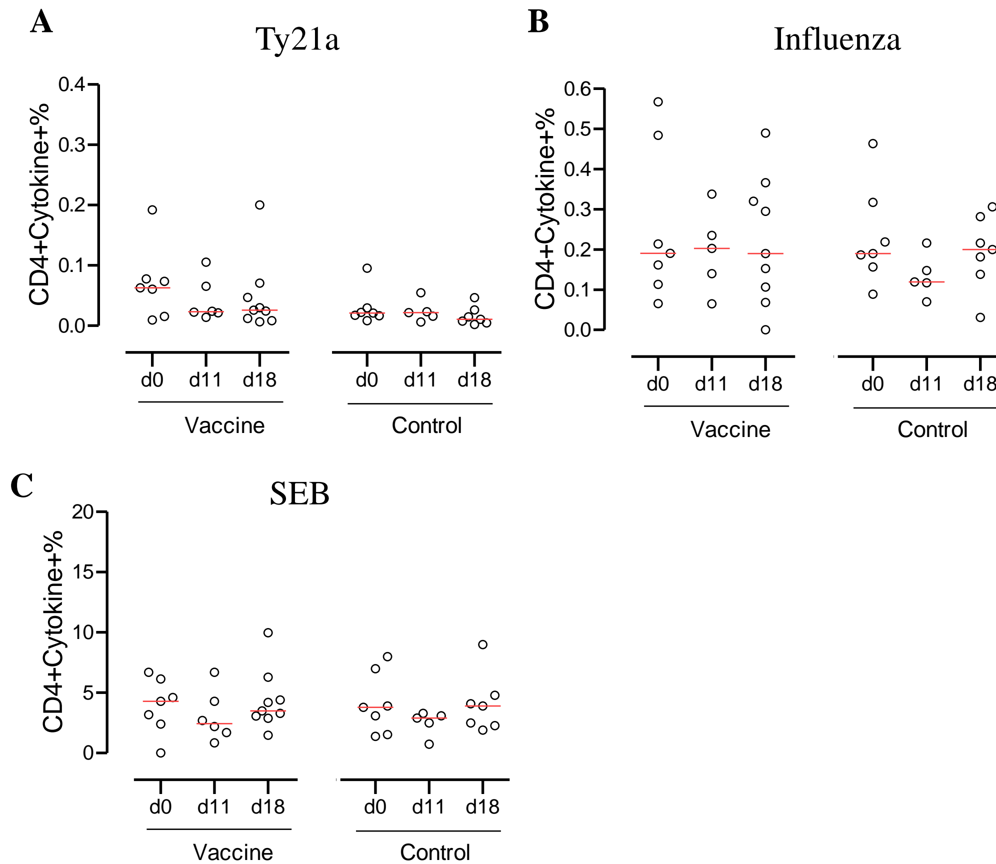

Figure 6. 2: Percentage of Ty21a-specific CD4+Cytokine+ T cells remain unchanged in

Ty21a vaccinated subjects at day 11 and 18

Percentage of CD4+ T cells producing a combination of cytokines (addition of single, double and triple cytokine producers [IFN-γ, TNF-α and IL-2], here termed as Cytokine+) in the peripheral blood among control (unvaccinated) and vaccinated subjects as indicated (6.2A-C). Blood was collected from study participants on day 0, 11 and 18 and PBMCs were stimulated with heat killed Ty21a, Influenza (control) and SEB (control) as indicated. Vaccinated subjects at day 0 (n=7), 11 (n=6), and 18 (n=9).

Unvaccinated subjects at day 0 (n=7), 11 (n=5) and 18 (n=7). The bars represent the median. Group immune responses were compared using Wilcoxon signed ranked test.

There was no statistical difference in the percentages of Ty21a-specific CD4+Cytokine+ T cells and CD8+Cytokine+ T cells at day 11 and 18 in comparison with day 0 in control (unvaccinated) subjects (CD4+Cytokine+ T cells, median; 0.02%, IQR [0.016-0.029] at day 0 vs 0.02%, IQR [0.010-0.039] at day 11, p=1.0 and 0.01% , IQR [0.004-0.026] at day 18, p=0.31) and (CD8+Cytokine+ T cells, median; 0.04%, IQR [0-0.11] at day 0 vs 0.03% , IQR [0.023-0.078] at day 11 , p=0.87 and 0.03%, IQR [0.014-0.04] at day 18 , p=1.0) respectively (Figure 6.2A and 6.3A).

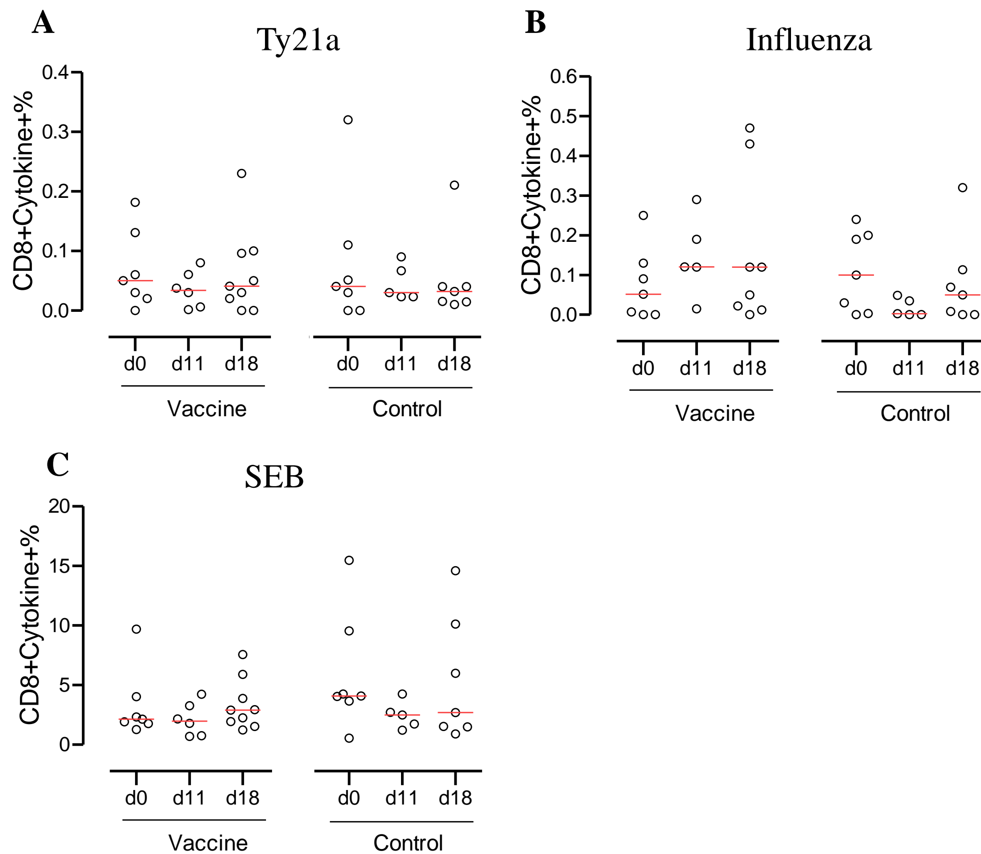

Figure 6. 3: Percentage of Ty21a-specific CD8+Cytokine+ T cells remain unchanged in Ty21a vaccinated subjects at day 11 and 18

Percentage of CD8+ T cells producing a combination of cytokines (addition of single, double and triple cytokine producers [IFN-γ, TNF-α and IL-2], here termed as Cytokine+) in the peripheral blood among control and vaccinated subjects as indicated (6.3A-C). Blood was collected from study participants on day 0, 11 and 18 and PBMCs were stimulated with heat killed Ty21a, Influenza (control) and SEB (control) as indicated. Vaccinated subjects at day 0 (n=7), 11 (n=6), and 18 (n=9). Unvaccinated

subjects at day 0 (n=7), 11 (n=5) and 18 (n=7). The bars represent the median. Group immune responses were compared using Wilcoxon signed ranked test.

In all T cell-stimulated control conditions (influenza and SEB) there was no evidence of statistical difference in percentage of CD4+Cytokine+ and CD8+Cytokine+ T cells at day 11 and 18 in comparison with day 0 in vaccinated subjects; Influenza stimulated condition (CD4+Cytokine+, median; 0.19%, IQR [0.11-0.48] at day 0 vs 0.20%, IQR [0.10-0.28], at day 11, p=0.87 and 0.19%, IQR [0.087- 0.34] at day 18, p=0.93) and SEB stimulated condition (CD4+Cytokine+, median; 4.28%, IQR [2.39-6.14] at day 0 vs 2.44%, IQR [1.48-4.89] at day 11, p=0.62 and 3.49%, IQR [2.97-5.34] at day 18, p=0.81)(Figure 6.2B-C). Influenza stimulated condition (CD8+Cytokine+, median; 0.05%, IQR [0-0.13] at day 0 vs 0.12%, IQR [0.02-0.24], at day 11, p=0.12 and 0.12%, IQR [0.01-0.45] at day 18, p=0.29) and SEB stimulated condition (CD8+Cytokine+, median; 2.14%, IQR [1.77-4.02] at day 0 vs 1.96%, IQR [0.73-3.5] at day 11, p=1.0 and 2.9%, IQR [1.72-4.8] at day 18, p=0.29) (Figure 6.3B-C).

There was no evidence of statistical difference in percentage of CD4+Cytokine+ and CD8+Cytokine+ T cells at day 11 and 18 in comparison with day 0 in control (unvaccinated) subjects; Influenza stimulated condition (CD4+Cytokine+, median; 0.19%, IQR [0.15-0.31] at day 0 vs 0.11%, IQR [0.09-0.18], at day 11, p=0.10 and 0.20%, IQR [0.13-0.28]at day 18, p= 0.21) and in SEB stimulated condition (CD4+Cytokine+, median; 3.79%, IQR [1.52-6.99] at day 0 vs 2.89%, IQR [1.61- 6.99] at day 11 p=0.37 and 3.89%, IQR [2.27-4.79] at day 18, p=0.84) (Figure 6.2B-C). In

Influenza stimulated condition (CD8+Cytokine+, median; 0.10%, IQR [0.002-0.20] at day 0 vs 0.002%, IQR [0-0.04], at day 11, p=0.25 and 0.05%, IQR [0-0.11] at day 18, p= 0.68) and in SEB stimulated condition (CD8+Cytokine+, median; 4.07%, 95% Cl [3.65-9.55] at day 0 vs 2.49%, IQR [1.47-3.48] at day 11 p=0.61 and 2.69%, 95% Cl [1.49-10.12] at day 18, p=0.31) (Figure 6.3B-C). Taken together these findings show that oral Ty21a vaccination did not induce an increase in peripheral CD4+ or CD8+ T cell immune responses to *S*. Typhi, detectable at 11 and 18 days post-vaccination.

6.4.2 Effector memory T cells highly express gut mucosal homing marker β7+ and CCR9+

Recently primed T cells within the gut associated lymphoid tissues (GALTs) migrate to sites of infection for antigen-specific effector functions, and migrate back to the gut mucosa through imprinted homing signature (Kantele*, et al.*, 1999). To investigate this in context of Ty21a vaccination, we quantified T cell subsets expressing gut mucosa homing markers (β7 and CCR9) and peripheral lymph nodes (PLN) homing marker (CD62L) using immunophenotying as described in Chapter 2 and figure 6.4 shows the representative gating strategy.

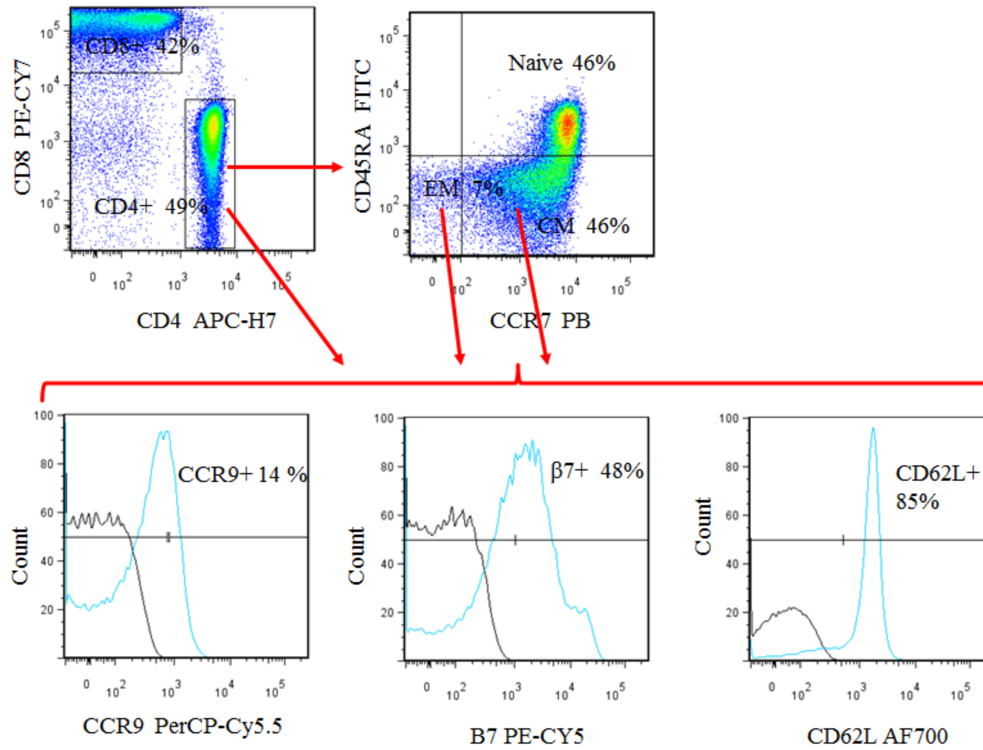

Figure 6. 4: Memory CD4+ T cells and homing marker gating strategy

From the CD4+ T cell gate (Figure 6.4 top left), memory CD4+ T cells were detected by the expression of CD45RA and CCR7. We defined me naïve CD4+ T cells as CD45RA+CCR7+, central memory CD4+ T cell as CD45RA-CCR7+ and effector memory CD4+ T cells as CD45RA-CCR7- (Figure 6.4 top right). CD4+ T cell subsets trafficking properties were characterized by expression of gut homing markers (β7 and CCR9) and peripheral lymphoid tissues homing marker CD62L (Figure 6.4 histogram plots). To determine the positive population FMO (fluorochrome Minus One) condition (FMO condition was stained with the same cocktail of fluorochrome antibodies as the test condition minus the antibody to the homing marker of interest i.e. anti-CCR9 PerCP

Cy 5.5) was used to set up the cut off line (represented by the black line) for the test condition.

There was a higher proportion of total CD8+ T cells expressing β7+ compared to CD4+ T cells (Median; CD4+β7+ 51%, IQR [45-57] vs CD8+β7+ 70%, IQR [57.5-77.5]) (Figure 6.5A). The proportion of CD4+ and CD8 T cells expressing CCR9 and CD62L were similar (Median; CD4+CCR9+ 28%, IQR [23-31] vs CD8+CCR9+ 32%, IQR [24.5-34.5]) and (Median; CD4+62L+ 30%, IQR [20.5-63.5] vs CD8+CD62L+ 29%, IQR [20-44.5]) respectively (Figure 6.5A).

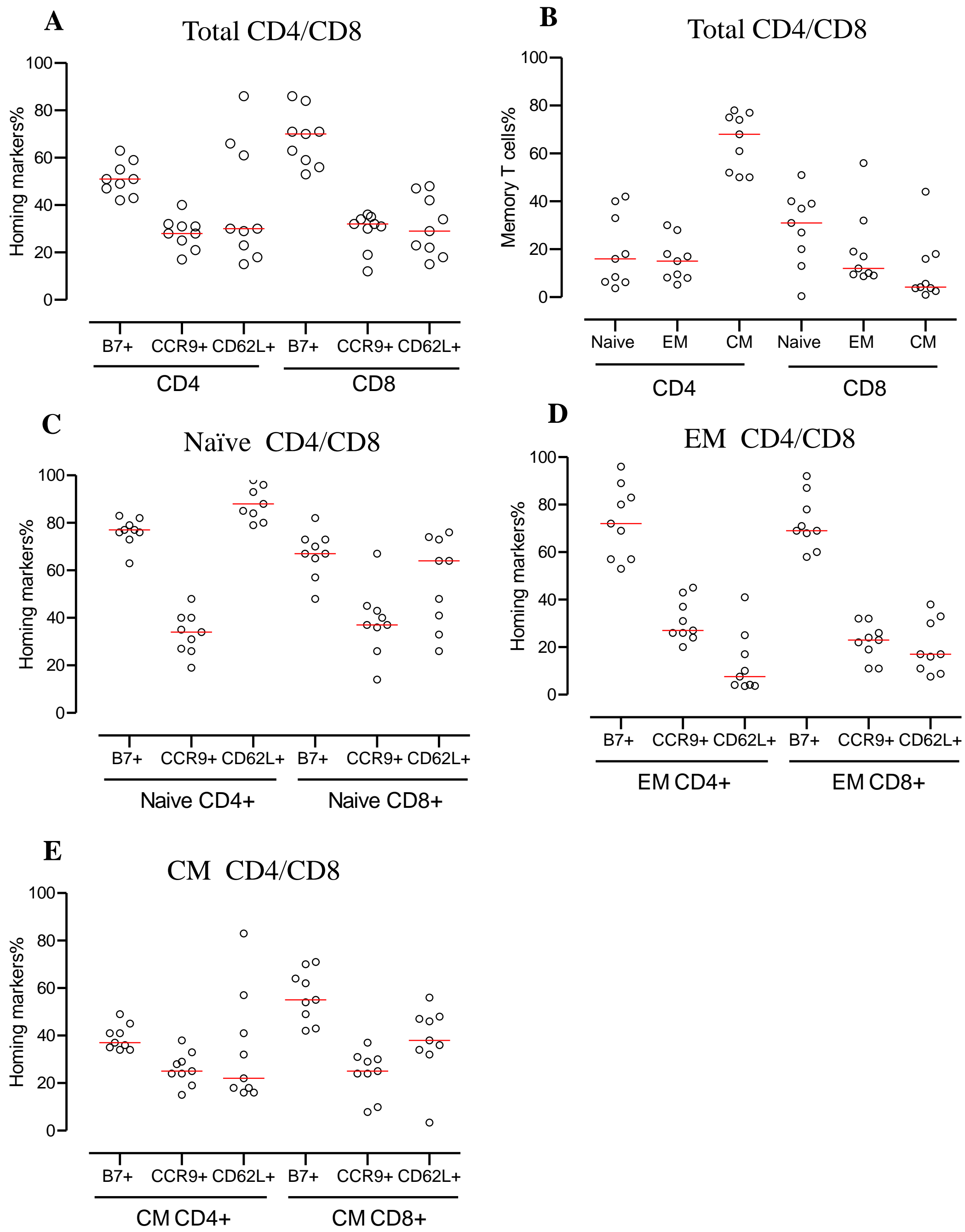

Figure 6. 5: Proportion of T cell subsets expressing homing markers

Percentage of total CD4+ and CD8+T cells in the peripheral blood imprinted for homing to the gut mucosa; CD4+β7+ and CCR9+, and the peripheral lymphoid tissues; CD62L+. (Figure 6.5A). Percentage of the total CD4 or CD8 population that were central memory (CM) CD4+ or CD8+ T cells (defined as CD45RA-CCR7+), effector memory (EM) CD4+ or CD8+ T cells (defined as CD45RA-CCR7-) and naïve CD4+ or CD8+ T cells (defined as CD45RA+CCR7+) (Figure 6.5B). Percentage of naïve (Figure 65.C), EM (Figure 6.5D), CM (Figure 6.5E) CD4+ or EM CD8 T cells in the peripheral blood imprinted for homing to the gut mucosa; CD4+β7+ and CCR9+, and the peripheral lymphoid tissues; CD62L+ (Figure 6.5C-E). Figure 6.5A-E, n=9. The bars represent the median.

Vaccination or pathogen exposure induces the generation of memory T cells and these can ably be distinguished phenotypically (Mackay*, et al.*, 1990, Bunce & Bell, 1997). There was a lower percentage of CM CD8+ T cells compared to CM CD4+ T cells (Median; CM CD4+ 68%, IQR [51-76] vs CM CD8+ 4%, IQR [3.1-44]) (Figure 6.5B). There was a higher percentage of naïve CD8+ T cells compared to naïve CD4+ T cells (Median; Naïve CD4+ 16%, IQR [8.1-23] vs naïve CD8+ 31%, IQR [16.5-39.5]) while the percentage of EM were similar (Median; EM CD4+ 16% , IQR [6.3-36.5] vs EM CD8+ 12%, IQR [9.25-25.5]) (Figure 6.5B).

There were variations in proportions of memory T cells subsets expressing β7+, more naïve CD4+ T cells were expressing β7+ (Median; 77% , IQR [74.5-80.5]) followed by

EM CD4+ (Median; 72% , IQR [57-86]) and less CM CD4+ T cells were expressing β7+ (Median; 37%, IQR [34.5-43]) (Figure 6.5C-E). The proportion of naïve and effector memory CD8+ T cells expressing β7+ were similar (naïve CD8+ median; 67%, IQR [61-73]) and (EM CD8+ median; 69%, IQR [64-82]) and less proportion of CM CD8+ T cells were expressing β7+ (Median; 55%, IQR [46-67]). However, the fluorescent intensity of β7+ was highest in EM CD4+ T cells followed by CM CD4+ T cells and least expressed in naïve CD4+ T cells (Figure 6.6A). The proportion of CD4+ and CD8+ T cells expressing CCR9+ was similar in all CD4+ and CD8 T cell subsets examined (Naïve, EM and CM) (Figure 6.5C-E). The fluorescent intensity of CCR9+ was highest in EM CD4+ T cells while it was similar in CM and naïve CD4+ T cells (Figure 6.6B). As expected, the proportion of CD4+ and CD8+ T cells expression of CD62L was highest in naïve T cells (median; naïve CD4+CD62L+88%, IQR [82-97] and naïve CD8+CD62L+64%, IQR [37-73]) followed by CM T cells (median; CM CD4+CD62L+22%, IQR [17-49] and CM CD8+CD62L+38%, IQR [33-47.5]) and less proportion of EM T cells were expressing CD62L (median; EM CD4+CD62L+ 7.6%, IQR [3.9-21] and EM CD8+CD62L+17%, IQR [9.9-31.5]). The fluorescent intensity of CD62L expression was highest in naïve CD4+ T cells, followed by CM CD4+ T cells and least expressed by EM CD4+ T cells (Figure 6.6C). Taken together these findings indicate that in the peripheral blood, some EM T cells are imprinted within the gut mucosa to highly express gut homing markers β7+ and CCR9+ and this permits these cells to migrate back to the gut mucosal tissues for mounting of effector functions.

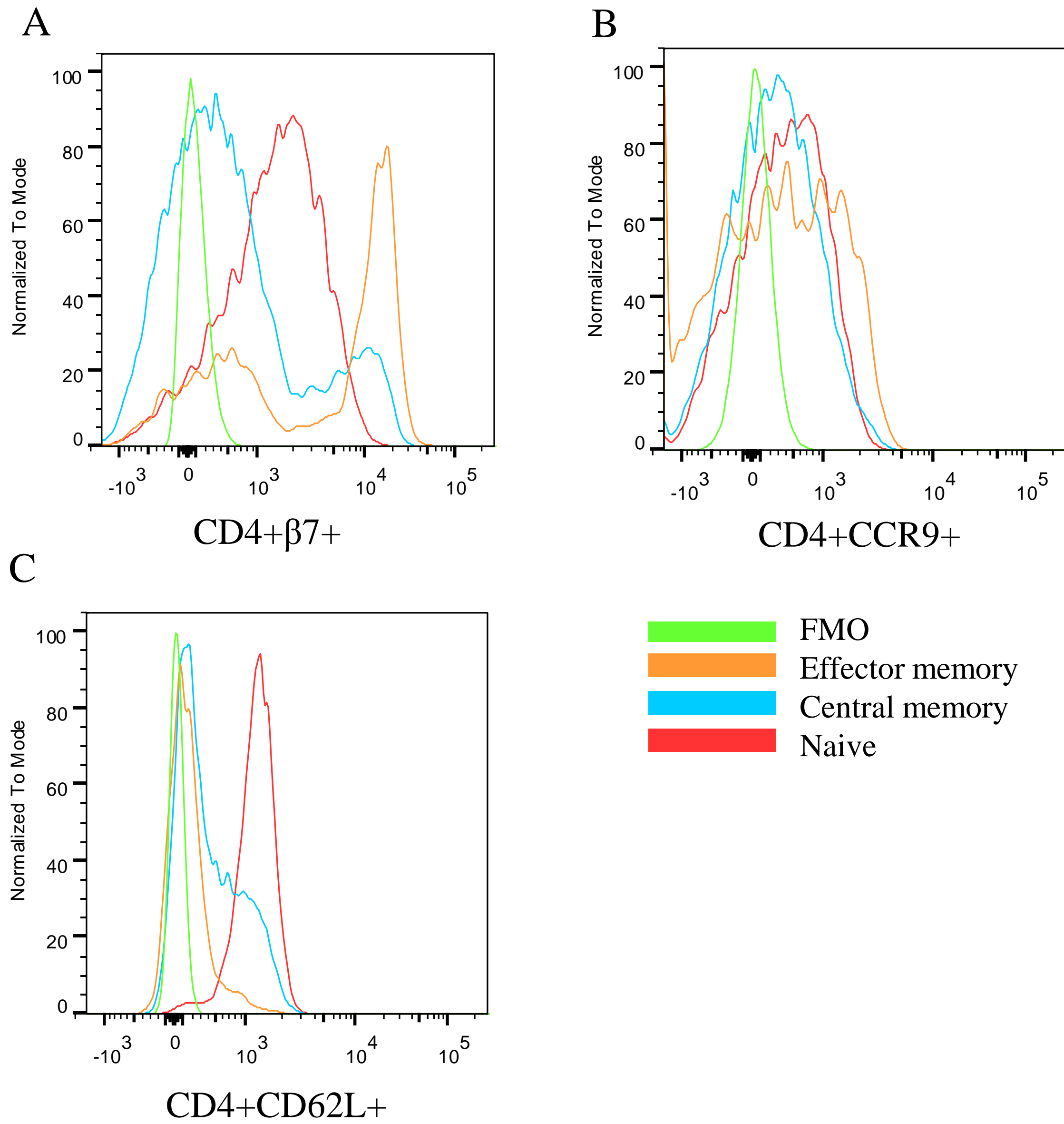

Figure 6. 6: Heterogeneous expression of homing markers on T cell subsets

Representative diagram showing expression fluorescent intensity of homing markers (β7+ CCR9+ and CD62L) on CD4+ T cells subsets (naïve, EM, CM).

6.4.3 Peripheral blood cytokine producing CD4+β7+ and CD8+β7+ T cells remain unaltered following oral Ty21a vaccination

An approach was then taken, thought to be more sensitive in detecting oral vaccine induced T cell immune responses, by quantifying Ty21a-specific T cells expressing gut homing markers (Lundin, *et al.*, 2002). There was no statistical difference in percentage of Ty21a-specific CD4+β7+Cytokine+ and CD8+β7+Cytokine+ T cells in the peripheral blood at day 11, and 18 compared to day 0 in vaccinated subjects (CD4+β7+Cytokine+, median; 0.035%, IQR [0.020-0.08] at day 0 vs 0.031%, IQR [0.021-0.092] at day 11, p=0.75 and 0.032%, IQR [0.001-0.10] at day 18, p=0.56) and (CD8+β7+Cytokine, median; 0.05%, IQR [0.03-0.06] at day 0 vs 0.036%, IQR [0-0.069] at day 11, p=0.25 and 0.055%, IQR [0.02-0.09] at day 18, p=0.68)(Figure 6.7A and B).

Similarly, there was no statistical difference in percentage of Ty21a-specific CD4+CCR9+Cytokine+ and CD8+CCR9+Cytokine+ T cells in the peripheral blood at day 11, and 18 compared to day 0 in vaccinated subjects (CD4+CCR9+Cytokine+, median; 0.022%, IQR [0.06-0.32] at day 0 vs 0.081%, IQR [0.01-0.23] at day 11, p=1.0 and 0.045%, IQR [0.01-0.34] at day 18, p=0.84) and (CD8+CCR9+Cytokine+, median; 0.055%, IQR [0-0.12] at day 0 vs 0.0% , IQR [0-0.04] at day 11, p=0.25 and 0.045%, IQR [0-0.13] at day 18, p=0.62) (Figure 6.7C and D). Taken together these findings show that oral Ty21a vaccination did not induce an increase in gut homing CD4+ and CD8+ T cell immune responses to *S*. Typhi, detectable at 11 and 18 days post-vaccination.

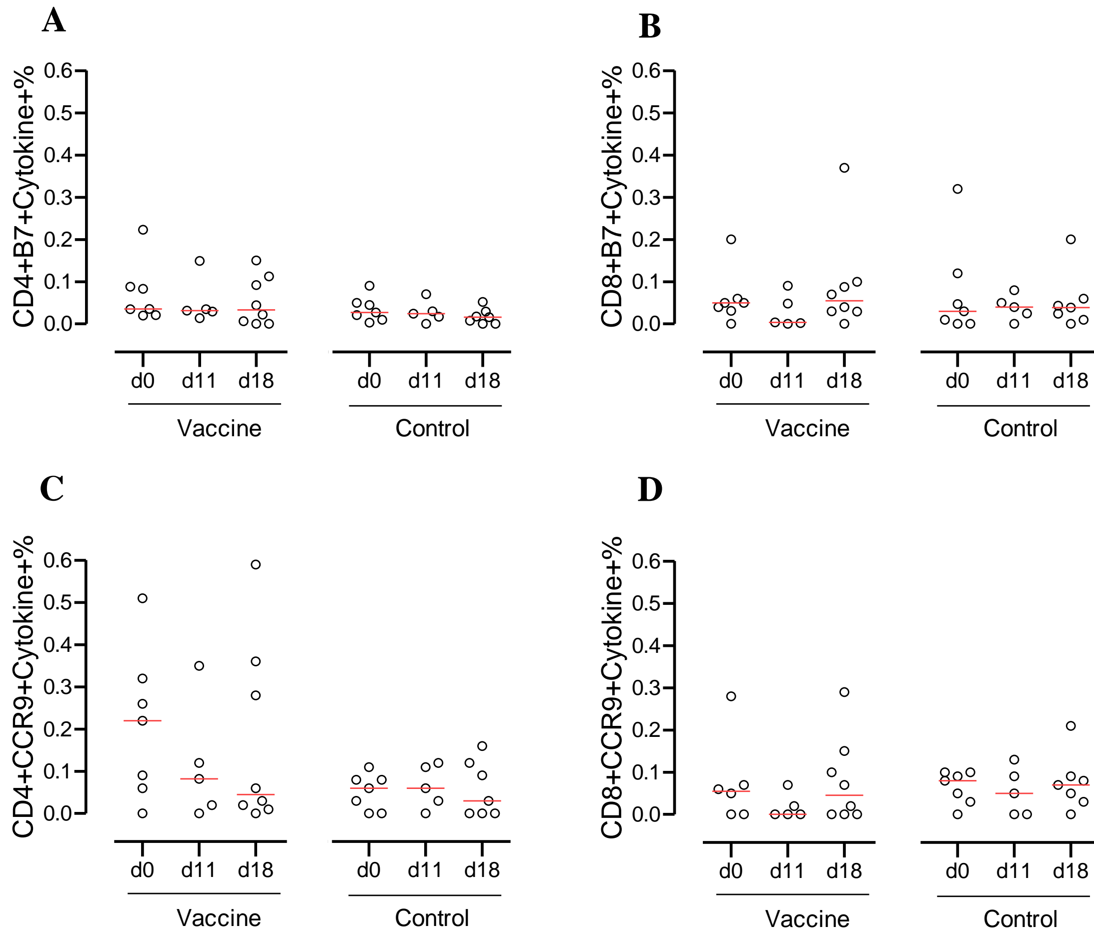

Figure 6. 7: Peripheral blood cytokine producing CD4+β7+ and CD8+β7+ T cells remain unaltered following oral Ty21a vaccination

Percentage of CD4+β7+ and CD8+ β7+ T cells (Figure 6.7A-B) andCD4+CCR9+ and CD8+CCR9+ T cells (Figure 6.7C-D) producing combination of cytokines (addition of single, double and triple cytokine producers[IFN-γ, TNF-α and IL-2], here termed as Cytokine+) in the peripheral blood among vaccinated and unvaccinated subjects as indicated. Blood was collected from study participants on day 0, 11 and 18 and PBMCs were stimulated with heat killed Ty21a. Vaccinated subjects at day 0 (n=7), 11 (n=5), and

18 (n=8). Unvaccinated subjects at day 0 (n=7), 11 (n=5) and 18 (n=7). The bars represent the median. Groups were compared using Wilcoxon signed ranked test.

6.4.4 Gut mucosa Ty21a-specific CD4+Cytokine+ and CD8+Cytokine+ T cells remain unaltered following oral Ty21a vaccination at day 18

Evaluation of oral Ty21a vaccine induced T cell and antibody immune response has mainly relied on peripheral blood immunological measurements. Direct measurement of oral Ty21a induced immune responses in the gut-mucosa tissue has not previously been undertaken. It was hypothesised that direct measurement of oral Ty21a induced immune responses within the gut mucosal tissue and might provide more insight than peripheral blood measurements because it is possible that some immunizing events occurring within the gut mucosa could be missed by using peripheral blood measurements only. Day 18 was chosen in order to allow time for priming and re-homing events to have occurred. Twelve to 15, D2-D3 duodenal single-bite cold biopsies at 20-25cm insertion were collected using Boston Scientific large capacity 'jumbo' forceps (Boston Scientific, USA) which passed through a standard 2.8mm endoscopic biopsy channel. Gut mucosal biopsies were collected from all study subjects on day 18 post vaccination. As described in chapter 2, mucosal mononuclear cells (MMNCs) isolated were examined for Ty21a specific T cell cytokine immune responses. Cytokine responses were examined on viable MMMCs using Vivid dye and no less than 90% lymphocytes viability was achieved. Contrary to this study hypothesis, there was no statistical difference in percentage of Ty21a-specific CD4+Cytokine+ and CD8+Cytokine+ T cells in vaccinated and

unvaccinated subjects in the gut mucosa at day 18 (CD4+Cytokine+, median; control 0.14%, IQR [0-0.41] vs vaccine 0.08% , IQR [0.002-0.18], p=0.44) and (CD8+Cytokine+, median; control 0.039% , IQR [0.02-0.21] vs vaccine 0.022%, IQR [0-0.08], p=0.39)(Figure 6.8A). It was observed that both influenza and SEB stimulated conditions in the gut mucosal performed as expected and immune responses were similar in vaccinated and unvaccinated subjects (Figure 6.8B-C).

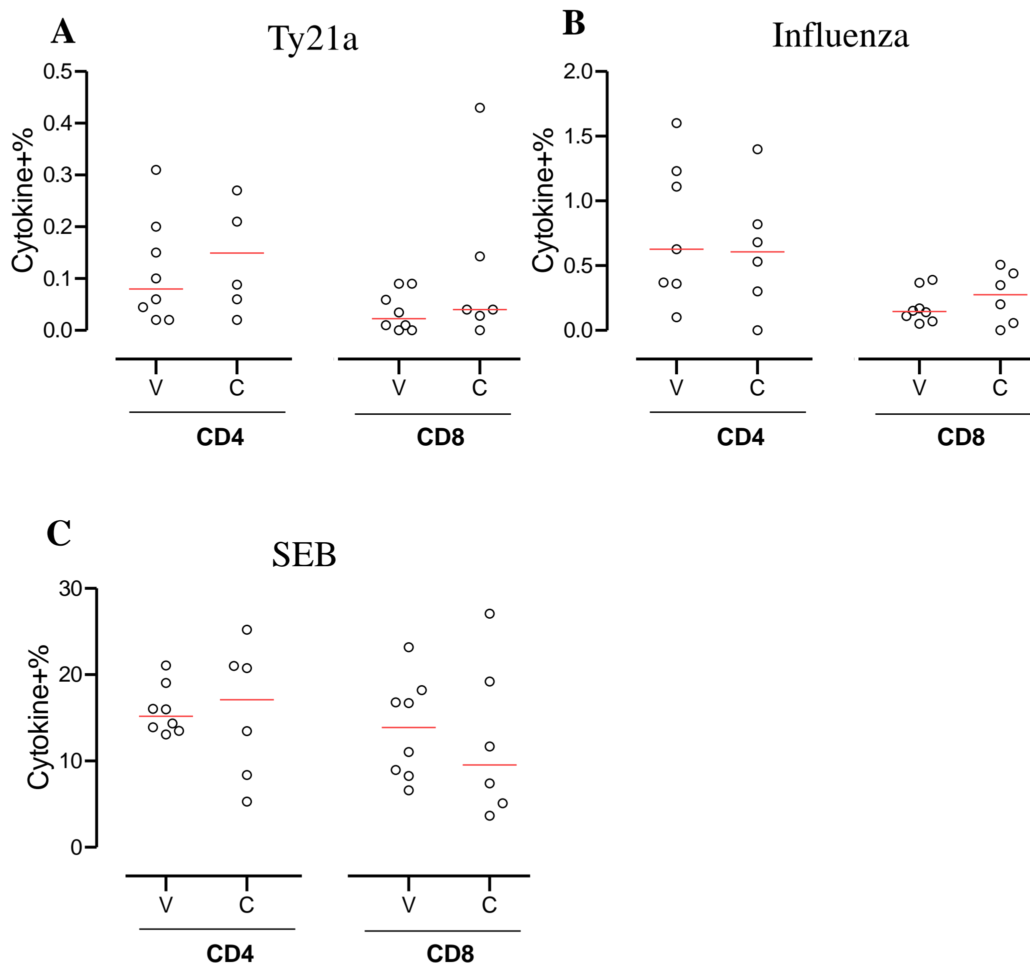

Figure 6. 8: Gut mucosa cytokine producing CD4+ and CD8+ T cells remain unaltered following oral Ty21a vaccination

Percentage of CD8+ T cells producing a combination of cytokines (addition of single, double and triple cytokine producers [IFN-γ, TNF-α and IL-2], here termed as Cytokine+) in the duodenum (gut mucosa) among control (represented by C) and vaccinated (represented by V) subjects as indicated (6.8A-C). Gut mucosa biopsy was collected from study participants on day 18 after vaccination, and MMNCs were stimulated with heat killed Ty21a, Influenza (control) and SEB (control) as indicated. Vaccinated subjects CD4+Cytokine+ Ty21a (n=8), Influenza (n=7), and SEB (n=8). Vaccinated subjects CD8+Cytokine+ Ty21a (n=8), Influenza (n=8), and SEB (n=8). Unvaccinated subjects CD4+Cytokine+ Ty21a (n=5), Influenza (n=6), and SEB (n=6). Unvaccinated subjects CD8+Cytokine+ Ty21a (n=6), Influenza (n=6), and SEB (n=6). The bars represent the median. Groups were compared using two-tailed nonparametric Mann Whitney test.

Taken together these findings suggest that oral Ty21a vaccination does not induce an increase in CD4+ and CD8+ T cell immune responses to *S*. Typhi detectable in the gut mucosa and peripheral blood compartments among vaccinated subjects at day 18.

6.5 **DISCUSSION**

Currently, assessment of T cell and antibody immune responses induced by oral vaccines such as Ty21a or natural *Salmonella* infection relies on indirect measurements of immune

responses, mainly within the peripheral blood. Commonly used immunological tools for measurement of T cell immune responses include; intracellular-cytokine staining, proliferation, ELISpot (Lundin*, et al.*, 2002, Salerno-Goncalves*, et al.*, 2002) while commonly used tools for measurement of antibody production include ELISA and ELISpot (Toapanta*, et al.*, 2014). Peripheral blood assessment of antigen-specific immune responses induced by oral vaccination or natural *Salmonella* infection might lead to under-appreciation of immunological events, particularly immunizing events occurring within the gut mucosa. In this interventional study comprising Ty21a vaccinees and unvaccinated subjects natural *Salmonella* infection was modelled and directly quantified Ty21a-specific CD4+Cytokine+ and CD8+Cytokine+ T cells within the gut mucosa and indirectly quantified similar T cells in the peripheral blood simultaneously. Unexpectedly, in the vaccinated group, peripheral blood Ty21a-specific CD4+Cytokine+ and CD8+Cytokine+ T cells were not statistically different at day 11 and day 18 post-vaccination compared to day 0. This study findings are not in keeping with previous reports (Salerno-Goncalves*, et al.*, 2002, Salerno-Goncalves*, et al.*, 2005).

In this study, the proportion of memory T cell subsets were characterised and showed that a effector memory CD4+ and CD8+ T cells highly express gut homing marker β7+ and CCR9. Observations were made that large proportion of naïve T cells also express gut homing markers β7+ and CCR9 and this was in keeping with previously studies (Cose, 2007). Furthermore, the fluorescent intensity of β7+ and CCR9+ expression of naïve T cells was lower compared to EM T cells. This may not mean that naïve and memory T

cells have the same migration pathways to the non-lymphoid tissues (sites of infection) since migration is dictated by the density of homing receptor, in this case memory T cells are likely to preferentially home to gut mucosal tissue over the naïve T cells (Cose, 2007). Previously, it has been demonstrated that a majority of Ty21a-specific CD4+ and CD8+ T cells are imprinted for gut mucosa homing, as evidenced by the expression of β7 (Lundin*, et al.*, 2002), suggesting that evaluation of gut homing T cells might improve the sensitivity of detecting antigen-specific T cells. Unexpectedly, using the gut homing marker β7 to identify Ty21a-specific cytokine-producing T cells, did not help to delineate immunological changes that might have occurred in vaccinated compared to non-vaccinated subjects. There were no statistical differences in percentage of Ty21a specific-CD4+β7+Cytokine+ and CD8+β7+Cytokine+ T cells at day 11 and day 18 compared to day 0 in vaccinated group. These findings are not consistent with previous reports (Lundin*, et al.*, 2002). There were also no statistical differences in percentage of Ty21a specific-CD4+CCR9+Cytokine+ and CD8+CCR9+Cytokine+ T cells at day 11 and day 18 compared to day 0 in the vaccinated group. Furthermore, even with direct assessment of gut mucosa immunological measurements, there was no evidence that oral Ty21a vaccination induced an increase in Ty21a-specific CD4+Cytokine+ and CD8+Cytokine+ T cell responses in vaccinated subjects compared to non-vaccinated subjects at day 18 post-vaccination. Why this study found no evidence of increased T cell responses following vaccination with Ty21a as previously described elsewhere (Salerno-Goncalves*, et al.*, 2002, Salerno-Goncalves*, et al.*, 2005) is the key question. The control stimulated condition's (Influenza and SEB) induced CD4+Cytokine+ and CD8+Cytokine+ T cells

responses performed as expected, so there was no evidence that cell isolation and culture conditions compromised the ability of cells to produce cytokine responses. In this study heat-inactivated whole-bacterial Ty21a stimulant was used in ICS experiments, it is possible that our stimulant could not induce robust stimulation comparable to live stimulants used by other groups (Thatte*, et al.*, 1993). It has been demonstrated before that whole-cell killed bacteria are less efficient stimulant compared to live whole cell bacteria (Thatte*, et al.*, 1993, Nyirenda*, et al.*, 2010). In this study, co-stimulatory antibody such as CD28/49d were not used as before (Nyirenda*, et al.*, 2010), and this might have increased the ability to detect different responses. Previous studies have shown that T cell responses to oral vaccination vary as to when they are evaluated in the peripheral blood. Antigen-specific T cell responses such as CD4+ T helper 1 cytokines and proliferation peaks around 7 to 14 days (Lundin*, et al.*, 2002). In this study, timing for assessment of antigen-specific responses at day 11 post-vaccination in our intervention study was in keeping previous work. Whether assessment at 18 day post-vaccination might have been late to detect immune response is not clear. Taken together these findings and previous reports, it is inconclusive whether or not Ty21a vaccination does induce the generation of Ty21a specific CD4+ and CD8+ T cells both within the gut mucosa and peripheral blood. To effectively trouble shoot this lack of T cell immune responses following Ty21a vaccination in future investigations a panel of antigen specific stimulants will be evaluated to identify the strong stimulant. These experiments will include, evaluation of antibody responses *S*. Typhi using ELISA on existing serum samples from this cohort, comparing live versus heat killed specific antigen, purified

antigens versus crude preparations and also determining the optimum stimulation concentration of these antigens. A number of immunological tools for assessment of antigen specific T cell immune responses will be compared including ICS short term stimulation versus long terms stimulation, ICS versus ELISpot and also proliferation assays. Optimum time for assessment of T cells responses following Ty21a vaccination will also be evaluated in time course experiments, whereby blood specimens from vaccinees collected at varying days following vaccination will be compared. Only one vaccine batch will be used and the vaccine concentration of live attenuated *S*. Typhi will be verified.

Currently, there is no human vaccine for NTS bacteraemia which is an important public health problem in SSA. Young children and HIV infected individuals are more susceptible to NTS bacteraemia and non-invasive techniques are required to evaluate immunity following natural or vaccine exposure. This model is important for understanding immunizing events that occur within the gut following natural NTS infection and evaluates immunological tools that could be used to assess immunity occurring within the gut and peripheral blood. Gut homing makers including CCR9 and β7 can potentially be used non-invasively to evaluate T cell immune responses in children and HIV infected individuals naturally infected with NTS. Better understanding of natural *Salmonella* immunizing events within the gut will help in designing an effective vaccine for NTS that is urgently required.

Summary

This study has demonstrated that in the peripheral blood, effector memory T cells highly express β7, suggesting these T cells migrates back to the gut mucosal for induction of antigen-specific immunity. In this study, using gut mucosal measurements and peripheral blood measurements including use of gut homing markers, failed to detect a significant increase in the percentage of Ty21a specific CD4+Cytokine+ and CD8+Cytokine+ T cell immune responses in blood at day 11 and 18 in vaccinees, and Ty21a specific CD4+Cytokine+ and CD8+Cytokine+ T cell immune responses at day 18 in gut mucosa in vaccinees compared to control subjects. Better immunological methods and appropriate study design could help to answer the question whether or not orally induced antibody and T cell immune responses occurring within the gut mucosa can be measured directly and also whether these responses can be evaluated non-invasively in the peripheral blood. Both young children and HIV infected individuals, who are more susceptible invasive NTS infection in SSA, will require use of non-invasive immunological techniques such as gut homing markers (CCR9 and β7) to evaluate immunizing events occurring within the gut mucosa.

CHAPTER 7: GENERAL DISCUSSION

7.1 Introduction

The research studies presented in this thesis were designed to investigate natural acquisition of antibody and T cell immunity to *Salmonella* in healthy and *Salmonella*-infected Malawian children. In healthy adults, in the UK, acquisition of live oral typhoid vaccine-induced T cell immunity was investigated to model immunizing events occurring with the gut mucosa following natural *Salmonella* infection. A range of immunological tools were used in these investigations including the intra-cellular cytokine staining assay (ICS), serum bactericidal activity (SBA) assay, ELISA and ELISpot. Microbiological tools including standard stool culture and real-time PCR for detection of *Salmonella* were also used.

These tools were implemented in several cross-sectional and longitudinal studies. Firstly, the development of antibody and T cell immune responses to *S*. Typhimurium in healthy Malawian children aged 0-60 months from Blantyre urban were investigated in a cross-sectional study. Secondly, the relationship between *Salmonella* exposure events within the gastrointestinal tract (GIT) or oropharynx and development of *Salmonella*-specific serum immunity in healthy Malawian children aged 6-18 months from Blantyre urban was investigated in a longitudinal study. Thirdly, the immunological importance of vaccine candidates for *Salmonella* disease were investigated in a cohort of children below 15 years, presenting to hospital with *Salmonella* bacteraemia at acute and convalescent phases and healthy family members. These vaccine candidates are, currently in pre-

clinical phase, including *Salmonella* derived-surface proteins (FliC, OMP and GMMA) and lipopolysaccharide (LPS) O antigens. Lastly, antibody and T cell immune responses induced by oral Ty21a typhoid vaccination were investigated within the gut mucosa and peripheral blood compartments in vaccinated and unvaccinated healthy adults from the UK to prospectively model immunizing events occurring with the gut mucosa following natural *Salmonella* infection.

This discussion focusses on lessons learnt and the implications of the research findings. Special emphasis is given to the development of antibody and T cell immunity to *Salmonella* resulting from natural *Salmonella* infection. Future investigations originating from this PhD study are then discussed.

7.2 Review of important findings from thesis

7.2.1 Development of adaptive immunity to nontyphoidal *Salmonella* in Malawian children

The first research study was aimed at characterizing the development of antibody and T cell immunity to *S*. Typhimurium in healthy Malawian children aged 0-60 months. At the time this research study idea was conceived, it was known that complement-fixing IgG and IgM antibodies to *Salmonella* play an important role in controlling *S*. Typhimurium bacteraemia. Both IgG and IgM antibody immunity to *S*. Typhimurium develops with age (MacLennan, *et al.*, 2008). How CD4+ T cell immunity to *S*. Typhimurium develops in

Malawian children was not known. It was hypothesized that CD4+ T cell immunity to *S*. Typhimurium develops in parallel with antibody immunity in the first 2 years of life.

Chapter 3 demonstrates that *S.* Typhimurium-specific CD4+ T cell immunity is detected early in life and reaches a peak at 14 months of age, suggesting early exposure to *S*. Typhimurium or cross-reactive bacteria antigens. In early life (≤ 14 months) *S*. Typhimurium-specific cytokine producing CD4+ T cells correlated positively with the anti-*S*. Typhimurium OMP and FliC IgG antibody titres, thus re-affirming that early exposure to *Salmonella* might be a requirement in the development of this immunity.

Salmonella infection primarily results from the ingestion of contaminated food, water and fomites (Kariuki*, et al.*, 2006). The pattern of *Salmonella* exposure events within the gastrointestinal tract (GIT) in Malawian children had not been investigated. *Salmonella* exposure within the GIT is thought to be driven by several factors such as breast feeding and supplementary feeding practices in childhood, environmental sanitation, food hygiene, dosage of the infecting *Salmonella* bacteria and the frequency of *Salmonella* exposure events (France*, et al.*, 1980, Kariuki*, et al.*, 2006, Bollaerts*, et al.*, 2008, Farooqui*, et al.*, 2009). These factors have not been investigated in Malawian children, in relation to natural *Salmonella*. Chapter 3 also demonstrates that *S.* Typhimurium specific-CD4+ T cells were lower in older children (24-60 months) in contrast to the younger age group (≤ 14 months) suggesting that *Salmonella* exposure events were low in the older children. Furthermore the decline in *Salmonella* exposure events might indicate differentiation of circulating specific-effector memory CD4+ T cells to central memory CD4+ T cells which are less readily detectable in the systemic circulation.

In agreement with what had previously been shown in Malawian children (MacLennan*, et al.*, 2008), Chapter 3 demonstrates that acquisition of serum bactericidal activity (SBA) to *S.* Typhimurium occurs with age. Development of the child's SBA to *S*. Typhimurium is delayed, and appears to start developing after the decline (in the first 8 months of life) of passively acquired maternal SBA. SBA to *S.* Typhimurium gradually develops from 8 months of age and peaks at 36 months of age in children aged 0-60 months. Importantly, *S*. Typhimurium-specific SBA correlated with specific IgG antibody titres targeting LPS O;4,5 antigen suggesting the importance of this antigen to the development of this immunity compared to other protein antigens, including *S*. Typhimurium derived OMP and FliC which were also examined in this study. In contrast to the observation made in an earlier study where serum from HIV infected adults had impaired SBA to *S*. Typhimurium (MacLennan*, et al.*, 2010), Chapter 3, shows that older children had robust SBA to *S*. Typhimurium. Lack of SBA to *S*. Typhimurium in HIV infected individuals has been attributed to high concentration of IgG antibodies targeting *S.* Typhimurium LPS O antigen and these appear to block bactericidal antibodies targeting *S.* Typhimurium OMP antigen (MacLennan*, et al.*, 2010, Trebicka*, et al.*, 2013).

The variation in T cell and SBA immunity to *S*. Typhimurium, in relation to frequency of *S*. Typhimurium bacteraemia in the same population and age group were evaluated. As demonstrated before (MacLennan*, et al.*, 2008), Chapter 3 demonstrates that *S.* Typhimurium bacteraemia in under-five children most frequently occurs in the first 2 years of life (80%) and the median age was 13 months. Observations were made that even though *S.* Typhimurium-specific CD4+Cytokine+ T cells were acquired early in

life, T cells alone appeared not to sufficiently control *S.* Typhimurium bacteraemia but sequential acquisition of SBA to *S.* Typhimurium was associated with a decline of bacteraemia cases. These findings are in agreement with the proposed hypothesis on how mice control systemic salmonellosis (Mastroeni, 2002), which suggests that high level of immune resistance to *Salmonella* infection is established in a step-wise fashion and involves both innate and adaptive immunity.

It is likely that the early development of T cell mediated response specific for *S.* Typhimurium protein antigens, and subsequent cognate interactions with B cells, promote antibody production against *S.* Typhimurium antigens, class-switching, affinity maturation and memory formation (Mohr, *et al.*, 2010). In view of the potentially complex nature of *Salmonella* antigen presentation during natural infection, it is possible that CD4+ T cells promote B cell immunity through a bystander (hapten-carrier) effect to B cells specific for *S*. Typhimurium-LPS. LPS alone is a T cell independent type 2 antigen, but when taken up by antigen presenting cells (APC) in combination with *S.* Typhimurium proteins, it has the potential to elicit the same response that polysaccharide-conjugate vaccines trigger and generate isotype-switched memory B-cell immunity (Mitchison, 1971).

Given the burden of invasive NTS disease in SSA (Reddy, *et al.*, 2010, Agnandji, *et al.*, 2011), a vaccine is urgently required. *S*. Typhimurium LPS O-antigen has considerable potential as a vaccine target and there are currently several groups developing conjugate

vaccines for this purpose to overcome the short-lived T-independent antibody response generated by polysaccharide alone (MacLennan, 2013). Immunization with *S.* Typhimurium-OMP and Typhimurium-FliC induce both T cells and antibodies in mice, and are therefore also being investigated as vaccine candidates, either separately (Gil-Cruz, *et al.*, 2009) or covalently linked to O-antigen as glycoconjugates (Simon & Levine, 2012).

Key immunological questions arising from this component of the thesis:

- When is the appropriate time for administration of a cross-protective *Salmonella* vaccine?
- What are antibody targets for both gut localised and systemic *Salmonella* infection?

7.2.2 *Salmonella* exposure and development of specific immunity in Malawian children

The second research study was aimed at characterizing the relationship between *Salmonella* exposure events within the GIT or oropharynx and development of *Salmonella*-specific serum immunity in Malawian children aged 6-18 months. At the time this study idea was conceived, observations were made (Chapter 3) that CD4+Cytokine+ T cells to *S.* Typhimurium in Malawian children were detected early in life peaking at 14 months of age, suggesting that Malawian children are exposed to *Salmonella* or cross-

reactive bacteria in early life. Furthermore the pattern of *Salmonella* exposure within the GIT in Malawian children was not known, when this study idea was conceived. It was hypothesized that *Salmonella* exposure within the GIT/or oropharynx facilitate the acquisition of *Salmonella*-specific serum immunity in Malawian children. In a cohort study, healthy Malawian children aged 6-18 months, were tested for *Salmonella* exposure within the GIT and oropharynx by standard culture and rt-PCR at 1 month intervals and also quantified serum immunity to *S.* Typhimurium at 3 months intervals.

Chapter 4 demonstrates that a total of 29 *Salmonella* positive stool out of 630 stool specimens, representing 4.6% were made. Furthermore, in this cohort, 46.8% (22/47) of children had at least one *Salmonella* exposure event within the GIT over a period of 1 year. A total of 29 *Salmonella* positive stools were detected by culture or PCR. Salmonellae isolates were principally *S*. Typhimurium 51.7% (15/29), followed by non-defined serovars 31% (9/29), *S*. Typhi 10.3% (3/29) and *S.* Enteritidis 7% (2/29). Chapter 4 demonstrates that some children had at least two *Salmonella* positive stool events by culture or PCR, 24% (7/29). 71% (5/7) of these were exposed to the same serovar *S.* Typhimurium. These findings indicate that *Salmonella* exposure events within the GIT in Malawian children are common and this underlines the need of public health interventions including improvements in sanitation, food hygiene and vaccination.

Observations were made that *S*. Typhimurium was the most commonly isolated *Salmonella* serovar from stool. *S.* Typhimurium is among the most frequent causes of

blood stream infection (BSI) in children <2 years of age (Chapter 3). *S.* Typhimurium isolates from blood in SSA are distinct from those isolated in the developed countries and are typically multi sequences locus type (MSLT) ST313 (Kingsley*, et al.*, 2009). They are multi-drug resistant (MDR), possess a degraded genome, and initial speculation suggested they might be human host restricted (Kingsley*, et al.*, 2009). Recently, however, another study demonstrated that ST313 can also cause severe and rapid invasive disease in chickens (Parsons*, et al.*, 2013). The question as to whether transmission of ST313 *S*. Typhimurium strains occurs from human to human or animals to human remains unanswered. In murine models, *S.* Typhimurium strain D23580 more efficiently spread to systemic organs from mesenteric lymph nodes compared to *S.* Typhimurium strain A130 (pre 2002 strain) (Msefula, 2009).

Whether these *S*. Typhimurium isolates (isolated in this cohort) from stool are phenotypically and genetically similar to *S*. Typhimurium (ST313) isolates causing bacteraemia in SSA is the key question and will be addressed by whole genome sequencing and other studies in future investigations. Furthermore, the predominance of *S*. Typhimurium isolates in this cohort, is consistent with the hypothesis stated in Chapter 3 that the early acquisition of CD4+ T cell immunity to *S*. Typhimurium might be driven by exposure to *S*. Typhimurium or cross-reactive bacteria within the GIT.

Several factors might contribute to *Salmonella* exposure in Malawian children including feeding practices, food hygiene and sanitary environment. As previously demonstrated

(Kalanda*, et al.*, 2006, Kerr*, et al.*, 2007), and Chapter 4 shows that 9% of children were introduced to supplementary food (such as porridge) as early as <3 months of age, and 91% were introduced to supplementary food by 6 months of age. At 6 months of age all children were being breastfed and only 9% were exclusively breastfeeding. Furthermore, only 17% of children had discontinued breast feeding by 18 months of age. Chapter 4 also demonstrates that a majority of families (82.7%), had access to tap water (classified as protected source) but 64% of babies were drinking non-boiled water as reported by their parents/guardians. Observations were made that boiling water for drinking and using water from the borehole appeared to reduce transmission of *Salmonella,* although these findings did not reach statistical significance. Even though, a majority of families had access to tap water, frequent water outages and high utility bills might be a barrier for this population access to tap water (MCI, 2012) and some might resort to using water from the nearby river or water stream for house hold chores.

Rivers and water streams in Blantyre city have been shown to be highly polluted and also contaminated with potential pathogen (Kumwenda*, et al.*, 2012, MCI, 2012). Children that had *Salmonella* exposure detected in their stool, appeared to be living in close proximity to the river or water streams (Chapter 4), suggesting that access to the river or water streams might contribute to transmission of *Salmonella* in these children. The contribution of environmental factors in the transmission of *Salmonella* infection (including water sources) in this population is not known. Using water from the river was a significant risk factor for transmission of *Salmonella* in this cohort (Chapter 4). It has previously been demonstrated that residents in lower grounds (low altitudes) are at risk of

Salmonella infection (Baker*, et al.*, 2011). In this cohort among children exposed to *Salmonella* and non-exposed, absolute altitude of location of residence did not reach statistical significance. Further investigations including more complex, geospatial analyses studies are required, to generate hypotheses stimulating future studies aimed at understanding the role of environmental factors (including water sources) in the transmission of *Salmonella* infection in this population.

Malaria infection was uncommonly detected in this cohort at the current study specific visit 0.9% (5/563) and administration of antimalarial drugs in the previous months was only reported in 1.4% (8/563). This was surprising considering that Malawi is among the malaria endemic countries (Mathanga*, et al.*, 2012), with asymptomatic parasitaemia rates ranging between 11.2-18.5% (Roca-Feltrer*, et al.*, 2012). In Blantyre, the *Plasmodium falciparum* parasite rate for children 2-<10 years is the lowest in Malawi (26%) (Bennett*, et al.*, 2013). Despite this observation, there was a trend that did not reach statistical significance that children that had taken antimalarial drugs were at higher risk of being exposed to *Salmonella*. Studies in mice models have demonstrated that malaria infection compromises neutrophil immunity and favours *Salmonella* to spread into the blood stream (Roux*, et al.*, 2010, Cunnington*, et al.*, 2012, Maclennan, 2014). Case fatality among children presenting to hospital with malaria and *Salmonella* BSI or any other BSI co-infection is higher compared to children that present with malaria infection alone (Bassat*, et al.*, 2009). Most children with malaria and BSI co-infection are diagnosed and treated for malaria infection but BSI including *Salmonella* bacteraemia

remains non-diagnosed and un-treated. These critical observations in SSA, made WHO recommend that all children with severe malaria should be treated with antibiotics in addition to anti-malarial drugs (Brent, *et al.*, 2006). There is need to study malaria and *Salmonella* co-infection in this population to inform the current clinical management guidelines.

In keeping with the hypothesis made in Chapter 4, that *Salmonella* exposure within the gut facilitates acquisition of serum immunity in children, there was evidence that *Salmonella* exposure within the GIT associated with acquisition of 'protective' SBA to *S.* Typhimurium strain D23580 and 037v4 over a period of 1 year (6 to 18 months). As demonstrated by a previous study in Malawian children (MacLennan, *et al.*, 2008), Chapter 4 also showed that serum bactericidal activity to *S.* Typhimurium strain D23580 increased with age. It was further observed that serum bactericidal activity developed in children in whom *Salmonella* exposure was not detected, and this was in a similar pattern as the exposed counterparts. This study could not be certain if exposed and non-exposed children were appropriately grouped considering low sensitivity of *Salmonella* detection techniques employed and *Salmonella* exposure observations at 1 month interval, and it is also not clear whether cross-reactive enteric bacteria also facilitate serum bactericidal activity acquisition. Whether natural IgG antibodies (Michael, *et al.*, 1962), which also develops with age, can fix-complement and facilitate serum bactericidal activity to *S.* Typhimurium strain D23580 is not clear. Taken together these findings suggest that there

is a relationship between *Salmonella* exposure events occurring within the GIT and development of SBA.

Maternal breast milk capacity to kill *S.* Typhimurium strain D23580 *ex vivo* was also explored in Chapter 4. The hypothesis was made that maternal breast milk kills *Salmonella ex vivo* and might indicate that breast milk provides resistance to *Salmonella* colonisation within the gut lumen among breast fed children. Contrary to this hypothesis, maternal breast milk could not kill *S.* Typhimurium strain D23580 *ex vivo*. Whether or not lack of direct maternal breast milk bactericidal activity to *S.* Typhimurium strain D23580 *ex vivo* could also be true with other *Salmonella* serovars and other enteric pathogens is not clear. The general view is that breast feeding has considerable benefits to babies including protection to enteric pathogens such as *Salmonella* (France*, et al.*, 1980, Borgnolo*, et al.*, 1996). It is possible that these findings do not necessary contradict these observation but this resistance to breast milk killing might be specific for *S.* Typhimurium strain D23580. *S.* Typhimurium strain D23580 is resistant to complement killing alone, and susceptible to IgG antibody fixed complement (MacLennan*, et al.*, 2008). Whether or not maternal breast milk antibodies (including IgG and IgA) fixes complement is not clear. Secretory IgA is more abundant in maternal breast milk and poorly activates complement (Michetti*, et al.*, 1992, Roos*, et al.*, 2001, Woof & Kerr, 2006). A number of factors contribute to maternal breast milk bacterial resistance including complement, lysozymes and free fatty acid (Ogundele, 1999). But it appears all this did not make substantial contribution in maternal breast milk killing assay. Taken

together these findings suggest that *in vivo* maternal breast milk alone might not be sufficient to control *S*. Typhimurium colonisation within the GIT, in breast fed babies.

Key immunological questions arising from this component of the thesis:

- What is the relationship between natural *Salmonella* exposure within the gut and the development of protective immunity from gut localised and blood stream *Salmonella* infection?
- What is the relationship between gut microbial flora changes and *Salmonella* exposure within the gut lumen?
- How do malaria parasites predispose children to either or both gut and systemic *Salmonella* infection?

7.2.3 Development of T cell and antibody immunity in response to invasive *Salmonella* infection

The third research study was aimed at determining the immunological importance of vaccine candidates, currently in pre-clinical phase, including *Salmonella* derived-surface proteins (FliC, OMP and GMMA) and LPS O antigens. The development of *Salmonella*-specific antibody secreting cells (ASC) and T cells induced by invasive *Salmonella* infection (*Salmonella* bacteraemia) has not been investigated in Malawian children. It was hypothesized that invasive *Salmonella* infection facilitates the acquisition of ASCs and T cells. The frequency of IgG or IgA ASCs and T cells specific for *Salmonella* was evaluated in a cohort of children, presenting to hospital with *Salmonella* bacteraemia at acute and convalescent phases and healthy family members.

Chapter 5 demonstrates that absolute IgG ASCs targeting *Salmonella* derived proteins (FliC, OMP, GMMA O+, GMMA O-) and polysaccharides (LPS O; 4,5 and LPS O; 9) were significantly increased in index cases compared to healthy family members. In contrast absolute IgA ASCs targeting *Salmonella* derived proteins (FliC, OMP, GMMA O+, GMMA O-) were higher in index cases compared to healthy family members although the difference did not reach statistically significance suggesting that IgG contribution to controlling *Salmonella* bacteraemia in the peripheral blood might be more compared to IgA. IgA antibodies constitute a key immune component that provides

primary immune defence to pathogens including *Salmonella* within the gut mucosa (Hapfelmeier*, et al.*, 2010).

Chapter 5 also showed that *Salmonella*-specific CD4+IFN-γ+ T cells were higher at acute phase compared to 1 month into recovery period. This decline in *Salmonella* specific CD4+ T cells might result from differentiation of effector CD4+ T cells into central memory CD4+ T cells which end up being recruited in the secondary lymphoid tissues (Sallusto*, et al.*, 1999, Campbell*, et al.*, 2001). This decline may also reflect compromised development of *Salmonella* specific T cell memory. A number of factors have been proposed to explain compromised development of *Salmonella* specific T cell memory, including administration of antibiotics and *Salmonella* immune escape mechanisms. Antibiotic administration has been implicated to compromise establishment of memory T cells (Griffin*, et al.*, 2009, Endt*, et al.*, 2012). In this study, children with *S.* Typhi bacteraemia were treated with ceftriaxone and ciprofloxacin. Whether or not this treatment regimen compromises development of memory T cells is not known. *Salmonella* has also the capacity to modulate immune responses and compromise development of memory T cells. For instance in mice, *Salmonella* compromise dendritic cells antigen presentation to T cells, down regulates T cell receptor expression, and *Salmonella* derived LPS and proteins are thought to be immune suppressive (Matsui, 1996, Tobar*, et al.*, 2006, Bueno*, et al.*, 2007, Srinivasan & McSorley, 2007). In humans the mechanisms that *Salmonella* uses to compromise the acquisition of memory T cells have not been explicitly investigated particularly during the course of *Salmonella* bacteraemia disease.

T cells stimulation with *Salmonella* derived GMMA O+ induced robust CD4+IFN-γ+ T cells responses in index cases and family members compared to T cells stimulation with *Salmonella* derived GMMA O-. These findings suggested that expression of LPS O antigen on GMMA acted as adjunct and augmented the generation of *Salmonella* specific CD4+IFN-γ+ T cells. In mice LPS is thought to a have profound effect on CD4+ T cell responses through LPS interaction with APC toll like receptor 4, which results in up-regulation of downstream signals including MHC-II expression (McAleer & Vella, 2008). In pre-clinical phase it has been demonstrated that GMMA is highly immunogenic (MacLennan, 2013, Koeberling, *et al.*, 2014). A clinical study is required to investigate the immunogenicity induced by *Salmonella* derived GMMA vaccination.

Key immunological questions arising from this component of the thesis:

- What immunological tests can be used to evaluate Ty21a vaccine induced protective immunity to nontyphoidal *Salmonella* serovars?
- How can better serological tests be developed to study *Salmonella* epidemiology in sub Saharan Africa?

7.2.4 Ty21a oral typhoid vaccine induced immunity in the gut mucosa and peripheral blood of healthy adults

The fourth research study was aimed at evaluating antibody and T cell immune responses induced by Ty21a oral typhoid vaccine within the gut mucosa and the peripheral blood in healthy adults from the United Kingdom. This study aimed to model natural *Salmonella* immunizing events occurring within the gut mucosa compartment, by prospectively using a defined exposure. A model for natural *Salmonella* infection will help to understand natural immunizing events that occurs in young children exposed to gut localised NTS and invasive NTS disease in SSA. Currently, there is no licenced vaccine for NTS, understanding the immunizing events that occur in this model will help to inform the design of future NTS vaccine candidates. Oral Ty21a oral typhoid vaccine was licensed in the 1980s and yet immune responses induced by this vaccine have not been directly examined within the gut mucosa and compared to peripheral blood immune responses. Whether the current practice of measuring immunity induced by oral vaccines such as Ty21a within the peripheral blood alone sufficiently characterizes the immunizing events within the gut mucosa is not clear. It has been demonstrated previously that Ty21a vaccination induces the generation of both T cell and antibody immunity and this vaccine is safe, well tolerated and confers up to three years cumulative efficacy of 51% (Olanratmanee*, et al.*, 1992, Fraser*, et al.*, 2007, Pasetti*, et al.*, 2011).

T lymphocytes trafficking markers particularly those of the gut mucosa including β7 and CCR9 were used to extrapolate cellular immune responses occurring within the gut mucosa. A majority of effector memory T cells highly express the β7 and CCR9

compared to central memory CD4+ T cells and naïve T cells, suggesting *Salmonella* specific CD4+ T cells primed within the GALT migrate back to the gut mucosa for mounting of effector immune responses. A majority of central memory CD4+ T cells and naïve CD4+ T cells express CD62L. These are in keeping with previous reports (Sallusto, *et al.*, 1999), expression of CD62L by the central memory CD4+ T cells allows them to reside within the secondary lymphoid tissues (SLT) while naïve CD4+ T cell are also able to home to SLT for antigen specific priming.

Vaccination with Ty21a did not induce an increase in production of Ty21a specific-CD4+Cytokine+ and CD8+Cytokine+T cells at day 11 and 18 post-vaccination in the peripheral blood. These findings are not consistent with previous reports (Salerno-Goncalves, *et al.*, 2002, Salerno-Goncalves, *et al.*, 2005). Evaluation of gut mucosa induced immune responses by examining Ty21a-specific T cell immune responses co-expressing gut homing markers β7 or CCR9 in the peripheral blood, also showed that vaccination with Ty21a did not induce an increase in production of Ty21a-specific CD4+β7+/CCR9+Cytokine+ and CD8+β7+/CCR9+Cytokine+T cells at day 11 and 18 post-vaccination. Even with direct evaluation of gut mucosa of immune responses, vaccination with Ty21a did not induce an increase in production of Ty21a-specific CD4+Cytokine+ and CD8+Cytokine+T cells at day 18 post-vaccination. The reason why this study did not detect differences in the magnitude of Ty21a-specific CD4+Cytokine+ and CD8+Cytokine T cells in peripheral blood and gut mucosa is the key question. A number of factors might have contributed to this outcome including poor antigen stimulation and inappropriate timing for blood and duodenum biopsy collection and not

ideal immunological tools. Due to lack of difference in magnitude of CD4+ and CD8+ T cell immune responses in the gut mucosa and peripheral blood, this study failed to demonstrate relevance of using either direct (gut mucosa biopsy) or indirect (peripheral blood) assessment of oral Ty21a induced immune responses. Better methods and materials including strong T cells stimulant, assessment of both effector and central memory T cell responses and appropriate timing for sampling and measurements will help to appropriately compare peripheral blood and gut mucosa immune responses induced by oral vaccination in future investigations. Gut homing makers including CCR9 and β7 can potentially be used non-invasively evaluate T cell and B cell immune responses in children and HIV infected individuals infected with NTS. Better understanding of natural *Salmonella* immunizing events within the gut will help in designing an effective vaccine for NTS that is urgently required in SSA.

Key immunological questions arising from this component of the thesis:

- What is the relationship between gut mucosa and peripheral blood B cell and T cell immunity to *Salmonella* infection?

7.3 **Future investigations**

7.3.1 Development of adaptive immunity to nontyphoidal *Salmonella* in Malawian children

In healthy Malawian children the development of antibodies and T cells to *S*. Typhimurium was somehow linked to age distribution or frequency of *S*. Typhimurium bacteraemia in under-five children. The study showed the age group as to when children are more vulnerable to *S*. Typhimurium bacteraemia. This was evidenced by lack of either CD4+ T cell or antibody immunity in a specific age group. Among under-five children, 80% of cases with *S.* Typhimurium bacteraemia cases occur in children under-two and the median is 13 months. *S.* Typhimurium-specific CD4+ T cell immunity peaks at 1 year of life while specific antibody immunity peaks at 3 years of age. Essentially the study demonstrates the age group of children that require public health intervention in form of vaccine. Development of a vaccine for *S.* Typhimurium that can promote the generation of antibody and T cell immunity in children below 2 years is required in Malawian children and SSA. Currently, even the two licenced vaccines for typhoid fever, no vaccine is recommended for administration in children below 2 year of age (Germanier & Fuer, 1975, Guzman*, et al.*, 2006, Khan*, et al.*, 2010), indicating the need for *Salmonella* vaccine for both *S*. Typhi and NTS in children below 2 years. As much as it is generally agreed that vaccination for NTS is required in under-two children, determining the appropriate time to administer a NTS vaccine is crucial. Children below 6 months of age are at least protected by passively acquired maternal antibodies but are more vulnerable from 6 months to 36 months of age, as they are introduced to

supplementary food and passively acquired maternal antibodies continue to decline. Administration of NTS vaccine within the current immunization schedule would be pragmatic but would the timing be appropriate to elicit the desired immune response (antibody and T cell immunity). To effectively determine the time optimum time to administer a cross-protective vaccine; children of varying ages (1-24 months) will be investigated for immunogenicity elicited by the vaccine. Strong immune responses would signal appropriate age group to administer the vaccine but this would have to take into account when children are most vulnerable to invasive *Salmonella* bacteraemia to ensure that those most at risk are covered.

The importance of *S*. Typhimurium derived FliC and OMP antigens were demonstrated in Chapter 3. These protein antigens positively correlated with age development of *S*. Typhimurium-specific CD4+Cytokine+ T cells. IgG antibody responses targeting *S*. Typhimurium derived LPS O;4,5 also correlated with the development of serum bactericidal activity to *S*. Typhimurium. Already these protein and polysaccharide antigens (FliC, OMP and LPS O;4,5) are being explored in pre-clinical phase as candidate vaccines (Udhayakumar & Muthukkaruppan, 1987, Cunningham*, et al.*, 2004, Gil-Cruz*, et al.*, 2009, Bobat*, et al.*, 2011, Grimont PA. D and Weill, Cited 2007) either as conjugated polysaccharide vaccine or not. Further studies are required to evaluate vaccine candidates in pre-clinical phase now including FliC, OMP and LPS O antigen in humans, vaccines that confer cross-protection to *Salmonella* serovars causing substantial

burden in SSA and induce generation of memory B and T cell immunity should be prioritized.

Antibody based vaccine for NTS is required as a means to control invasive NTS disease but moving forward with this would require determining key antibody targets that mediate SBA. During infection *Salmonella* does express a range of antigens or genes in various tissues for its survival (Lee*, et al.*, Miller*, et al.*, 1989, Ohl & Miller, 2001, Charles*, et al.*, 2010). Therefore it is crucial to determine whether antibody target expressed by NTS are similar or not within the gut mucosa and systemic circulation. To effectively determine key antibody targets at various stage of infection or in various tissues; children infected with either or both gut localised and systemic *Salmonella* disease will be investigated during the acute and convalesces to determine antibody target profile using both immunological tools and molecular tools (proteomics, microarrays) as described before (Lee*, et al.*, Charles*, et al.*, 2010). This will help to inform the development of subunit vaccine for *Salmonella*.

7.3.2 ***Salmonella* exposure and development of specific-antibody immunity in Malawian children**

Chapter 4 demonstrates that among children aged 6-18 months, 46.8% were exposed to *Salmonella* at least once within the GIT over a period of 1 year. Importantly, this demonstrates that *Salmonella* exposure within the GIT associated with the development

of ‘protective’ serum bactericidal activity (SBA) to *S.* Typhimurium D23580 in children. Whether or not natural *Salmonella* exposure within the gut confers protection to subsequent *Salmonella* infection within the gut and peripheral blood is a key question. Potentially protective SBA in this study was defined as SBA of at least -1 log10 change in *Salmonella* cfu/ml. Although acquisition of SBA or IgG antibodies to *Salmonella* correlates with the decline in bacteraemia cases of both NTS and *S*. Typhi in children (MacLennan*, et al.*, 2008, Lindow*, et al.*, 2011), correlates of protection for *Salmonella* bacteraemia are poorly defined. Recently, SBA assay has been proposed as key immunological tool for evaluation of *Salmonella* vaccines efficacy (Boyd*, et al.*, 2014). To address this question a large cohort of healthy children (age 0-24 months) will be conducted. Children will be tested for *Salmonella* exposure within the GIT and blood by culture and PCR and this will be related with acquisition of potentially protective SBA as described in Chapter 4 and as before (MacLennan*, et al.*, 2008, Boyd*, et al.*, 2014). Children will be examined at scheduled visits and also whenever they experience gastrointestinal symptoms or bacteraemia symptoms. A total of 29 *Salmonella* positive stool were detected by culture or PCR. Salmonellae isolates were principally *S*. Typhimurium 51.7% (15/29), followed by non-defined serovars 31% (9/29), *S*. Typhi 10.3% (3/29) and *S*. Enteritidis 7% (2/29). Chapter 4 demonstrates that some children had at least twice *Salmonella* positive stool by culture or PCR, 24% (7/29). Importantly, a majority of these 71% (5/7) were exposed to the same serovar *S*. Typhimurium. As hypothesised before, it is possible that protection develops following multiple *Salmonella* exposure events (Saul*, et al.*, 2013, Pitzer*, et al.*, 2014). Whether protection to *Salmonella*

is established following multiples exposure events will also be explored by comparing SBA amongst children exposed to *Salmonella* once and multiple times. Whether protection to *Salmonella* is established following exposure to a same *Salmonella* serovar or strain will also be investigated by comparing children SBA amongst those exposed to *Salmonella* serovar or strain and those exposed to multiple serovars or strains. Isolates from this cohort will be characterised phenotypically and also molecularly.

Normal flora of the GIT contributes to resistance of enteric pathogens including *Salmonella* from colonizing the gut lumen (Endt*, et al.*, 2010, Stecher & Hardt, 2011). The changes in microbial flora could result from a number of factors including; breast feeding practices, administration of antibiotics and the virulence of the infecting pathogen (Lolekha, 1986, Pavia*, et al.*, 1990, Bollaerts*, et al.*, 2008, Gradel*, et al.*, 2008, Endt*, et al.*, 2010, Kaiser*, et al.*, 2012). It has been demonstrated before that usage of antibiotics (particularly broad spectrum) disrupts the composition of gut microbial flora and *Salmonella* may take advantage of this to invade the gut lumen barrier (Lolekha, 1986, Pavia*, et al.*, 1990, Gradel*, et al.*, 2008, Endt*, et al.*, 2010, Kaiser*, et al.*, 2012). The variation of gut microbial flora composition in breast fed children and non-breast fed has been shown before (Gonzalez*, et al.*, 2013). Chapter 4 demonstrates *Salmonella* exposure events within the period of 1 year (6 to 18 months). Whether or not microbial flora changes in the gut lumen predispose children to *Salmonella* colonisation is a key question. To investigate whether the *Salmonella* exposure events detected in this cohort are associated with the changes in normal flora, archive stool samples (*Salmonella*

exposed and non-exposed) collected in this cohort will be investigated for microbiome changes by shotgun sequencing as described before (Yatsunenko, *et al.*, 2012) and this will be related to *Salmonella* exposure events. Antibiotic usage and breast feeding practices will also be investigated whether they contribute to changes in gut microbiome in relation to *Salmonella* exposure events in this cohort. Studying the microbiome changes by shotgun sequencing in relation to the *Salmonella* exposure events in this cohort will help to explain factors that render children susceptible to both gut localized and disseminated *Salmonella* infections.

Malawi is among the malaria endemic countries (Mathanga, *et al.*, 2012), with asymptomatic parasitaemia rates ranging between 11.2-18.5% (Roca-Feltrer, *et al.*, 2012). In Blantyre, the *Plasmodium falciparum* parasite rate for children 2-<10 years is the lowest in Malawi (26%) (Bennett, *et al.*, 2013). Case fatality among children presenting to hospital with malaria and *Salmonella* BSI or any other BSI co-infection is higher compared to children that present with malaria infection alone (Bassat, *et al.*, 2009). In this cohort, there was a trend that did not reach statistical significance that children that had taken antimalarial drugs were at higher risk of being exposed to *Salmonella*. It has been shown in mice models that malaria infection compromises neutrophil immunity and favours *Salmonella* to spread into the blood stream (Roux, *et al.*, 2010, Cunnington, *et al.*, 2012, Maclennan, 2014). Neutrophils are key players in providing the primary immunity to rapidly dividing NTS within the gut mucosa and hence preventing the spread of NTS into the systemic organs (Tam, *et al.*, 2008).

Correlates of protection for NTS bacteraemia have not been clearly defined in humans. IgG antibodies to *Salmonella* LPS are important for protection against NTS bacteraemia (Chapter 3) and also previously described (MacLennan*, et al.*, 2008, Lindow*, et al.*, 2011). To investigate whether malaria parasites impairs neutrophils or monocytes effector function to *Salmonella*, it is not feasible to recruit children presenting with malaria and NTS co-infection, hence investigations will involve development of in-vitro malaria and NTS co-infection model to study bacterial growth kinetics during malaria infection. Malawian children aged (6-60 months) suffering from uncomplicated malaria will be investigated for impairment of neutrophil mediated immunity to NTS or *Streptococcus pneumoniae* (control) during acute and early convalescent phases. Briefly, whole blood or neutrophils plus red blood cells (RBCs) from subjects (malaria infected or healthy controls) will be challenged with NTS or *Streptococcus pneumoniae* (control) and examined for growth inhibition on LB agar plates. The contribution of antibody mediated neutrophil killing will be examined in anti-NTS-IgG antibody depleted and non-depleted plasma or serum as previously described (Trebicka, et al., 2013). Neutrophils will also be examined for respiratory burst as previously described (Gondwe, et al., 2010). Serum levels of IL-10 will be quantified by ELISA to explore the contribution of regulatory cytokines to this immunity. This study will therefore help to explain or define the immunological basis of immune impairment favouring bacterial BSI during malaria infection and associated poor health outcomes. These investigations would potentially provide rational basis for modifying the clinical management of malaria infection in children from SSA by revising the current management guidelines to

include routine provision of antibiotics to children suffering from malaria. It can further help to define those at greater risk and might require targeted antimicrobial treatment.

7.3.3 Development of T cell and antibody mediated immunity in response to invasive *Salmonella* infection

Among children that had *S*. Typhi bacteraemia, IgG ASCs targeting LPS O;4, 5 antigen and LPS O;9 correlated positively suggesting that IgG antibodies targeting LPS O antigen induced by *S*. Typhi infection cross-react with *S*. Typhimurium LPS O (Chapter 5). Already there is evidence that Ty21a and CVD 909 induce opsonophagocytic functional antibodies in humans that cross-react with *S*. Paratyphi A and *S*. Paratyphi B (Wahid, *et al.*, 2014). It is possible that currently licensed vaccines (for instance Ty21a) could confer cross protection to other *Salmonella* serovars in populations where the disease burden is considerable but there is no specific NTS vaccine available. To address the question of whether or not current licenced *Salmonella* vaccines for *S.* Typhi (Ty21a) confer cross-protection to NTS serovars in humans, a clinical study will be conducted to investigate the importance of cross-reactive antibodies in conferring protection. In a cohort of children older than 5 years vaccinated or not with Ty21a antibody immune responses to a range of *Salmonella* serovars (*S*. Typhimurium, *S.* Typhi and S. Enteritidis) derived antigens will be quantified by ELISA. Furthermore, SBA to a range of *Salmonella* serovars will be quantified to determine whether Ty21a vaccination facilitates development of cross-protective SBA. SBA will be examined as described in

Chapter 3 and before (MacLennan*, et al.*, 2008, Boyd*, et al.*, 2014). This study will inform the development of cross-protective vaccine for *Salmonella* and also provide the basis for use of currently licenced vaccines (such as Ty21a) in areas where NTS is an important public problem but there is no specific vaccine available.

The burden of *Salmonella* in SSA is huge, and yet this is under appreciated partly due to lack of data on disease burden. Estimating the true burden of *Salmonella* has been challenging because data is patchy and confined largely to sentinel facility-based surveillance (Crump & Heyderman, 2014). The pattern of invasive NTS strains (*S.* Typhimurium and *S.* Enteritidis) transmission in SSA is not clear and whether it is from human to human or from animals to human (Kariuki*, et al.*, 2006) remains to be determined. Furthermore, the reservoirs for invasive NTS strains remain unknown. This is further hampered by lack for diagnostics for *Salmonella*. Whether immunological tools can help to understand the epidemiology of *Salmonella* in SSA is a key question. Chapter 5 demonstrates that the frequency of *Salmonella*-specific CD4+IFN-γ+ T cells was generally higher in healthy family members compared to index cases at both the acute phase and at 1 month into recovery period. These findings suggest that these healthy family members were previously exposed to *Salmonella* and further underlying the need for epidemiological study to understand the pattern of *Salmonella* disease transmission amongst cases and household contacts in this population. Children that had *S*. Typhi bacteraemia their IgG ASCs targeting FliC, OMP and LPS O antigens were significantly elevated compared to healthy family members suggesting that these

antibody targets can be explored for use as diagnostic markers (Chapter 5). But the challenge with the available diagnostic tools including Widal test for diagnosis of typhoid is the lack of specificity. Antibodies targeting LPS O;9 cross-react with LPS O;4,5 hence might not be ideal for development of diagnostic tools. *Salmonella* derived OMP (crude preparation) might also not be ideal for development of diagnostic tools due to cross-reactivity between *S.* Typhimurium and *S*. Typhi.

To effectively develop immunological tool for diagnosis of *Salmonella* and also for epidemiological studies (burden of disease), unique antibody targets for *Salmonella* in acute and convalescent patients will need to be determined using of advance technologies including proteomic and micro-array technologies (Lee*, et al.*, Charles*, et al.*, 2010). These unique targets for antibodies will then be investigated as diagnostic markers and be used to determine the burden of disease in cases and house hold contacts. Additional tests including blood and stool (culture and PCR) will be conducted to validate these tools.

7.3.4 **Ty21a oral typhoid vaccine induced immunity in the peripheral blood and gut mucosa of healthy adults**

The gut mucosa immunity mediated by both innate cell and adaptive cells has long been recognised to provide primary defence to enteric pathogens that potentially disseminate into the peripheral blood (Tam*, et al.*, 2008, Hapfelmeier*, et al.*, 2010). Little is known about the natural immunizing events occurring within the gut mucosa (GALTs) mainly due to anatomical barrier to obtain samples from the gut. Use of invasive methods to

evaluate immune response within is not recommended for other groups of interest like children and HIV infected individuals. There is evidence that oral vaccine induces both B cell and T cell immunity within the gut mucosa and antigen specific cells home back to the gut mucosa as dictated by expression of β7 and CCR9 as shown in Chapter 6 and as shown before (Lundin*, et al.*, 2002, Salerno-Goncalves*, et al.*, 2002). There is evidence of compartmentalization of effector immune responses within the gut mucosa and the peripheral blood depending on the route of immunization for instance oral or systemic vaccination (Quiding-Jarbrink*, et al.*, 1997). Whether or not there is a relationship between B cell and T cell immune responses to *Salmonella* infection within the gut mucosa and peripheral blood is a key question. To answer this question, a clinical study will be conducted where *Salmonella* infected children with either gut localised or bacteraemia will be recruited. These children will be sampled blood to quantify *Salmonella* specific IgG or IgA ASC and memory T cells expressing gut homing markers including β7, CCR9 and CD62L. By comparing the nature of immune responses in children with gut localised *Salmonella* infection versus those with blood stream infection, the study will help to understand relationship between gut mucosa and peripheral blood immune responses. This will also help to inform vaccine design and also determine the suitable route of vaccination either oral or systemic.

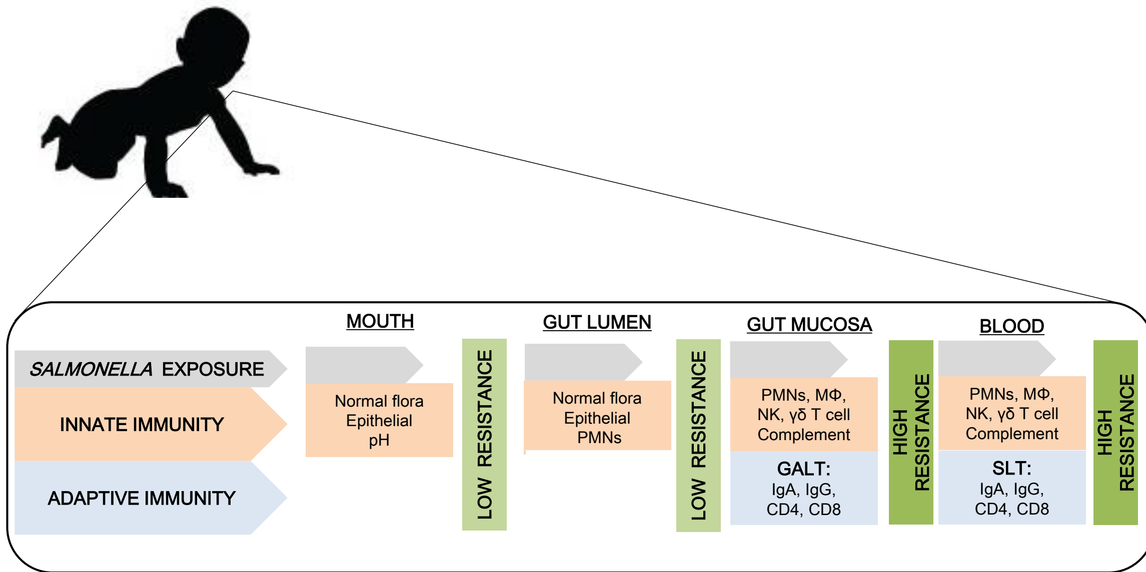

Figure 7. 1: Natural acquisition of immunity to *Salmonella* in children

Naïve children are more vulnerable to *Salmonella* infections as their immunity relies on innate arm of immunity to resist infections. Natural *Salmonella* exposure with the gut mucosa and peripheral blood facilitates the development of adaptive immunity (antibodies and T cells) within the gut associate lymphoid tissues (GALTs) and secondary lymphoid tissues (SLT). This permits establishment of high level of resistance to subsequent to *Salmonella* within the gut mucosa and peripheral blood compartments.

7.4 Conclusion

Many young Malawian children are naturally exposed to *Salmonella*. *Salmonella* exposure has previously been assumed to occur within the GIT, and may occasionally occur systemically beginning from young age. The studies included in this thesis suggest

that *Salmonella* exposure induces the sequential generation of T cells and antibodies directed against *Salmonella* that might provide protection to subsequent *Salmonella* infection. T cells might provide help for the generation of antibodies, and antibodies are crucial for serum bactericidal activity to *Salmonella*. These studies further show for the first time that natural exposure to *Salmonella* within the GIT is associated with the acquisition of potentially-protective serum bactericidal to invasive *Salmonella* strains in Malawian children. The acquisition of both antibody and T cell immunity is thought to control invasive *Salmonella* disease (see Figure 7.1). The studies also specifically explore the contribution of exposure to invasive *Salmonella* to responses to specific potential vaccine targets. Important risk factors for *Salmonella* infection include young age (< 2 years) and this study provides preliminary data to suggest that environmental factors such as contaminated river or stream water are also important risk factors. Studies directly investigating the mucosal cellular response to *Salmonella* were inconclusive, and further work to understand the nature of mucosal immunology and responses to *Salmonella* is required. Public health interventions are urgently required in SSA including vaccination strategies that are effective in young children, improvements in sanitation, access to clean and safe water and food hygiene.

REFERENCES

Agbaje M, Begum RH, Oyekunle MA, Ojo OE & Adenubi OT (2011) Evolution of Salmonella nomenclature: a critical note. *Folia Microbiol (Praha)* **56**: 497-503.

Aggarwal A, Ghosh A, Gomber S, Mitra M & Parikh AO (2011) Efficacy and safety of azithromycin for uncomplicated typhoid fever: an open label non-comparative study. *Indian Pediatr* **48**: 553-556.

Agnandji ST & Lell B & Soulanoudjingar SS*, et al.* (2011) First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. *N Engl J Med* **365**: 1863-1875.

Alos L, Navarrete P, Morente V*, et al.* (2005) Immunoarchitecture of lymphoid tissue in HIV-infection during antiretroviral therapy correlates with viral persistence. *Mod Pathol* **18**: 127-136.

Babirekere-Iriso E, Musoke P & Kekitiinwa A (2006) Bacteraemia in severely malnourished children in an HIV-endemic setting. *Ann Trop Paediatr* **26**: 319-328.

Bahwere P, Levy J, Hennart P*, et al.* (2001) Community-acquired bacteremia among hospitalized children in rural central Africa. *Int J Infect Dis* **5**: 180-188.

Bailey JW, Williams J, Bain BJ, Parker-Williams J, Chiodini PL & General Haematology Task Force of the British Committee for Standards in H (2013) Guideline: the laboratory diagnosis of malaria. General Haematology Task Force of the British Committee for Standards in Haematology. *Br J Haematol* **163**: 573-580.

Baker S, Holt KE, Clements AC*, et al.* (2011) Combined high-resolution genotyping and geospatial analysis reveals modes of endemic urban typhoid fever transmission. *Open Biol* **1**: 110008.

Bar-Meir M, Raveh D, Yinnon AM, Benenson S, Rudensky B & Schlesinger Y (2005) Non-Typhi Salmonella gastroenteritis in children presenting to the emergency department: characteristics of patients with associated bacteraemia. *Clin Microbiol Infect* **11**: 651-655.

Bassat Q, Guinovart C, Sigauque B*, et al.* (2009) Severe malaria and concomitant bacteraemia in children admitted to a rural Mozambican hospital. *Trop Med Int Health* **14**: 1011-1019.

Beasley WJ, Joseph SW & Weiss E (1981) Improved serodiagnosis of Salmonella enteric fevers by an enzyme-linked immunosorbent assay. *J Clin Microbiol* **13**: 106-114.

Bennett A, Kazembe L, Mathanga DP, Kinyoki D, Ali D, Snow RW & Noor AM (2013) Mapping malaria transmission intensity in Malawi, 2000-2010. *Am J Trop Med Hyg* **89**: 840-849.

Berkley JA, Lowe BS, Mwangi I*, et al.* (2005) Bacteremia among children admitted to a rural hospital in Kenya. *N Engl J Med* **352**: 39-47.

Berlanda Scorza F, Colucci AM, Maggiore L*, et al.* (2012) High yield production process for Shigella outer membrane particles. *PLoS One* **7**: e35616.

Berlin C, Berg EL, Briskin MJ*, et al.* (1993) Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell* **74**: 185-195.

Beutler B (2000) Tlr4: central component of the sole mammalian LPS sensor. *Curr Opin Immunol* **12**: 20-26.

Bhuiyan S, Sayeed A, Khanam F*, et al.* (2014) Cellular and cytokine responses to Salmonella enterica serotype Typhi proteins in patients with typhoid fever in Bangladesh. *Am J Trop Med Hyg* **90**: 1024-1030.

Bhutta ZA, Capeding MR, Bavdekar A*, et al.* (2014) Immunogenicity and safety of the Vi-CRM197 conjugate vaccine against typhoid fever in adults, children, and infants in south and southeast Asia: results from two randomised, observer-blind, age de-escalation, phase 2 trials. *Lancet Infect Dis* **14**: 119-129.

Biggs HM, Lester R, Nadjm B*, et al.* (2014) Invasive salmonella infections in areas of high and low malaria transmission intensity in Tanzania. *Clin Infect Dis* **58**: 638-647.

Blaschitz C & Raffatellu M (2010) Th17 cytokines and the gut mucosal barrier. *J Clin Immunol* **30**: 196-203.

Bobat S, Flores-Langarica A, Hitchcock J*, et al.* (2011) Soluble flagellin, FliC, induces an Ag-specific Th2 response, yet promotes T-bet-regulated Th1 clearance of Salmonella typhimurium infection. *Eur J Immunol* **41**: 1606-1618.

Bollaerts K, Aerts M, Faes C, Grijspeerdt K, Dewulf J & Mintiens K (2008) Human salmonellosis: estimation of dose-illness from outbreak data. *Risk Anal* **28**: 427-440.

Booth C, Inusa B & Obaro SK (2010) Infection in sickle cell disease: a review. *Int J Infect Dis* **14**: e2-e12.

Borgnolo G, Barbone F, Scornavacca G, Franco D, Vinci A & Iuculano F (1996) A case-control study of Salmonella gastrointestinal infection in Italian children. *Acta Paediatr* **85**: 804-808.

Boyd MA, Tennant SM, Saague VA*, et al.* (2014) Serum bactericidal assays to evaluate typhoidal and nontyphoidal Salmonella vaccines. *Clin Vaccine Immunol* **21**: 712-721.

Breiman RF, Cosmas L, Njuguna H*, et al.* (2012) Population-based incidence of typhoid fever in an urban informal settlement and a rural area in Kenya: implications for typhoid vaccine use in Africa. *PLoS One* **7**: e29119.

Brenner FW, Villar RG, Angulo FJ, Tauxe R & Swaminathan B (2000) Salmonella nomenclature. *J Clin Microbiol* **38**: 2465-2467.

Brent AJ, Oundo JO, Mwangi I, Ochola L, Lowe B & Berkley JA (2006) Salmonella bacteremia in Kenyan children. *Pediatr Infect Dis J* **25**: 230-236.

Bronzan RN, Taylor TE, Mwenechanya J*, et al.* (2007) Bacteremia in Malawian children with severe malaria: prevalence, etiology, HIV coinfection, and outcome. *J Infect Dis* **195**: 895-904.

Buchwald DS & Blaser MJ (1984) A review of human salmonellosis: II. Duration of excretion following infection with nontyphi Salmonella. *Rev Infect Dis* **6**: 345-356.

Bueno SM, Gonzalez PA, Schwebach JR & Kalergis AM (2007) T cell immunity evasion by virulent Salmonella enterica. *Immunol Lett* **111**: 14-20.

Bunce C & Bell EB (1997) CD45RC isoforms define two types of CD4 memory T cells, one of which depends on persisting antigen. *J Exp Med* **185**: 767-776.

Calis JC, Phiri KS, Faragher EB*, et al.* (2008) Severe anemia in Malawian children. *N Engl J Med* **358**: 888-899.

Campbell JJ, Murphy KE, Kunkel EJ*, et al.* (2001) CCR7 expression and memory T cell diversity in humans. *J Immunol* **166**: 877-884.

Carlsson HE, Lindberg AA, Hammarstrom S & Ljunggren A (1975) Quantitation of Salmonella O-antibodies in human sera by enzyme-linked immunosorbent assay (ELISA). *Int Arch Allergy Appl Immunol* **48**: 485-494.

Centers for Disease C & Prevention (2012) Notes from the field: Salmonella Typhi infections associated with contaminated water--Zimbabwe, October 2011-May 2012. *MMWR Morb Mortal Wkly Rep* **61**: 435.

Charles RC, Sheikh A, Krastins B*, et al.* (2010) Characterization of anti-Salmonella enterica serotype Typhi antibody responses in bacteremic Bangladeshi patients by an immunoaffinity proteomics-based technology. *Clin Vaccine Immunol* **17**: 1188-1195.

Cheminay C, Chakravortty D & Hensel M (2004) Role of neutrophils in murine salmonellosis. *Infect Immun* **72**: 468-477.

Chen Z & O'Shea JJ (2008) Regulation of IL-17 production in human lymphocytes. *Cytokine* **41**: 71-78.

Chilton PM, Hadel DM, To TT, Mitchell TC & Darveau RP (2013) Adjuvant activity of naturally occurring monophosphoryl lipopolysaccharide preparations from mucosa-associated bacteria. *Infect Immun* **81**: 3317-3325.

Chipeta J, Komada Y, Zhang XL, Deguchi T, Sugiyama K, Azuma E & Sakurai M (1998) CD4+ and CD8+ cell cytokine profiles in neonates, older children, and adults: increasing T helper type 1 and T cytotoxic type 1 cell populations with age. *Cell Immunol* **183**: 149-156.

Choo KE, Davis TM, Ismail A & Ong KH (1997) Longevity of antibody responses to a Salmonella typhi-specific outer membrane protein: interpretation of a dot enzyme immunosorbent assay in an area of high typhoid fever endemicity. *Am J Trop Med Hyg* **57**: 656-659.

Church J & Maitland K (2014) Invasive bacterial co-infection in African children with Plasmodium falciparum malaria: a systematic review. *BMC Med* **12**: 31.

Clark TW, Daneshvar C, Pareek M, Perera N & Stephenson I (2010) Enteric fever in a UK regional infectious diseases unit: a 10 year retrospective review. *J Infect* **60**: 91-98.

Colwell DE, Michalek SM, Briles DE, Jirillo E & McGhee JR (1984) Monoclonal antibodies to Salmonella lipopolysaccharide: anti-O-polysaccharide antibodies protect C3H mice against challenge with virulent Salmonella typhimurium. *J Immunol* **133**: 950-957.

Conlan JW (1997) Critical roles of neutrophils in host defense against experimental systemic infections of mice by Listeria monocytogenes, Salmonella typhimurium, and Yersinia enterocolitica. *Infect Immun* **65**: 630-635.

Cose S (2007) T-cell migration: a naive paradigm? *Immunology* **120**: 1-7.

Cravioto A (1990) [Presence of specific factors in mother's milk against strains of diarrhea-causing Escherichia coli in humans]. *Gac Med Mex* **126**: 35-42; discussion 42-33.

Cravioto A, Reyes RE, Trujillo F*, et al.* (1990) Risk of diarrhea during the first year of life associated with initial and subsequent colonization by specific enteropathogens. *Am J Epidemiol* **131**: 886-904.

Crosa JH, Brenner DJ, Ewing WH & Falkow S (1973) Molecular relationships among the Salmonelleae. *J Bacteriol* **115**: 307-315.

Crump JA & Mintz ED (2010) Global trends in typhoid and paratyphoid Fever. *Clin Infect Dis* **50**: 241-246.

Crump JA & Heyderman RS (2014) Invasive Salmonella infections in Africa. *Trans R Soc Trop Med Hyg* **108**: 673-675.

Crump JA, Griffin PM & Angulo FJ (2002) Bacterial contamination of animal feed and its relationship to human foodborne illness. *Clin Infect Dis* **35**: 859-865.

Crump JA, Luby SP & Mintz ED (2004) The global burden of typhoid fever. *Bull World Health Organ* **82**: 346-353.

Crump JA, Ram PK, Gupta SK, Miller MA & Mintz ED (2008) Part I. Analysis of data gaps pertaining to Salmonella enterica serotype Typhi infections in low and medium human development index countries, 1984-2005. *Epidemiol Infect* **136**: 436-448.

Cunliffe NA, Ngwira BM, Dove W*, et al.* (2010) Epidemiology of rotavirus infection in children in Blantyre, Malawi, 1997-2007. *J Infect Dis* **202 Suppl**: S168-174.

Cunningham AF, Khan M, Ball J, Toellner KM, Serre K, Mohr E & MacLennan IC (2004) Responses to the soluble flagellar protein FliC are Th2, while those to FliC on Salmonella are Th1. *Eur J Immunol* **34**: 2986-2995.

Cunningham AF, Gaspal F, Serre K*, et al.* (2007) Salmonella induces a switched antibody response without germinal centers that impedes the extracellular spread of infection. *J Immunol* **178**: 6200-6207.

Cunnington AJ, de Souza JB, Walther M & Riley EM (2012) Malaria impairs resistance to Salmonella through heme- and heme oxygenase-dependent dysfunctional granulocyte mobilization. *Nat Med* **18**: 120-127.

de Jong HK, Parry CM, van der Poll T & Wiersinga WJ (2012) Host-pathogen interaction in invasive Salmonellosis. *PLoS Pathog* **8**: e1002933.

De Rosa SC (2012) Vaccine applications of flow cytometry. *Methods* **57**: 383-391.

Dixon JM (1965) Effect of antibiotic treatment on duration of excretion of Salmonella typhimurium by children. *Br Med J* **2**: 1343-1345.

Dougan G, John V, Palmer S & Mastroeni P (2011) Immunity to salmonellosis. *Immunol Rev* **240**: 196-210.

Effa EE & Bukirwa H (2011) WITHDRAWN: Azithromycin for treating uncomplicated typhoid and paratyphoid fever (enteric fever). *Cochrane Database Syst Rev* CD006083.

Endt K, Maier L, Kappeli R, Barthel M, Misselwitz B, Kremer M & Hardt WD (2012) Peroral ciprofloxacin therapy impairs the generation of a protective immune response in a mouse model for Salmonella enterica serovar Typhimurium diarrhea, while parenteral ceftriaxone therapy does not. *Antimicrob Agents Chemother* **56**: 2295-2304.

Endt K, Stecher B, Chaffron S*, et al.* (2010) The microbiota mediates pathogen clearance from the gut lumen after non-typhoidal Salmonella diarrhea. *PLoS Pathog* **6**: e1001097.

Ernst RK, Guina T & Miller SI (2001) Salmonella typhimurium outer membrane remodeling: role in resistance to host innate immunity. *Microbes Infect* **3**: 1327-1334.

Fanaro S, Chierici R, Guerrini P & Vigi V (2003) Intestinal microflora in early infancy: composition and development. *Acta Paediatr Suppl* **91**: 48-55.

Farooqui A, Khan A & Kazmi SU (2009) Investigation of a community outbreak of typhoid fever associated with drinking water. *BMC Public Health* **9**: 476.

Feasey NA, Dougan G, Kingsley RA, Heyderman RS & Gordon MA (2012) Invasive non-typhoidal salmonella disease: an emerging and neglected tropical disease in Africa. *Lancet* **379**: 2489-2499.

Feasey NA, Archer BN, Heyderman RS, Sooka A, Dennis B, Gordon MA & Keddy KH (2010) Typhoid fever and invasive nontyphoid salmonellosis, Malawi and South Africa. *Emerg Infect Dis* **16**: 1448-1451.

Feasey NA, Houston A, Mukaka M*, et al.* (2014) A reduction in adult blood stream infection and case fatality at a large African hospital following antiretroviral therapy roll-out. *PLoS One* **9**: e92226.

Fierer J (2001) Polymorphonuclear leukocytes and innate immunity to Salmonella infections in mice. *Microbes Infect* **3**: 1233-1237.

Forest CG, Ferraro E, Sabbagh SC & Daigle F (2010) Intracellular survival of Salmonella enterica serovar Typhi in human macrophages is independent of Salmonella pathogenicity island (SPI)-2. *Microbiology* **156**: 3689-3698.

France GL, Marmer DJ & Steele RW (1980) Breast-feeding and Salmonella infection. *Am J Dis Child* **134**: 147-152.

Frankel G, Newton SM, Schoolnik GK & Stocker BA (1989) Unique sequences in region VI of the flagellin gene of Salmonella typhi. *Mol Microbiol* **3**: 1379-1383.

Fraser A, Paul M, Goldberg E, Acosta CJ & Leibovici L (2007) Typhoid fever vaccines: systematic review and meta-analysis of randomised controlled trials. *Vaccine* **25**: 7848-7857.

Freer G & Rindi L (2013) Intracellular cytokine detection by fluorescence-activated flow cytometry: basic principles and recent advances. *Methods* **61**: 30-38.

Galan JE (1999) Interaction of Salmonella with host cells through the centisome 63 type III secretion system. *Curr Opin Microbiol* **2**: 46-50.

Gat O, Galen JE, Tennant S*, et al.* (2011) Cell-associated flagella enhance the protection conferred by mucosally-administered attenuated Salmonella Paratyphi A vaccines. *PLoS Negl Trop Dis* **5**: e1373.

Germanier R & Fuer E (1975) Isolation and characterization of Gal E mutant Ty 21a of Salmonella typhi: a candidate strain for a live, oral typhoid vaccine. *J Infect Dis* **131**: 553-558.

Gil-Cruz C, Bobat S, Marshall JL*, et al.* (2009) The porin OmpD from nontyphoidal Salmonella is a key target for a protective B1b cell antibody response. *Proc Natl Acad Sci U S A* **106**: 9803-9808.

Gilks CF, Brindle RJ, Otieno LS*, et al.* (1990) Life-threatening bacteraemia in HIV-1 seropositive adults admitted to hospital in Nairobi, Kenya. *Lancet* **336**: 545-549.

Gondwe EN, Molyneux ME, Goodall M, Graham SM, Mastroeni P, Drayson MT & MacLennan CA (2010) Importance of antibody and complement for oxidative burst and killing of invasive nontyphoidal Salmonella by blood cells in Africans. *Proc Natl Acad Sci U S A* **107**: 3070-3075.

Gonzalez R, Maldonado A, Martin V*, et al.* (2013) Breast milk and gut microbiota in African mothers and infants from an area of high HIV prevalence. *PLoS One* **8**: e80299.

Gordon MA (2008) Salmonella infections in immunocompromised adults. *J Infect* **56**: 413-422.

Gordon MA & Graham SM (2008) Invasive salmonellosis in Malawi. *J Infect Dev Ctries* **2**: 438-442.

Gordon MA, Jack DL, Dockrell DH, Lee ME & Read RC (2005) Gamma interferon enhances internalization and early nonoxidative killing of Salmonella enterica serovar Typhimurium by human macrophages and modifies cytokine responses. *Infect Immun* **73**: 3445-3452.

Gordon MA, Gordon SB, Musaya L, Zijlstra EE, Molyneux ME & Read RC (2007) Primary macrophages from HIV-infected adults show dysregulated cytokine responses to Salmonella, but normal internalization and killing. *AIDS* **21**: 2399-2408.

Gordon MA, Banda HT, Gondwe M, *et al.* (2002) Non-typhoidal salmonella bacteraemia among HIV-infected Malawian adults: high mortality and frequent recrudescence. *AIDS* **16**: 1633-1641.

Gordon MA, Graham SM, Walsh AL, *et al.* (2008) Epidemics of invasive Salmonella enterica serovar enteritidis and S. enterica Serovar typhimurium infection associated with multidrug resistance among adults and children in Malawi. *Clin Infect Dis* **46**: 963-969.

Gordon MA, Kankwatira AM, Mwafulirwa G, *et al.* (2010) Invasive non-typhoid salmonellae establish systemic intracellular infection in HIV-infected adults: an emerging disease pathogenesis. *Clin Infect Dis* **50**: 953-962.

Gradel KO, Dethlefsen C, Ejlertsen T, Schonheyder HC & Nielsen H (2008) Increased prescription rate of antibiotics prior to non-typhoid Salmonella infections: a one-year nested case-control study. *Scand J Infect Dis* **40**: 635-641.

Graham SM (2010) Nontyphoidal salmonellosis in Africa. *Curr Opin Infect Dis* **23**: 409-414.

Graham SM & English M (2009) Non-typhoidal salmonellae: a management challenge for children with community-acquired invasive disease in tropical African countries. *Lancet* **373**: 267-269.

Graham SM, Walsh AL, Molyneux EM, Phiri AJ & Molyneux ME (2000) Clinical presentation of non-typhoidal Salmonella bacteraemia in Malawian children. *Trans R Soc Trop Med Hyg* **94**: 310-314.

Graham SM, Hart CA, Molyneux EM, Walsh AL & Molyneux ME (2000) Malaria and Salmonella infections: cause or coincidence? *Trans R Soc Trop Med Hyg* **94**: 227.

Graham SM, Molyneux EM, Walsh AL, Cheesbrough JS, Molyneux ME & Hart CA (2000) Nontyphoidal Salmonella infections of children in tropical Africa. *Pediatr Infect Dis J* **19**: 1189-1196.

Griffin A, Baraho-Hassan D & McSorley SJ (2009) Successful treatment of bacterial infection hinders development of acquired immunity. *J Immunol* **183**: 1263-1270.

Griffin AJ & McSorley SJ (2011) Development of protective immunity to Salmonella, a mucosal pathogen with a systemic agenda. *Mucosal Immunol* **4**: 371-382.

Grimont PA. D and Weill FX (Cited 2007) WHO Collaborating Centre for Reference and Research on Salmonellae Antigenic formulae of Salmonella serovars 2007 9th edition.

Groisman EA (2001) The pleiotropic two-component regulatory system PhoP-PhoQ. *J Bacteriol* **183**: 1835-1842.

Groisman EA & Ochman H (1996) Pathogenicity islands: bacterial evolution in quantum leaps. *Cell* **87**: 791-794.

Guadalupe M, Reay E, Sankaran S, Prindiville T, Flamm J, McNeil A & Dandekar S (2003) Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J Virol* **77**: 11708-11717.

Guinovart C, Navia MM, Tanner M & Alonso PL (2006) Malaria: burden of disease. *Curr Mol Med* **6**: 137-140.

Guo L, Lim KB, Gunn JS, Bainbridge B, Darveau RP, Hackett M & Miller SI (1997) Regulation of lipid A modifications by Salmonella typhimurium virulence genes phoP-phoQ. *Science* **276**: 250-253.

Guo L, Lim KB, Poduje CM, Daniel M, Gunn JS, Hackett M & Miller SI (1998) Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. *Cell* **95**: 189-198.

Guzman CA, Borsutzky S, Griot-Wenk M*, et al.* (2006) Vaccines against typhoid fever. *Vaccine* **24**: 3804-3811.

Hajna AA (1945) Triple-Sugar Iron Agar Medium for the Identification of the Intestinal Group of Bacteria. *J Bacteriol* **49**: 516-517.

Hand WL & King NL (1977) Deficiency of serum bactericidal activity against Salmonella typhimurium in sickle cell anaemia. *Clin Exp Immunol* **30**: 262-270.

Hanson LA, Ashraf R, Cruz JR*, et al.* (1990) Immunity related to exposition and bacterial colonization of the infant. *Acta Paediatr Scand Suppl* **365**: 38-45.

Hapfelmeier S, Lawson MA, Slack E*, et al.* (2010) Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. *Science* **328**: 1705-1709.

Heale JP, Pollard AJ, Stokes RW, Simpson D, Tsang A, Massing B & Speert DP (2001) Two distinct receptors mediate nonopsonic phagocytosis of different strains of Pseudomonas aeruginosa. *J Infect Dis* **183**: 1214-1220.

Heath WR, Belz GT, Behrens GM*, et al.* (2004) Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol Rev* **199**: 9-26.

Heffernan EJ, Harwood J, Fierer J & Guiney D (1992) The Salmonella typhimurium virulence plasmid complement resistance gene rck is homologous to a family of virulence-related outer membrane protein genes, including pagC and ail. *J Bacteriol* **174**: 84-91.

Heffernan EJ, Reed S, Hackett J, Fierer J, Roudier C & Guiney D (1992) Mechanism of resistance to complement-mediated killing of bacteria encoded by the Salmonella typhimurium virulence plasmid gene rck. *J Clin Invest* **90**: 953-964.

Hendriksen RS, Leekitcharoenphon P, Lukjancenko O*, et al.* (2014) Genomic Signature of Multi-Drug Resistant Salmonella Typhi related to a Massive Outbreak in Zambia during 2010 - 2012. *J Clin Microbiol*.

Hensel M, Shea JE, Waterman SR*, et al.* (1998) Genes encoding putative effector proteins of the type III secretion system of Salmonella pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. *Mol Microbiol* **30**: 163-174.

Hessel L, Debois H, Fletcher M & Dumas R (1999) Experience with Salmonella typhi Vi capsular polysaccharide vaccine. *Eur J Clin Microbiol Infect Dis* **18**: 609-620.

Hindle Z, Chatfield SN, Phillimore J*, et al.* (2002) Characterization of Salmonella enterica derivatives harboring defined aroC and Salmonella pathogenicity island 2 type III secretion system (ssaV) mutations by immunization of healthy volunteers. *Infect Immun* **70**: 3457-3467.

Hodges K & Gill R (2010) Infectious diarrhea: Cellular and molecular mechanisms. *Gut Microbes* **1**: 4-21.

Hoffman SL, Punjabi NH, Rockhill RC, Sutomo A, Rivai AR & Pulungsih SP (1984) Duodenal string-capsule culture compared with bone-marrow, blood, and rectal-swab cultures for diagnosing typhoid and paratyphoid fever. *J Infect Dis* **149**: 157-161.

Hohmann EL, Oletta CA, Killeen KP & Miller SI (1996) phoP/phoQ-deleted Salmonella typhi (Ty800) is a safe and immunogenic single-dose typhoid fever vaccine in volunteers. *J Infect Dis* **173**: 1408-1414.

Hooper LV & Gordon JI (2001) Commensal host-bacterial relationships in the gut. *Science* **292**: 1115-1118.

Hormaeche CE (1990) Dead salmonellae or their endotoxin accelerate the early course of a Salmonella infection in mice. *Microb Pathog* **9**: 213-218.

House D, Ho VA, Diep TS*, et al.* (2008) Antibodies to the Vi capsule of Salmonella Typhi in the serum of typhoid patients and healthy control subjects from a typhoid endemic region. *J Infect Dev Ctries* **2**: 308-312.

Hueck CJ (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* **62**: 379-433.

Iwajomo OH, Moons P, Nkhata R*, et al.* (2014) Delayed reconstitution of B cell immunity to pneumococcus in HIV-infected Malawian children on antiretroviral therapy. *J Infect*.

Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C & Song SY (2004) Retinoic acid imprints gut-homing specificity on T cells. *Immunity* **21**: 527-538.

Jahnmatz M, Kesa G, Netterlid E, Buisman AM, Thorstensson R & Ahlborg N (2013) Optimization of a human IgG B-cell ELISpot assay for the analysis of vaccine-induced B-cell responses. *J Immunol Methods* **391**: 50-59.

Janssen R, Van Wengen A, Verhard E, De Boer T, Zomerdijk T, Ottenhoff TH & Van Dissel JT (2002) Divergent role for TNF-alpha in IFN-gamma-induced killing of Toxoplasma gondii and Salmonella typhimurium contributes to selective susceptibility of patients with partial IFN-gamma receptor 1 deficiency. *J Immunol* **169**: 3900-3907.

Jensen PE, Weber DA, Thayer WP, Chen X & Dao CT (1999) HLA-DM and the MHC class II antigen presentation pathway. *Immunol Res* **20**: 195-205.

Johansson-Lindbom B, Svensson M, Wurbel MA, Malissen B, Marquez G & Agace W (2003) Selective generation of gut tropic T cells in gut-associated lymphoid tissue (GALT): requirement for GALT dendritic cells and adjuvant. *J Exp Med* **198**: 963-969.

Jouanguy E, Doffinger R, Dupuis S, Pallier A, Altare F & Casanova JL (1999) IL-12 and IFN-gamma in host defense against mycobacteria and salmonella in mice and men. *Curr Opin Immunol* **11**: 346-351.

Kafulafula G, Hoover DR, Taha TE, *et al.* (2010) Frequency of gastroenteritis and gastroenteritis-associated mortality with early weaning in HIV-1-uninfected children born to HIV-infected women in Malawi. *J Acquir Immune Defic Syndr* **53**: 6-13.

Kaiser P, Diard M, Stecher B & Hardt WD (2012) The streptomycin mouse model for Salmonella diarrhea: functional analysis of the microbiota, the pathogen's virulence factors, and the host's mucosal immune response. *Immunol Rev* **245**: 56-83.

Kaisho T & Akira S (2000) Critical roles of Toll-like receptors in host defense. *Crit Rev Immunol* **20**: 393-405.

Kalanda BF, Verhoeff FH & Brabin BJ (2006) Breast and complementary feeding practices in relation to morbidity and growth in Malawian infants. *Eur J Clin Nutr* **60**: 401-407.

Kaltsidis H, Cheeseman H, Kopycinski J, *et al.* (2011) Measuring human T cell responses in blood and gut samples using qualified methods suitable for evaluation of HIV vaccine candidates in clinical trials. *J Immunol Methods* **370**: 43-54.

Kannanganat S, Ibegbu C, Chennareddi L, Robinson HL & Amara RR (2007) Multiple-cytokine-producing antiviral CD4 T cells are functionally superior to single-cytokine-producing cells. *J Virol* **81**: 8468-8476.

Kantele A, Zivny J, Hakkinen M, Elson CO & Mestecky J (1999) Differential homing commitments of antigen-specific T cells after oral or parenteral immunization in humans. *J Immunol* **162**: 5173-5177.

Kantele A, Pakkanen SH, Siitonen A, Karttunen R & Kantele JM (2012) Live oral typhoid vaccine Salmonella Typhi Ty21a - a surrogate vaccine against non-typhoid salmonella? *Vaccine* **30**: 7238-7245.

Kariuki S, Revathi G, Kiiru J, Lowe B, Berkley JA & Hart CA (2006) Decreasing prevalence of antimicrobial resistance in non-typhoidal Salmonella isolated from children with bacteraemia in a rural district hospital, Kenya. *Int J Antimicrob Agents* **28**: 166-171.

Kariuki S, Revathi G, Kariuki N*, et al.* (2006) Invasive multidrug-resistant non-typhoidal Salmonella infections in Africa: zoonotic or anthroponotic transmission? *J Med Microbiol* **55**: 585-591.

Kariuki S, Revathi G, Kiiru J*, et al.* (2010) Typhoid in Kenya is associated with a dominant multidrug-resistant Salmonella enterica serovar Typhi haplotype that is also widespread in Southeast Asia. *J Clin Microbiol* **48**: 2171-2176.

Kerr RB, Berti PR & Chirwa M (2007) Breastfeeding and mixed feeding practices in Malawi: timing, reasons, decision makers, and child health consequences. *Food Nutr Bull* **28**: 90-99.

Khan MI, Ochiai RL & Clemens JD (2010) Population impact of Vi capsular polysaccharide vaccine. *Expert Rev Vaccines* **9**: 485-496.

Khanam F, Sheikh A, Sayeed MA*, et al.* (2013) Evaluation of a typhoid/paratyphoid diagnostic assay (TPTest) detecting anti-Salmonella IgA in secretions of peripheral blood lymphocytes in patients in Dhaka, Bangladesh. *PLoS Negl Trop Dis* **7**: e2316.

Khoo SK, Petillo D, Parida M, Tan AC, Resau JH & Obaro SK (2011) Host response transcriptional profiling reveals extracellular components and ABC (ATP-binding cassette) transporters gene enrichment in typhoid fever-infected Nigerian children. *BMC Infect Dis* **11**: 241.

Kingsley RA, Msefula CL, Thomson NR*, et al.* (2009) Epidemic multiple drug resistant Salmonella Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype. *Genome Res* **19**: 2279-2287.

Kirkpatrick BD, McKenzie R, O'Neill JP*, et al.* (2006) Evaluation of Salmonella enterica serovar Typhi (Ty2 aroC-ssaV-) M01ZH09, with a defined mutation in the Salmonella pathogenicity island 2, as a live, oral typhoid vaccine in human volunteers. *Vaccine* **24**: 116-123.

Koeberling O, Ispasanie E, Hauser J*, et al.* (2014) A broadly-protective vaccine against meningococcal disease in sub-Saharan Africa based on generalized modules for membrane antigens (GMMA). *Vaccine* **32**: 2688-2695.

Kolyva S, Waxin H & Popoff MY (1992) The Vi antigen of Salmonella typhi: molecular analysis of the viaB locus. *J Gen Microbiol* **138**: 297-304.

Krushkal J, Bat O & Gigli I (2000) Evolutionary relationships among proteins encoded by the regulator of complement activation gene cluster. *Mol Biol Evol* **17**: 1718-1730.

Kumwenda S, Tsakama M, Kalulu K & Kambala C (2012) Determination of Biological, Physical and Chemical Pollutants in Mudi River, Blantyre, Malawi. *Journal of Basic and Applied Scientific Research* **2**: 6833-6839.

Kunkel EJ, Kim CH, Lazarus NH, Vierra MA, Soler D, Bowman EP & Butcher EC (2003) CCR10 expression is a common feature of circulating and mucosal epithelial tissue IgA Ab-secreting cells. *J Clin Invest* **111**: 1001-1010.

Kunkel EJ, Campbell JJ, Haraldsen G*, et al.* (2000) Lymphocyte CC chemokine receptor 9 and epithelial thymus-expressed chemokine (TECK) expression distinguish the small intestinal immune compartment: Epithelial expression of tissue-specific chemokines as an organizing principle in regional immunity. *J Exp Med* **192**: 761-768.

Lalmanach AC & Lantier F (1999) Host cytokine response and resistance to Salmonella infection. *Microbes Infect* **1**: 719-726.

Le Minor L, Veron M & Popoff M (1982) [A proposal for Salmonella nomenclature]. *Ann Microbiol (Paris)* **133**: 245-254.

Lee SJ, Liang L, Juarez S*, et al.* Identification of a common immune signature in murine and human systemic Salmonellosis. *Proc Natl Acad Sci U S A* **109**: 4998-5003.

Lee SJ, Liang L, Juarez S*, et al.* (2012) Identification of a common immune signature in murine and human systemic Salmonellosis. *Proc Natl Acad Sci U S A* **109**: 4998-5003.

Leifson E (1939) New selenite selective enrichment medium for the isolation of typhoid and paratyphoid bacilli. *Am. J. Hyg* 423-432.

Levine MM, Black RE & Lanata C (1982) Precise estimation of the numbers of chronic carriers of Salmonella typhi in Santiago, Chile, an endemic area. *J Infect Dis* **146**: 724-726.

Lin AY, Lin CY, Chen CT & Chen WL (2008) Host defense against Salmonella and rotaviral gastroenteritis: a serial study of transcriptional factors and cytokines. *J Microbiol Immunol Infect* **41**: 265-271.

Lindow JC, Fimlaid KA, Bunn JY & Kirkpatrick BD (2011) Antibodies in action: role of human opsonins in killing Salmonella enterica serovar Typhi. *Infect Immun* **79**: 3188-3194.

Lolekha S (1986) Consequences of treatment of gastrointestinal infections. *Scand J Infect Dis Suppl* **49**: 154-159.

Looney RJ & Steigbigel RT (1986) Role of the Vi antigen of Salmonella typhi in resistance to host defense in vitro. *J Lab Clin Med* **108**: 506-516.

Lundin BS, Johansson C & Svennerholm AM (2002) Oral immunization with a Salmonella enterica serovar typhi vaccine induces specific circulating mucosa-homing CD4(+) and CD8(+) T cells in humans. *Infect Immun* **70**: 5622-5627.

Lutterloh E, Likaka A, Sejvar J, *et al.* (2012) Multidrug-resistant typhoid fever with neurologic findings on the Malawi-Mozambique border. *Clin Infect Dis* **54**: 1100-1106.

Mackay CR, Marston WL & Dudler L (1990) Naive and memory T cells show distinct pathways of lymphocyte recirculation. *J Exp Med* **171**: 801-817.

Mackenzie G, Ceesay SJ, Hill PC, *et al.* (2010) A decline in the incidence of invasive non-typhoidal Salmonella infection in The Gambia temporally associated with a decline in malaria infection. *PLoS One* **5**: e10568.

MacLennan C, Fieschi C, Lammas DA, *et al.* (2004) Interleukin (IL)-12 and IL-23 are key cytokines for immunity against Salmonella in humans. *J Infect Dis* **190**: 1755-1757.

MacLennan CA (2012) Host defense against malaria favors Salmonella. *Nat Med* **18**: 21-22.

MacLennan CA (2013) Vaccines for low-income countries. *Semin Immunol* **25**: 114-123.

Maclennan CA (2014) Editorial commentary: out of Africa: links between invasive nontyphoidal salmonella disease, typhoid Fever, and malaria. *Clin Infect Dis* **58**: 648-650.

Maclennan CA, Martin LB & Micoli F (2014) Vaccines against invasive Salmonella disease: Current status and future directions. *Hum Vaccin Immunother* **10**.

MacLennan CA, Gondwe EN, Msefula CL*, et al.* (2008) The neglected role of antibody in protection against bacteremia caused by nontyphoidal strains of Salmonella in African children. *J Clin Invest* **118**: 1553-1562.

MacLennan CA, Gilchrist JJ, Gordon MA*, et al.* (2010) Dysregulated humoral immunity to nontyphoidal Salmonella in HIV-infected African adults. *Science* **328**: 508-512.

Madsen LB, Ustrup M, Hansen KS, Nyasulu PS, Bygbjerg IC & Konradsen F (2014) Estimating the costs of implementing the rotavirus vaccine in the national immunisation programme: the case of Malawi. *Trop Med Int Health* **19**: 177-185.

Mantis NJ, Rol N & Corthesy B (2011) Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal Immunol* **4**: 603-611.

Martin LB (2012) Vaccines for typhoid fever and other salmonelloses. *Curr Opin Infect Dis* **25**: 489-499.

Mastroeni P (2002) Immunity to systemic Salmonella infections. *Curr Mol Med* **2**: 393-406.

Mastroeni P & Menager N (2003) Development of acquired immunity to Salmonella. *J Med Microbiol* **52**: 453-459.

Mastroeni P, Villarreal-Ramos B & Hormaeche CE (1992) Role of T cells, TNF alpha and IFN gamma in recall of immunity to oral challenge with virulent salmonellae in mice vaccinated with live attenuated aro- Salmonella vaccines. *Microb Pathog* **13**: 477-491.

Mastroeni P, Villarreal-Ramos B & Hormaeche CE (1993) Adoptive transfer of immunity to oral challenge with virulent salmonellae in innately susceptible BALB/c mice requires both immune serum and T cells. *Infect Immun* **61**: 3981-3984.

Mastroeni P, Simmons C, Fowler R, Hormaeche CE & Dougan G (2000) Igh-6(-/-) (B-cell-deficient) mice fail to mount solid acquired resistance to oral challenge with virulent Salmonella enterica serovar typhimurium and show impaired Th1 T-cell responses to Salmonella antigens. *Infect Immun* **68**: 46-53.

Mastroeni PU, S. Chandra, A. MacLennan, C. Doffinger, R. Kumararatne, D. (2003) Resistance and Susceptibility to *Salmonella* infections: lessons from mice and patients with immunodeficiencies. *Rev Med Micro* **14**: 1-10.

Mathanga DP, Walker ED, Wilson ML, Ali D, Taylor TE & Laufer MK (2012) Malaria control in Malawi: current status and directions for the future. *Acta Trop* **121**: 212-217.

Matheson N, Kingsley RA, Sturgess K, Aliyu SH, Wain J, Dougan G & Cooke FJ (2010) Ten years experience of Salmonella infections in Cambridge, UK. *J Infect* **60**: 21-25.

Matsui K (1996) A purified protein from Salmonella typhimurium inhibits proliferation of murine splenic anti-CD3 antibody-activated T-lymphocytes. *FEMS Immunol Med Microbiol* **14**: 121-127.

McAleer JP & Vella AT (2008) Understanding how lipopolysaccharide impacts CD4 T-cell immunity. *Crit Rev Immunol* **28**: 281-299.

MCI (2012) WATER AND SANITATION NEEDS ASSESSMENT FOR BLANTYRE CITY, MALAWI. *MCI SOCIAL SECTOR WORKING PAPER SERIES*.

MCI (2013) HEALTH NEEDS ASSESSMENT FOR BLANTYRE CITY, MALAWI. *MCI SOCIAL SECTOR WORKING PAPER SERIES*.

McSorley SJ & Jenkins MK (2000) Antibody is required for protection against virulent but not attenuated Salmonella enterica serovar typhimurium. *Infect Immun* **68**: 3344-3348.

McSorley SJ, Cookson BT & Jenkins MK (2000) Characterization of CD4+ T cell responses during natural infection with Salmonella typhimurium. *J Immunol* **164**: 986-993.

McSorley SJ, Ehst BD, Yu Y & Gewirtz AT (2002) Bacterial flagellin is an effective adjuvant for CD4+ T cells in vivo. *J Immunol* **169**: 3914-3919.

Meadow WL, Schneider H & Beem MO (1985) Salmonella enteritidis bacteremia in childhood. *J Infect Dis* **152**: 185-189.

Michael JG, Whitby JL & Landy M (1962) Studies on natural antibodies to gram-negative bacteria. *J Exp Med* **115**: 131-146.

Michetti P, Mahan MJ, Slauch JM, Mekalanos JJ & Neutra MR (1992) Monoclonal secretory immunoglobulin A protects mice against oral challenge with the invasive pathogen Salmonella typhimurium. *Infect Immun* **60**: 1786-1792.

Miles AA, Misra SS & Irwin JO (1938) The estimation of the bactericidal power of the blood. *J Hyg (Lond)* **38**: 732-749.

Miller CP, Bohnhoff M & Drake BL (1954) The effect of antibiotic therapy on susceptibility to an experimental enteric infection. *Trans Assoc Am Physicians* **67**: 156-161.

Miller SI & Mekalanos JJ (1990) Constitutive expression of the phoP regulon attenuates Salmonella virulence and survival within macrophages. *J Bacteriol* **172**: 2485-2490.

Miller SI, Kukral AM & Mekalanos JJ (1989) A two-component regulatory system (phoP phoQ) controls Salmonella typhimurium virulence. *Proc Natl Acad Sci U S A* **86**: 5054-5058.

Mitchison NA (1971) The carrier effect in the secondary response to hapten-protein conjugates. II. Cellular cooperation. *Eur J Immunol* **1**: 18-27.

Mittrucker HW & Kaufmann SH (2000) Immune response to infection with Salmonella typhimurium in mice. *J Leukoc Biol* **67**: 457-463.

Mittrucker HW, Kohler A, Mak TW & Kaufmann SH (1999) Critical role of CD28 in protective immunity against Salmonella typhimurium. *J Immunol* **163**: 6769-6776.

Mittrucker HW, Raupach B, Kohler A & Kaufmann SH (2000) Cutting edge: role of B lymphocytes in protective immunity against Salmonella typhimurium infection. *J Immunol* **164**: 1648-1652.

Miyake M, Zhao L, Ezaki T, *et al.* (1998) Vi-deficient and nonfimbriated mutants of Salmonella typhi agglutinate human blood type antigens and are hyperinvasive. *FEMS Microbiol Lett* **161**: 75-82.

Mohr E, Cunningham AF, Toellner KM, *et al.* (2010) IFN-{gamma} produced by CD8 T cells induces T-bet-dependent and -independent class switching in B cells in responses to alum-precipitated protein vaccine. *Proc Natl Acad Sci U S A* **107**: 17292-17297.

Molyneux EM, Mankhambo LA, Phiri A*, et al.* (2009) The outcome of non-typhoidal salmonella meningitis in Malawian children, 1997-2006. *Ann Trop Paediatr* **29**: 13-22.

Monack DM, Mueller A & Falkow S (2004) Persistent bacterial infections: the interface of the pathogen and the host immune system. *Nat Rev Microbiol* **2**: 747-765.

Morpeth SC, Ramadhani HO & Crump JA (2009) Invasive non-Typhi Salmonella disease in Africa. *Clin Infect Dis* **49**: 606-611.

Mouy R, Fischer A, Vilmer E, Seger R & Griscelli C (1989) Incidence, severity, and prevention of infections in chronic granulomatous disease. *J Pediatr* **114**: 555-560.

Msefula CL, Kingsley RA, Gordon MA*, et al.* (2012) Genotypic homogeneity of multidrug resistant S. Typhimurium infecting distinct adult and childhood susceptibility groups in Blantyre, Malawi. *PLoS One* **7**: e42085.

Msefula CM (2009) Aspects of pathogenesis of nontyphoidal Salmonella Bacteraemia in Malawian children: A study of pathogen virulence, antibiotic resistance and carriage. Thesis, University of Liverpool.

Murray GL, Attridge SR & Morona R (2006) Altering the length of the lipopolysaccharide O antigen has an impact on the interaction of Salmonella enterica serovar Typhimurium with macrophages and complement. *J Bacteriol* **188**: 2735-2739.

Musher DM & Rubenstein AD (1973) Permanent carriers of nontyphosa salmonellae. *Arch Intern Med* **132**: 869-872.

Muti M, Gombe N, Tshimanga M, Takundwa L, Bangure D, Mungofa S & Chonzi P (2014) Typhoid outbreak investigation in Dzivaresekwa, suburb of Harare City, Zimbabwe, 2011. *Pan Afr Med J* **18**: 309.

Nambiar M, Harish BN, Mangilal V, Pai D & Parija SC (2009) Immunoblot analysis of sera in uncomplicated typhoid fever & with typhoid ileal perforation. *Indian J Med Res* **129**: 432-437.

Navaneethan U & Giannella RA (2008) Mechanisms of infectious diarrhea. *Nat Clin Pract Gastroenterol Hepatol* **5**: 637-647.

Neil KP, Sodha SV, Lukwago L*, et al.* (2012) A large outbreak of typhoid fever associated with a high rate of intestinal perforation in Kasese District, Uganda, 2008-2009. *Clin Infect Dis* **54**: 1091-1099.

Nkuo-Akenji TK, Ntemgwa ML & Ndip RN (2001) Asymptomatic salmonellosis and drug susceptibility in the Buea District, Cameroon. *Cent Afr J Med* **47**: 254-257.

Nyirenda TS, Seeley AE, Mandala WL, Drayson MT & MacLennan CA (2010) Early interferon-gamma production in human lymphocyte subsets in response to nontyphoidal Salmonella demonstrates inherent capacity in innate cells. *PLoS One* **5**: e13667.

O'Brien AD & Metcalf ES (1982) Control of early Salmonella typhimurium growth in innately Salmonella-resistant mice does not require functional T lymphocytes. *J Immunol* **129**: 1349-1351.

Ogundele M (2001) Role and significance of the complement system in mucosal immunity: particular reference to the human breast milk complement. *Immunol Cell Biol* **79**: 1-10.

Ogundele MO (1999) Complement-mediated bactericidal activity of human milk to a serum-susceptible strain of E. coli 0111. *J Appl Microbiol* **87**: 689-696.

Ogundele MO (2000) Activation and deposition of human breast-milk complement C3 opsonins on serum sensitive Escherichia coli 0111. *J Reprod Immunol* **48**: 99-105.

Ohl ME & Miller SI (2001) Salmonella: a model for bacterial pathogenesis. *Annu Rev Med* **52**: 259-274.

Okoro CK, Kingsley RA, Connor TR*, et al.* (2012) Intracontinental spread of human invasive Salmonella Typhimurium pathovariants in sub-Saharan Africa. *Nat Genet* **44**: 1215-1221.

Olanratmanee T, Levine M, Losonsky G, Thisyakorn V & Cryz SJ, Jr. (1992) Safety and immunogenicity of Salmonella typhi Ty21a liquid formulation vaccine in 4- to 6-year-old Thai children. *J Infect Dis* **166**: 451-452.

Pafumi C, Leanza V, Carbonaro A*, et al.* (2011) CD34(+) stem cells from umbilical cord blood. *Clin Pract* **1**: e79.

Papasavvas E, Azzoni L, Foulkes A*, et al.* (2011) Increased microbial translocation in </= 180 days old perinatally human immunodeficiency virus-positive infants as compared with human immunodeficiency virus-exposed uninfected infants of similar age. *Pediatr Infect Dis J* **30**: 877-882.

Park SH, Ryu S & Kang DH (2012) Development of an improved selective and differential medium for isolation of Salmonella spp. *J Clin Microbiol* **50**: 3222-3226.

Parkhill J, Dougan G, James KD*, et al.* (2001) Complete genome sequence of a multiple drug resistant Salmonella enterica serovar Typhi CT18. *Nature* **413**: 848-852.

Parry CM, Hien TT, Dougan G, White NJ & Farrar JJ (2002) Typhoid fever. *N Engl J Med* **347**: 1770-1782.

Parsons BN, Humphrey S, Salisbury AM, Mikoleit J, Hinton JC, Gordon MA & Wigley P (2013) Invasive non-typhoidal Salmonella typhimurium ST313 are not host-restricted and have an invasive phenotype in experimentally infected chickens. *PLoS Negl Trop Dis* **7**: e2487.

Pasetti MF, Simon JK, Sztein MB & Levine MM (2011) Immunology of gut mucosal vaccines. *Immunol Rev* **239**: 125-148.

Pavia AT, Shipman LD, Wells JG, Puhr ND, Smith JD, McKinley TW & Tauxe RV (1990) Epidemiologic evidence that prior antimicrobial exposure decreases resistance to infection by antimicrobial-sensitive Salmonella. *J Infect Dis* **161**: 255-260.

Pegues DA, Hantman MJ, Behlau I & Miller SI (1995) PhoP/PhoQ transcriptional repression of Salmonella typhimurium invasion genes: evidence for a role in protein secretion. *Mol Microbiol* **17**: 169-181.

Peters RP, Zijlstra EE, Schijffelen MJ*, et al.* (2004) A prospective study of bloodstream infections as cause of fever in Malawi: clinical predictors and implications for management. *Trop Med Int Health* **9**: 928-934.

Pickard D, Wain J, Baker S*, et al.* (2003) Composition, acquisition, and distribution of the Vi exopolysaccharide-encoding Salmonella enterica pathogenicity island SPI-7. *J Bacteriol* **185**: 5055-5065.

Pitzer VE, Bowles CC, Baker S, Kang G, Balaji V, Farrar JJ & Grenfell BT (2014) Predicting the impact of vaccination on the transmission dynamics of typhoid in South Asia: a mathematical modeling study. *PLoS Negl Trop Dis* **8**: e2642.

Pitzinger B, Steffen R & Tschopp A (1991) Incidence and clinical features of traveler's diarrhea in infants and children. *Pediatr Infect Dis J* **10**: 719-723.

Polonsky JA, Martinez-Pino I, Nackers F*, et al.* (2014) Descriptive Epidemiology of Typhoid Fever during an Epidemic in Harare, Zimbabwe, 2012. *PLoS One* **9**: e114702.

Pulickal AS, Gautam S, Clutterbuck EA*, et al.* (2009) Kinetics of the natural, humoral immune response to Salmonella enterica serovar Typhi in Kathmandu, Nepal. *Clin Vaccine Immunol* **16**: 1413-1419.

Quiding-Jarbrink M, Nordstrom I, Granstrom G*, et al.* (1997) Differential expression of tissue-specific adhesion molecules on human circulating antibody-forming cells after systemic, enteric, and nasal immunizations. A molecular basis for the compartmentalization of effector B cell responses. *J Clin Invest* **99**: 1281-1286.

Raffatellu M, Chessa D, Wilson RP, Dusold R, Rubino S & Baumler AJ (2005) The Vi capsular antigen of Salmonella enterica serotype Typhi reduces Toll-like receptor-dependent interleukin-8 expression in the intestinal mucosa. *Infect Immun* **73**: 3367-3374.

Raffatellu M, Santos RL, Verhoeven DE*, et al.* (2008) Simian immunodeficiency virus-induced mucosal interleukin-17 deficiency promotes Salmonella dissemination from the gut. *Nat Med* **14**: 421-428.

Ramarathinam L, Niesel DW & Klimpel GR (1993) Salmonella typhimurium induces IFN-gamma production in murine splenocytes. Role of natural killer cells and macrophages. *J Immunol* **150**: 3973-3981.

Rapola S, Jantti V, Haikala R*, et al.* (2000) Natural development of antibodies to pneumococcal surface protein A, pneumococcal surface adhesin A, and pneumolysin in relation to pneumococcal carriage and acute otitis media. *J Infect Dis* **182**: 1146-1152.

Rautemaa R & Meri S (1999) Complement-resistance mechanisms of bacteria. *Microbes Infect* **1**: 785-794.

Reddy EA, Shaw AV & Crump JA (2010) Community-acquired bloodstream infections in Africa: a systematic review and meta-analysis. *Lancet Infect Dis* **10**: 417-432.

Reeves MW, Evins GM, Heiba AA, Plikaytis BD & Farmer JJ, 3rd (1989) Clonal nature of Salmonella typhi and its genetic relatedness to other salmonellae as shown by multilocus enzyme electrophoresis, and proposal of Salmonella bongori comb. nov. *J Clin Microbiol* **27**: 313-320.

Rescigno M, Urbano M, Valzasina B*, et al.* (2001) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol* **2**: 361-367.

Reynolds JV, O'Farrelly C, Feighery C*, et al.* (1996) Impaired gut barrier function in malnourished patients. *Br J Surg* **83**: 1288-1291.

Robbins JD & Robbins JB (1984) Reexamination of the protective role of the capsular polysaccharide (Vi antigen) of Salmonella typhi. *J Infect Dis* **150**: 436-449.

Roca-Feltrer A, Kwizombe CJ, Sanjoaquin MA*, et al.* (2012) Lack of decline in childhood malaria, Malawi, 2001-2010. *Emerg Infect Dis* **18**: 272-278.

Rock KL & Shen L (2005) Cross-presentation: underlying mechanisms and role in immune surveillance. *Immunol Rev* **207**: 166-183.

Roger LC, Costabile A, Holland DT, Hoyles L & McCartney AL (2010) Examination of faecal Bifidobacterium populations in breast- and formula-fed infants during the first 18 months of life. *Microbiology* **156**: 3329-3341.

Roos A, Bouwman LH, van Gijlswijk-Janssen DJ, Faber-Krol MC, Stahl GL & Daha MR (2001) Human IgA activates the complement system via the mannan-binding lectin pathway. *J Immunol* **167**: 2861-2868.

Roux CM, Butler BP, Chau JY*, et al.* (2010) Both hemolytic anemia and malaria parasite-specific factors increase susceptibility to Nontyphoidal Salmonella enterica serovar typhimurium infection in mice. *Infect Immun* **78**: 1520-1527.

Rowe B, Ward LR & Threlfall EJ (1997) Multidrug-resistant Salmonella typhi: a worldwide epidemic. *Clin Infect Dis* **24 Suppl 1**: S106-109.

Saade F, Gorski SA & Petrovsky N (2012) Pushing the frontiers of T-cell vaccines: accurate measurement of human T-cell responses. *Expert Rev Vaccines* **11**: 1459-1470.

Sabbagh SC, Forest CG, Lepage C, Leclerc JM & Daigle F (2010) So similar, yet so different: uncovering distinctive features in the genomes of Salmonella enterica serovars Typhimurium and Typhi. *FEMS Microbiol Lett* **305**: 1-13.

Salazar-Gonzalez RM, Maldonado-Bernal C, Ramirez-Cruz NE*, et al.* (2004) Induction of cellular immune response and anti-Salmonella enterica serovar typhi bactericidal antibodies in healthy volunteers by immunization with a vaccine candidate against typhoid fever. *Immunol Lett* **93**: 115-122.

Salerno-Goncalves R & Sztein MB (2009) Priming of Salmonella enterica serovar typhi-specific CD8(+) T cells by suicide dendritic cell cross-presentation in humans. *PLoS One* **4**: e5879.

Salerno-Goncalves R, Pasetti MF & Sztein MB (2002) Characterization of CD8(+) effector T cell responses in volunteers immunized with Salmonella enterica serovar Typhi strain Ty21a typhoid vaccine. *J Immunol* **169**: 2196-2203.

Salerno-Goncalves R, Wahid R & Sztein MB (2005) Immunization of volunteers with Salmonella enterica serovar Typhi strain Ty21a elicits the oligoclonal expansion of CD8+ T cells with predominant Vbeta repertoires. *Infect Immun* **73**: 3521-3530.

Sallusto F, Lenig D, Forster R, Lipp M & Lanzavecchia A (1999) Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401**: 708-712.

Sansonetti PJ (2004) War and peace at mucosal surfaces. *Nat Rev Immunol* **4**: 953-964.

Saul A, Smith T & Maire N (2013) Stochastic simulation of endemic Salmonella enterica serovar Typhi: the importance of long lasting immunity and the carrier state. *PLoS One* **8**: e74097.

Schreiber F, Lynn DJ, Houston A*, et al.* (2011) The human transcriptome during nontyphoid Salmonella and HIV coinfection reveals attenuated NFkappaB-mediated inflammation and persistent cell cycle disruption. *J Infect Dis* **204**: 1237-1245.

Schultz M (2008) Theobald Smith. *Emerg Infect Dis* **14**: 1940–1942.

Secundino I, Lopez-Macias C, Cervantes-Barragan L*, et al.* (2006) Salmonella porins induce a sustained, lifelong specific bactericidal antibody memory response. *Immunology* **117**: 59-70.

Sejvar J, Lutterloh E, Naiene J*, et al.* (2012) Neurologic manifestations associated with an outbreak of typhoid fever, Malawi--Mozambique, 2009: an epidemiologic investigation. *PLoS One* **7**: e46099.

Sekirov I, Russell SL, Antunes LC & Finlay BB (2010) Gut microbiota in health and disease. *Physiol Rev* **90**: 859-904.

Sepako E, Glennie SJ, Jambo KC*, et al.* (2014) Incomplete recovery of pneumococcal CD4 T cell immunity after initiation of antiretroviral therapy in HIV-infected malawian adults. *PLoS One* **9**: e100640.

Shapiro RL, Lockman S, Kim S*, et al.* (2007) Infant morbidity, mortality, and breast milk immunologic profiles among breast-feeding HIV-infected and HIV-uninfected women in Botswana. *J Infect Dis* **196**: 562-569.

Sharifi Mood B, Mohraz M, Mansouri SD*, et al.* (2004) Recurrent non-typhoidal salmonella bacteremia in a patient with interleukin -12p40 deficiency. *Iran J Allergy Asthma Immunol* **3**: 197-200.

Sharma A & Qadri A (2004) Vi polysaccharide of Salmonella typhi targets the prohibitin family of molecules in intestinal epithelial cells and suppresses early inflammatory responses. *Proc Natl Acad Sci U S A* **101**: 17492-17497.

Sheikh A, Bhuiyan MS, Khanam F*, et al.* (2009) Salmonella enterica serovar Typhi-specific immunoglobulin A antibody responses in plasma and antibody in lymphocyte supernatant specimens in Bangladeshi patients with suspected typhoid fever. *Clin Vaccine Immunol* **16**: 1587-1594.

Sheikh A, Khanam F, Sayeed MA*, et al.* (2011) Interferon-gamma and proliferation responses to Salmonella enterica Serotype Typhi proteins in patients with S. Typhi Bacteremia in Dhaka, Bangladesh. *PLoS Negl Trop Dis* **5**: e1193.

Silverman M & Simon M (1980) Phase variation: genetic analysis of switching mutants. *Cell* **19**: 845-854.

Simon M, Zieg J, Silverman M, Mandel G & Doolittle R (1980) Phase variation: evolution of a controlling element. *Science* **209**: 1370-1374.

Simon R & Levine MM (2012) Glycoconjugate vaccine strategies for protection against invasive Salmonella infections. *Hum Vaccin Immunother* **8**: 494-498.

Simon R, Tennant SM, Wang JY, *et al.* (2011) Salmonella enterica serovar enteritidis core O polysaccharide conjugated to H:g,m flagellin as a candidate vaccine for protection against invasive infection with S. enteritidis. *Infect Immun* **79**: 4240-4249.

Sinha K, Mastroeni P, Harrison J, de Hormaeche RD & Hormaeche CE (1997) Salmonella typhimurium aroA, htrA, and aroD htrA mutants cause progressive infections in athymic (nu/nu) BALB/c mice. *Infect Immun* **65**: 1566-1569.

Sirsat SA (2013) Can Salmonella Reside in the Human Oral Cavity? . *Agric. Food Anal. Bacteriol.* **3**: 30-38.

Soininen A, Pursiainen H, Kilpi T & Kayhty H (2001) Natural development of antibodies to pneumococcal capsular polysaccharides depends on the serotype: association with pneumococcal carriage and acute otitis media in young children. *J Infect Dis* **184**: 569-576.

Srinivasan A & McSorley SJ (2006) Activation of Salmonella-specific immune responses in the intestinal mucosa. *Arch Immunol Ther Exp (Warsz)* **54**: 25-31.

Srinivasan A & McSorley SJ (2007) Pivotal advance: exposure to LPS suppresses CD4+ T cell cytokine production in Salmonella-infected mice and exacerbates murine typhoid. *J Leukoc Biol* **81**: 403-411.

Stecher B & Hardt WD (2011) Mechanisms controlling pathogen colonization of the gut. *Curr Opin Microbiol* **14**: 82-91.

Stefanich EG, Danilenko DM, Wang H, *et al.* (2011) A humanized monoclonal antibody targeting the beta7 integrin selectively blocks intestinal homing of T lymphocytes. *Br J Pharmacol* **162**: 1855-1870.

Steffen R (2005) Epidemiology of traveler's diarrhea. *Clin Infect Dis* **41 Suppl 8**: S536-540.

Stoycheva MV & Murdjeva MA (2004) Cytokines in Salmonella infections. *Folia Med (Plovdiv)* **46**: 5-10.

Streeter PR, Rouse BT & Butcher EC (1988) Immunohistologic and functional characterization of a vascular addressin involved in lymphocyte homing into peripheral lymph nodes. *J Cell Biol* **107**: 1853-1862.

Strid MA, Dalby T, Molbak K & Krogfelt KA (2007) Kinetics of the human antibody response against Salmonella enterica Serovars Enteritidis and Typhimurium determined by lipopolysaccharide enzyme-linked immunosorbent assay. *Clin Vaccine Immunol* **14**: 741-747.

Su LH & Chiu CH (2007) Salmonella: clinical importance and evolution of nomenclature. *Chang Gung Med J* **30**: 210-219.

Sundquist M, Rydstrom A & Wick MJ (2004) Immunity to Salmonella from a dendritic point of view. *Cell Microbiol* **6**: 1-11.

Sur D, Ochiai RL, Bhattacharya SK, *et al.* (2009) A cluster-randomized effectiveness trial of Vi typhoid vaccine in India. *N Engl J Med* **361**: 335-344.

Sztein MB (2007) Cell-mediated immunity and antibody responses elicited by attenuated Salmonella enterica Serovar Typhi strains used as live oral vaccines in humans. *Clin Infect Dis* **45 Suppl 1**: S15-19.

Szu SC (2013) Development of Vi conjugate - a new generation of typhoid vaccine. *Expert Rev Vaccines* **12**: 1273-1286.

Tacket CO & Levine MM (2007) CVD 908, CVD 908-htrA, and CVD 909 live oral typhoid vaccines: a logical progression. *Clin Infect Dis* **45 Suppl 1**: S20-23.

Tacket CO, Levine MM & Robbins JB (1988) Persistence of antibody titres three years after vaccination with Vi polysaccharide vaccine against typhoid fever. *Vaccine* **6**: 307-308.

Tacket CO, Pasetti MF, Sztein MB, Livio S & Levine MM (2004) Immune responses to an oral typhoid vaccine strain that is modified to constitutively express Vi capsular polysaccharide. *J Infect Dis* **190**: 565-570.

Tacket CO, Ferreccio C, Robbins JB*, et al.* (1986) Safety and immunogenicity of two Salmonella typhi Vi capsular polysaccharide vaccines. *J Infect Dis* **154**: 342-345.

Tacket CO, Sztein MB, Wasserman SS*, et al.* (2000) Phase 2 clinical trial of attenuated Salmonella enterica serovar typhi oral live vector vaccine CVD 908-htrA in U.S. volunteers. *Infect Immun* **68**: 1196-1201.

Takemori T, Kaji T, Takahashi Y, Shimoda M & Rajewsky K (2014) Generation of memory B cells inside and outside germinal centers. *Eur J Immunol* **44**: 1258-1264.

Tam MA, Rydstrom A, Sundquist M & Wick MJ (2008) Early cellular responses to Salmonella infection: dendritic cells, monocytes, and more. *Immunol Rev* **225**: 140-162.

Thatte J, Rath S & Bal V (1993) Immunization with live versus killed Salmonella typhimurium leads to the generation of an IFN-gamma-dominant versus an IL-4-dominant immune response. *Int Immunol* **5**: 1431-1436.

Thomas JE, Bunn JE, Kleanthous H, Monath TP, Harding M, Coward WA & Weaver LT (2004) Specific immunoglobulin A antibodies in maternal milk and delayed Helicobacter pylori colonization in Gambian infants. *Clin Infect Dis* **39**: 1155-1160.

Thompson LJ, Dunstan SJ, Dolecek C*, et al.* (2009) Transcriptional response in the peripheral blood of patients infected with Salmonella enterica serovar Typhi. *Proc Natl Acad Sci U S A* **106**: 22433-22438.

Toapanta FR, Simon JK, Barry EM, Pasetti MF, Levine MM, Kotloff KL & Sztein MB (2014) Gut-Homing Conventional Plasmablasts and CD27(-) Plasmablasts Elicited after a Short Time of Exposure to an Oral Live-Attenuated Shigella Vaccine Candidate in Humans. *Front Immunol* **5**: 374.

Tobar JA, Carreno LJ, Bueno SM, Gonzalez PA, Mora JE, Quezada SA & Kalergis AM (2006) Virulent Salmonella enterica serovar typhimurium evades adaptive immunity by preventing dendritic cells from activating T cells. *Infect Immun* **74**: 6438-6448.

Toobak H, Rasooli I, Talei D, Jahangiri A, Owlia P & Darvish Alipour Astaneh S (2013) Immune response variations to Salmonella enterica serovar Typhi recombinant porin proteins in mice. *Biologicals* **41**: 224-230.

Trebicka E, Jacob S, Pirzai W, Hurley BP & Cherayil BJ (2013) Role of anti-lipopolysaccharide antibodies in serum bactericidal activity against Salmonella enterica serovar Typhimurium in healthy adults and children in the United States. *Clin Vaccine Immunol*.

Uchiya K, Barbieri MA, Funato K, Shah AH, Stahl PD & Groisman EA (1999) A Salmonella virulence protein that inhibits cellular trafficking. *EMBO J* **18**: 3924-3933.

Udhayakumar V & Muthukkaruppan VR (1987) Protective immunity induced by outer membrane proteins of Salmonella typhimurium in mice. *Infect Immun* **55**: 816-821.

Uhnoo I, Olding-Stenkvist E & Kreuger A (1986) Clinical features of acute gastroenteritis associated with rotavirus, enteric adenoviruses, and bacteria. *Arch Dis Child* **61**: 732-738.

Uppington H, Menager N, Boross P, Wood J, Sheppard M, Verbeek S & Mastroeni P (2006) Effect of immune serum and role of individual Fcgamma receptors on the intracellular distribution and survival of Salmonella enterica serovar Typhimurium in murine macrophages. *Immunology* **119**: 147-158.

van Bruggen R, Zweers D, van Diepen A, van Dissel JT, Roos D, Verhoeven AJ & Kuijpers TW (2007) Complement receptor 3 and Toll-like receptor 4 act sequentially in uptake and intracellular killing of unopsonized Salmonella enterica serovar Typhimurium by human neutrophils. *Infect Immun* **75**: 2655-2660.

van Damme P, Kafeja F, Anemona A*, et al.* (2011) Safety, immunogenicity and dose ranging of a new Vi-CRM(1)(9)(7) conjugate vaccine against typhoid fever: randomized clinical testing in healthy adults. *PLoS One* **6**: e25398.

van den Bergh ET, Gasem MH, Keuter M & Dolmans MV (1999) Outcome in three groups of patients with typhoid fever in Indonesia between 1948 and 1990. *Trop Med Int Health* **4**: 211-215.

Vazquez-Torres A, Jones-Carson J, Baumler AJ*, et al.* (1999) Extraintestinal dissemination of Salmonella by CD18-expressing phagocytes. *Nature* **401**: 804-808.

Vidal SM, Malo D, Vogan K, Skamene E & Gros P (1993) Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. *Cell* **73**: 469-485.

Wahid R, Salerno-Goncalves R, Tacket CO, Levine MM & Sztein MB (2007) Cell-mediated immune responses in humans after immunization with one or two doses of oral live attenuated typhoid vaccine CVD 909. *Vaccine* **25**: 1416-1425.

Wahid R, Zafar SJ, McArthur MA, Pasetti MF, Levine MM & Sztein MB (2014) Live oral Salmonella enterica serovar Typhi vaccines Ty21a and CVD 909 induce opsonophagocytic functional antibodies in humans that cross-react with S. Paratyphi A and S. Paratyphi B. *Clin Vaccine Immunol* **21**: 427-434.

Wahid R, Pasetti MF, Maciel M, Jr., Simon JK, Tacket CO, Levine MM & Sztein MB (2011) Oral priming with Salmonella Typhi vaccine strain CVD 909 followed by parenteral boost with the S. Typhi Vi capsular polysaccharide vaccine induces CD27+IgD-S. Typhi-specific IgA and IgG B memory cells in humans. *Clin Immunol* **138**: 187-200.

Wain J & Hosoglu S (2008) The laboratory diagnosis of enteric fever. *J Infect Dev Ctries* **2**: 421-425.

Wain J, House D, Zafar A*, et al.* (2005) Vi antigen expression in Salmonella enterica serovar Typhi clinical isolates from Pakistan. *J Clin Microbiol* **43**: 1158-1165.

Wain J, Pham VB, Ha V*, et al.* (2001) Quantitation of bacteria in bone marrow from patients with typhoid fever: relationship between counts and clinical features. *J Clin Microbiol* **39**: 1571-1576.

Walters MS, Routh J, Mikoleit M*, et al.* (2014) Shifts in geographic distribution and antimicrobial resistance during a prolonged typhoid fever outbreak--Bundibugyo and Kasese Districts, Uganda, 2009-2011. *PLoS Negl Trop Dis* **8**: e2726.

Warren J, Mastroeni P, Dougan G, Noursadeghi M, Cohen J, Walport MJ & Botto M (2002) Increased susceptibility of C1q-deficient mice to Salmonella enterica serovar Typhimurium infection. *Infect Immun* **70**: 551-557.

Watson PR, Paulin SM, Bland AP, Jones PW & Wallis TS (1995) Characterization of intestinal invasion by Salmonella typhimurium and Salmonella dublin and effect of a mutation in the invH gene. *Infect Immun* **63**: 2743-2754.

Weening EH, Barker JD, Laarakker MC, Humphries AD, Tsolis RM & Baumler AJ (2005) The Salmonella enterica serotype Typhimurium lpf, bcf, stb, stc, std, and sth fimbrial operons are required for intestinal persistence in mice. *Infect Immun* **73**: 3358-3366.

Weiss KF (1965) Inhibitory Action of Selenite on Escherichia coli, Proteus vulgaris, and Salmonella thompson. *J. Bacteriol.* **90**: 857-862.

WHO (2003) The diagnosis, treatment and prevention of typhoid fever. ed.^eds.), p.^pp. 7-18.

WHO (2008) Typhoid vaccines: WHO position paper. **83**: 49–60.

Wilson RP, Raffatellu M, Chessa D, Winter SE, Tukel C & Baumler AJ (2008) The Vi-capsule prevents Toll-like receptor 4 recognition of Salmonella. *Cell Microbiol* **10**: 876-890.

Winter SE, Raffatellu M, Wilson RP, Russmann H & Baumler AJ (2008) The Salmonella enterica serotype Typhi regulator TviA reduces interleukin-8 production in intestinal epithelial cells by repressing flagellin secretion. *Cell Microbiol* **10**: 247-261.

Wolf PR & Ploegh HL (1995) How MHC class II molecules acquire peptide cargo: biosynthesis and trafficking through the endocytic pathway. *Annu Rev Cell Dev Biol* **11**: 267-306.

Woof JM & Kerr MA (2006) The function of immunoglobulin A in immunity. *J Pathol* **208**: 270-282.

Wright RC (1986) The seasonality of bacterial quality of water in a tropical developing country (Sierra Leone). *J Hyg (Lond)* **96**: 75-82.

Xu HR, Hsu HS, Moncure CW & King RA (1993) Correlation of antibody titres induced by vaccination with protection in mouse typhoid. *Vaccine* **11**: 725-729.

Yatsunenko T, Rey FE, Manary MJ*, et al.* (2012) Human gut microbiome viewed across age and geography. *Nature* **486**: 222-227.

Ygberg S & Nilsson A (2012) The developing immune system - from foetus to toddler. *Acta Paediatr* **101**: 120-127.

Yong DC, Thompson JS & Prytula A (1985) Rapid microbiochemical method for presumptive identification of gastroenteritis-associated members of the family Enterobacteriaceae. *J Clin Microbiol* **21**: 914-918.

Yrlid U, Svensson M, Johansson C & Wick MJ (2000) Salmonella infection of bone marrow-derived macrophages and dendritic cells: influence on antigen presentation and initiating an immune response. *FEMS Immunol Med Microbiol* **27**: 313-320.

Zaki SA & Karande S (2011) Multidrug-resistant typhoid fever: a review. *J Infect Dev Ctries* **5**: 324-337.

Zeng H, Carlson AQ, Guo Y*, et al.* (2003) Flagellin is the major proinflammatory determinant of enteropathogenic Salmonella. *J Immunol* **171**: 3668-3674.

Zhang XL, Jeza VT & Pan Q (2008) Salmonella typhi: from a human pathogen to a vaccine vector. *Cell Mol Immunol* **5**: 91-97.

APPENDIX

MAJOR ARTICLE

Sequential Acquisition of T Cells and Antibodies to Nontyphoidal *Salmonella* in Malawian Children

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Background. *Salmonella* Typhimurium (STm) remain a prominent cause of bacteremia in sub-Saharan Africa. Complement-fixing antibodies to STm develop by 2 years of age. We hypothesized that STm-specific $CD4^+$ T cells develop alongside this process.

Methods. Eighty healthy Malawian children aged 0–60 months were recruited. STm-specific $CD4^+$ T cells producing interferon γ, tumor necrosis factor α, and interleukin 2 were quantified using intracellular cytokine staining. Antibodies to STm were measured by serum bactericidal activity (SBA) assay, and anti-STm immunoglobulin G antibodies by enzyme-linked immunosorbent assay.

Results. Between 2006 and 2011, STm bacteremias were detected in 449 children <5 years old. STm-specific $CD4^+$ T cells were acquired in infancy, peaked at 14 months, and then declined. STm-specific SBA was detectable in newborns, declined in the first 8 months, and then increased to a peak at age 35 months. Acquisition of SBA correlated with acquisition of anti–STm–lipopolysaccharide (LPS) immunoglobulin G ($r = 0.329$ [95% confidence interval, .552–.062]; $P = .01$) but not anti–STm–outer membrane protein or anti–STm-flagellar protein (FliC).

Conclusions. Acquisition of STm-specific $CD4^+$ T cells in early childhood is consistent with early exposure to STm or cross-reactive protein antigens priming this T-cell development. STm-specific $CD4^+$ T cells seem insufficient to protect against invasive nontyphoidal *Salmonella* disease, but sequential acquisition of SBA to STm LPS is associated with a decline in its incidence.

Keywords. Salmonella; T cell; antibody; cytokine; children.

Nontyphoidal *Salmonella* (NTS), mainly *Salmonella enterica* serovars Typhimurium (STm) and Enteritidis, commonly causes bacteremia among young children in sub-Saharan Africa [1, 2]. Although NTS bacteremia is undergoing considerable epidemiological change [3, 4], the case fatality in children continues to exceed 20% [1]. Important risk factors for NTS bacteremia include age <2 years, malnutrition, severe malarial anemia, and human immunodeficiency virus (HIV) infection [1]. In resource-poor settings, lack of diagnostic services, increasing multidrug resistance, and the nonspecific nature of clinical presentation all compromise effective diagnosis and treatment of these children [1].

Evidence from whole-genome sequencing of STm, the most common NTS serovar isolated in Malawi, suggests that a pathovar characterized by multilocus sequence type 313 dominates invasive NTS (iNTS) disease in Africa [5]. Rarely seen in industrialized countries, sequence type 313 has undergone genomic degradation which suggests both the loss of an enteric lifestyle and possible human-host adaptation [6, 7]. Mouse models of disease caused by this facultative intracellular pathogen implicate innate immune cell

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phagocytosis, T-cell immunity, and antibody-mediated immunity [8, 9]. If iNTS is to be controlled effectively through public health interventions or vaccination, human studies are needed to establish the key immune components that constitute naturally acquired immunity in young children.

Most Malawian children acquire anti-*Salmonella* immunoglobulin G (IgG) and immunoglobulin M antibody and bactericidal activity against NTS by 2 years of age [10]. Antibodies targeting NTS can effect bacterial killing through activation of complement cascade and assembly of the membrane attack complex [10]. Antibodies opsonize NTS and, together with C3b deposition, facilitate internalization by phagocytes and subsequent killing of NTS through oxidative burst [11]. These immune processes are critical for preventing extracellular growth and dissemination of NTS [10]. Although it is known that $CD4^+$ T cells orchestrate macrophage effector functions through interferon (IFN) γ and tumor necrosis factor (TNF) α [12, 13] and that HIV-infected individuals with low CD4 counts are particularly susceptible to iNTS disease [14], the contribution of $CD4^+$ T-cell–mediated control of NTS in humans has not been well studied. We therefore explored the hypothesis that in the first 2 years of life $CD4^+$ T-cell immune responses to STm develop in parallel with the development of anti-STm antibodies. Contrary to our expectations, we have found that although acquisition of STm-specific $CD4^+$ T-cell immunity occurs together with antibody to STm protein antigens, these are evident before the development of serum bactericidal activity. This STm-specific $CD4^+$ T-cell immunity seems insufficient to protect against iNTS disease in Malawian children, which declines in incidence in parallel with the later development of antibodies targeting STm LPS O-antigen.

METHODS

Setting and Bloodstream Infection Surveillance

Queen Elizabeth Central Hospital is a 1250-bed teaching hospital and the largest government hospital in Malawi, providing free health care to Blantyre district (population approximately 1 million). It is the only inpatient pediatric facility for non–fee-paying patients in Blantyre. The Malawi-Liverpool-Wellcome Trust Clinical Research Programme has undertaken routine bloodstream infection surveillance of febrile children presenting to Queen Elizabeth Central Hospital since 1997. Blood cultures are obtained from febrile children whose thick films are negative for malaria parasites or who are critically ill, irrespective of malaria infection. Blood culture is undertaken using a pediatric bottle (BacT/Alert PF BioMerieux), and isolates identified using standard techniques [15].

Healthy Study Participants

A total of 80 healthy children (Table 1), in 8 predefined age categories ranging from 0 to 60 months, were prospectively recruited at a large community health center in Blantyre, Malawi, from March 2009 to January 2011. Children with malaria parasitemia, a positive HIV antibody test, severe anemia (hemoglobin <7 g/dL), malnutrition (weight-for-height *z* score ≤2), or other chronic illness were excluded from the study. Ethical approval for the study (protocol P.08/09/815) was obtained from College of Medicine Research Ethics Committee, and written informed consent was obtained from the parent or guardian of every participating child.

Characterization of $CD4^+$ Memory T-Cell Subsets

Whole blood was collected in ethylenediaminetetraacetic acid–anticoagulated tubes; 200 µL of blood was stained with antibodies (CD3 –allophycocyanin (APC), CD4-Pacific Blue, CD45RO–fluorescein isothiocyanate, and CCR7-phycoerythrin [all Becton Dickson]) and red blood cells lysed with 2 mL of 1× fluorescence-activated cell sorting (FACS) lysing solution (Becton Dickson). Cells were washed with phosphate-buffered saline (PBS; Sigma Aldrich) and fixed in 200 µL of 1% formaldehyde/PBS. Up to 20 000 events on a $CD4^+$ T-lymphocyte gate were acquired immediately with a CyAN ADP flow cytometer (Beckman Coulter) and analyzed using FlowJo software (version 7.6.5, Tree Star). Lymphocytes were gated by their forward scatter and side scatter characteristics. We defined naive T cells

Table 1. General Characteristics and Nutritional and Hematological Profile

Parameter	Female Participants	Male Participants	All Participants
Participants, No. (%)	35 (43.7)	45 (56.3)	80 (100)
Age, median (range), mo	13.2 (0–52.5)	10 (0–47)	10.2 (0–52.5)
Weight, median (range), kg	9.5 (3.5–17)[a]	10 (6–16.9)[b]	ND
Height, median (range), cm	73.5 (48–97)[a]	74 (52–95)[b]	ND
Weight for height *z* score, median (range)	0.89 (−1.9–4.6)[a]	1.4 (−2–4)[b]	ND
Lymphocyte count, median (range), ×10³/µL	6.3 (2.9–13.46)	5.3 (2.2–10.4)	5.4 (2.2–13.6)
Hemoglobin, median (range), g/dL	11.5 (7.6–18.1)	11.2 (8.0–17.7)	11.4 (7.6–18.1)

Abbreviation: ND, not determined.

[a] Twenty-five children aged 1–60 months were included.

[b] Thirty-five children aged 1–60 months were included.

as CD4$^+$CD45RO$^-$CCR7$^+$, effector memory (EM) T cells as CD4$^+$CD45RO$^+$CCR7$^-$, and central memory (CM) T cells as CD4$^+$CD45RO$^+$CCR7$^+$.

Detection of CD4$^+$ T cells Producing Cytokines

After whole blood for intracellular cytokine staining assay was collected in sodium heparin tubes, 450 µL of blood was stimulated with 50 µL of a bead-beaten STm strain D23580 [16] at the final concentration of 1 µg/mL or phorbol 12-myristate 13-acetate (PMA) at 1 µg/mL and ionomycin at 10 µg/mL (all Sigma Aldrich), and costimulated with anti-CD28/49d (Becton Dickson) for 6 hours at 37°C. At 2 hours, intracellular cytokine release was inhibited with BD Golgi Stop (Becton Dickson), and 200-µL samples were lysed with 2 mL of 1× FACS lysing solution and then permeabilized with 500 µL of 1× permeabilizing solution (Becton Dickson). Cells were washed with PBS/0.5% bovine serum albumin (BSA) buffer (Sigma Aldrich) and stained with 3 µL of surface antibodies (CD3-APC cyanine 7 and CD4-PB) and 5 µL of intracellular cytokine antibodies (IFN-γ–phycoerythrin, TNF-α–fluorescein isothiocyanate, and interleukin (IL) 2–APC [all Becton Dickson]). Cells were fixed and events acquired as described above. CD3$^+$CD4$^+$ T cells producing IFN-γ, TNF-α, and IL-2 were defined as CD3$^+$CD4$^+$IFN-γ$^+$, CD3$^+$CD4$^+$TNF-α$^+$, and CD3$^+$CD4$^+$IL-2$^+$. Further analysis for polyfunctional CD4$^+$ T cells producing single, double, and triple cytokines were analyzed by Boolean gates using FlowJo software.

Quantification of STm-Specific Serum Bactericidal Activity

Serum bactericidal activity (SBA) assays were performed as described elsewhere [10]. Briefly, serum or PBS was mixed with STm D23580 [5], adjusted to 1.0×10^6 CFU/mL, and incubated at 37°C for 180 minutes. Test samples were serially diluted and plated in triplicate on Luria-Bertani agar. *Salmonella* colony counts were done after 24 hours of incubation, and results were reported as $\log_{10}$ change in NTS count (CFU/mL) from the baseline.

Quantifying Anti-NTS IgG Antibody by Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay plates (Nunc-Immuno) were coated overnight using 100 µL of carbonate-bicarbonate buffer (Sigma Aldrich) per well containing the following antigens adjusted to 5 µg/mL: STm-LPS (Alexis Biochemicals), STm–outer membrane protein (OMP) and STm-flagellar protein (FliC) (kind gift from Adam Cunningham and Ian Henderson [17]), and *Escherichia coli*–LPS 0127:B8 (Sigma Aldrich). Plates were washed with wash buffer (PBS plus 0.05% Tween 20) and blocked with 200 µL of blocking buffer (PBS plus 1% BSA) per well for 1 hour at 37°C. Test serum at 1:20 in dilution buffer (PBS plus 0.05% Tween 20 plus 1% BSA) was serially diluted 3-fold and incubated at 37°C for 1 hour. After washing, 100 µL of 1:2000 secondary goat anti-human IgG-AP antibodies (Southern Biotech) were added and incubated for 1 hour at 37°C. Finally, after washing, 100 µL of SigmaFast p-nitrophenyl phosphate substrate was added to each plate and read after 30 minutes with a Bio Tek reader ELx800 (Bio Tek Instruments) at 405 nm.

Statistical Analyses

We distinguished phases of the immune response as follows. Nonlinear regression models were fit to data relating STm-specific T cells and SBA responses with age. The inflection points of the resultant curves were taken to represent the boundaries of qualitatively different phases of immune response. We call the first period before the boundary the *early response*, and the subsequent period the *late response*. The immune responses within these early and late periods were then modeled using linear regression. GraphPad Prism software (version 5.0) was used to generate graphs and analyze the data.

RESULTS

Age Distribution of STm Bloodstream Infection in Children <5 Years Old in Malawi

Between January 2006 and December 2011, STm bacteremia was detected in 449 children <5 years of age presenting to Queen Elizabeth Central Hospital, of whom 359 (80%) were <2 years old. The median age at STm bloodstream infection was 13 months (range, 0–60 months; Figure 1).

Development of Memory CD4$^+$ T-Cell Subsets in Children <5 Years Old

To provide a context for the subsequent assessment of functional T-cell memory, we first assessed the overall development of T-cell subsets in this Malawian population. Newborns are pathogen inexperienced [18], and therefore CD4$^+$ T cells develop memory with age, enabling them to mount rapid immune responses to previously encountered pathogens. Naive, EM, and

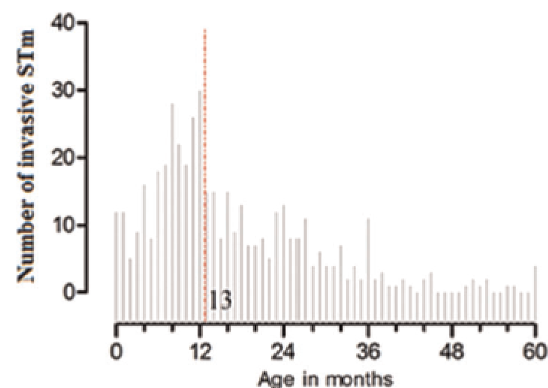

Figure 1. Age distribution of *Salmonella* Typhimurium (STm) bacteremia in children <5 years old at Queen Elizabeth Central Hospital, Blantyre, Malawi, 2006–2011 (N = 449); dashed line represents median age (13 months).

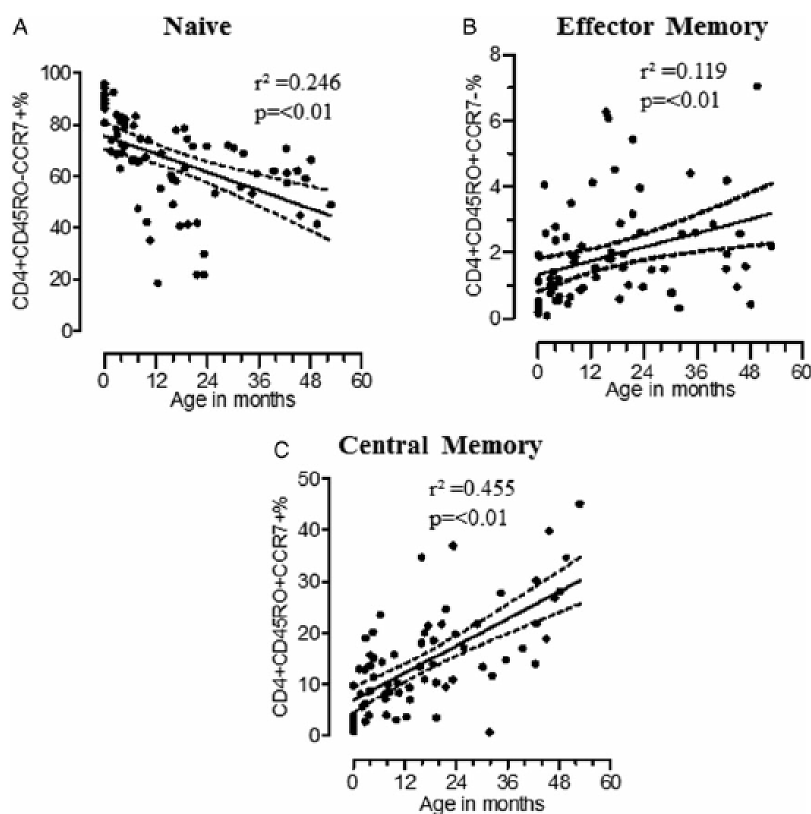

Figure 2. Development of memory CD4+ T-cell subsets in the first 5 years of life. Percentage are shown of naive CD4+ T cells: CD4+CD45RO−CCR7− (*A*; n = 73), effector memory CD4+ T cells: CD4+CD45RO+CCR7− (*B*; n = 73), and central memory CD4+ T cells: CD4+CD45RO+CCR7+) (*C*; n = 73) were plotted against age. Memory CD4+ T cells were determined by linear regression, represented by solid central lines, and 95% confidence intervals are represented by dashed lines.

CM CD4+ T cells can be differentiated by their extracellular expression of CD45RO and CCR7 [19, 20]. As expected [21], we found that the proportion of CD4+CD45RO−CCR7+ naive T cells decreased with age ($r^2 = 0.246$; slope, −0.58 [95% confidence interval (CI), −.83 to −.34]; $P \leq .01$; Figure 2*A*). The proportion of CD4+CD45RO+CCR7− EM ($r^2 = 0.119$; slope, 0.035 [95% CI, .012–.057]; $P \leq .01$) and CD4+CD45RO+CCR7+ CM ($r^2 = 0.455$; slope, 0.43 [95% CI, .32–.55]; $P \leq .01$) T cells increased with age (Figure 2*B* and 2*C*).

Early Acquisition of STm-Specific CD4+ T-Cell Immune Responses

We next sought to explore the hypothesis that CD4+ T-cell immune responses to STm develop in parallel with acquisition of antibody-mediated immunity. Contrary to our hypothesis, we found that STm-specific CD4+ T cells producing cytokines were present early in life, peaked at 14 months and then declined (Figure 3*A*). This was further analyzed by using the nonlinear model peak points to define early and late STm-specific CD4+ T cells. This showed early acquisition of STm-specific CD4+ T-cell immunity ($r^2 = 0.129$; slope, 0.021 [95% CI, .002–.041]; $P = .031$), followed by a decrease in older children ($r^2 = 0.157$; slope, −0.005 [95% CI, −.009 to −.0006]; $P = .024$; Figure 3*B* and 3*C*). These changes in intracellular cytokine profiles mirrored changes in IFN-γ– and TNF-α– rather than IL-2–secreting cells (see Supplementary Figure 1). STm-specific CD4+ cytokine responses did not correlate with PMA-stimulated CD4+ T-cell cytokine responses ($r = 0.109$ [95% CI, −.128 to .371]; $P = .426$; Table 2), indicating that these responses to STm antigens were not simply due to a general maturation of the immune system (Figure 3*D* and Supplementary Figure 2). Generation of antigen-specific multiple cytokine-producing cells is widely thought to indicate maturation of antigen-specific CD4+ T-cell responses [22]. Maturation of STm-specific T-cell responses in these healthy children (either double or triple cytokine producers) peaked mostly between 13–24 months

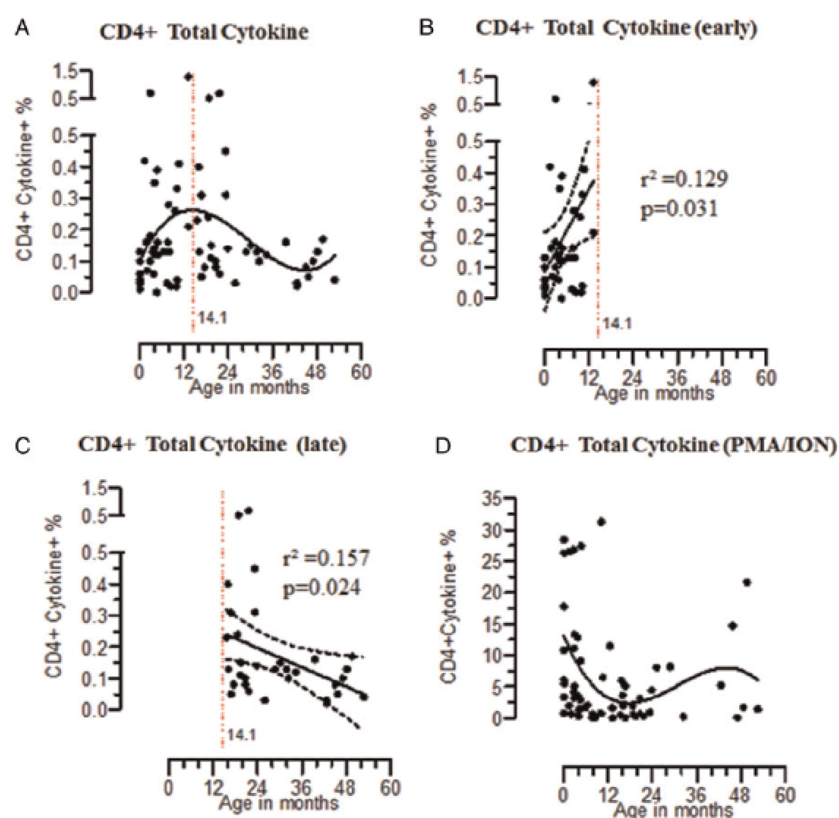

Figure 3. Early acquisition of *Salmonella* Typhimurium (STm)–specific CD4⁺ T-cell immune responses. Percentage are shown for STm-specific CD4⁺ T cells producing total (*A*; n = 68), early (*B*; n = 36), and late (*C*; n = 32) cytokine and phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulated CD4⁺ T cells producing total cytokine (*D*; n = 62). Nonlinear polynomial regression models of third order were fit to data relating specific T-cell cytokine response to age. STm-specific T cells response within early and late periods was determined by linear regression, represented by solid central lines; dashed lines represent 95% confidence intervals.

Table 2. Association Between Immune Variables

Parameter (s)	XY Pairs	Spearman *r*	95% CI	*P* Value
NTS vs PMA CD4⁺ cytokine⁺	55	0.109	−.128 to .371	.426
SBA vs anti–STm-LPS IgG antibody titers	55	0.329	.552–.062	.01
SBA vs anti–STm-OMP IgG antibody titers	57	0.044	−.226 to .308	.741
SBA vs anti–STm-FliC IgG antibody titers	58	−0.001	−.266 to .264	.992
SBA vs anti–*E. coli*-LPS IgG antibody titers	50	0.031	−.257 to .314	.830
CD4⁺ cytokine⁺ vs anti–STm-OMP IgG antibody titers	65	0.137	−.117 to .375	.275
CD4⁺ cytokine⁺ vs anti–STm-FliC IgG antibody titers	67	0.174	−.075 to .404	.157
CD4⁺ cytokine⁺ vs anti–STm-OMP IgG antibody titers (early)[a]	39	0.405	.088–.647	.01
CD4⁺ cytokine⁺ vs anti–STm-FliC IgG antibody titers (early)[a]	38	0.394	.080–.637	.01
CD4⁺ cytokine⁺ vs anti–STm-LPS IgG antibody titers (early)[a]	36	−0.257	−.547 to .087	.129

Abbreviations: CI, confidence interval; *E. coli*, *Escherichia coli*; FliC, flagellar protein; IgG, immunoglobulin G; LPS, lipopolysaccharide; NTS, nontyphoidal *Salmonella*; OMP, outer membrane protein; PMA, phorbol 12-myristate 13-acetate; SBA, serum bactericidal activity; STm, *Salmonella* Typhimurium.

[a] *Early* refers to parameters of participants aged <14 months.

and subsequently declined, whereas for IL-2$^+$TNF-α$^+$CD4 T cells, the response was sustained (data not shown).

Delayed Acquisition of STm-Specific SBA

To confirm previous observations made in Blantyre by MacLennan et al, we used the same SBA assay and clinical STm strain D23580 [10]. In line with the previous findings, STm-specific SBA declined in the first 8 months of life and then increased to a peak at 35 months (Figure 4*A*). To further analyze these trends in NTS-specific SBA, we divided the periods into early and late phases according to peak and nadir points, as before. We found that STm-specific SBA declined in the first 8 months of life ($r^2 = 0.323$; slope, 0.292 [95% CI, .125–.459]; $P \leq .01$) and then increased between 8 and 35 months ($r^2 = 0.319$; slope, −0.121 [95% CI, −.193 to −.048]; $P \leq .01$; Figure 4*B* and 4*C*). This STm-specific increase in SBA occurred later than that seen in T-cell immunity to STm (Figures 3–5).

Correlation of STm-Specific SBA With Presence of Antibodies Targeting STm-LPS

Previous work in HIV-infected Malawian adults showed that excess anti-LPS IgG antibodies can inhibit complement-mediated killing of NTS in vitro, whereas antibodies to OMPs can mediate bactericidal activity [17]. To clarify the antigenic targets of the STm-specific antibody in children, we measured serum antibodies to STm LPS, OMP, FliC, and *E. coli* 0127:B8 LPS. We found that anti–STm-LPS IgG antibody titers mirrored the pattern seen with SBA assay (Figure 4*A* and Supplementary Figure 3*A*). Anti–STm-OMP antibody titers were lowest at birth and increasing with age, whereas anti–STm-FliC IgG and anti–*E. coli*–LPS IgG antibody titers showed no particular trend with age (Supplementary Figure 3*B*–3*D*). The correlation between SBA and anti–STm-LPS IgG titers ($r = 0.329$ [95% CI, .552–.062]; $P = .01$), and the lack of correlation between SBA and anti–*E. coli*-LPS titers suggests that SBA is due to STm-specific rather

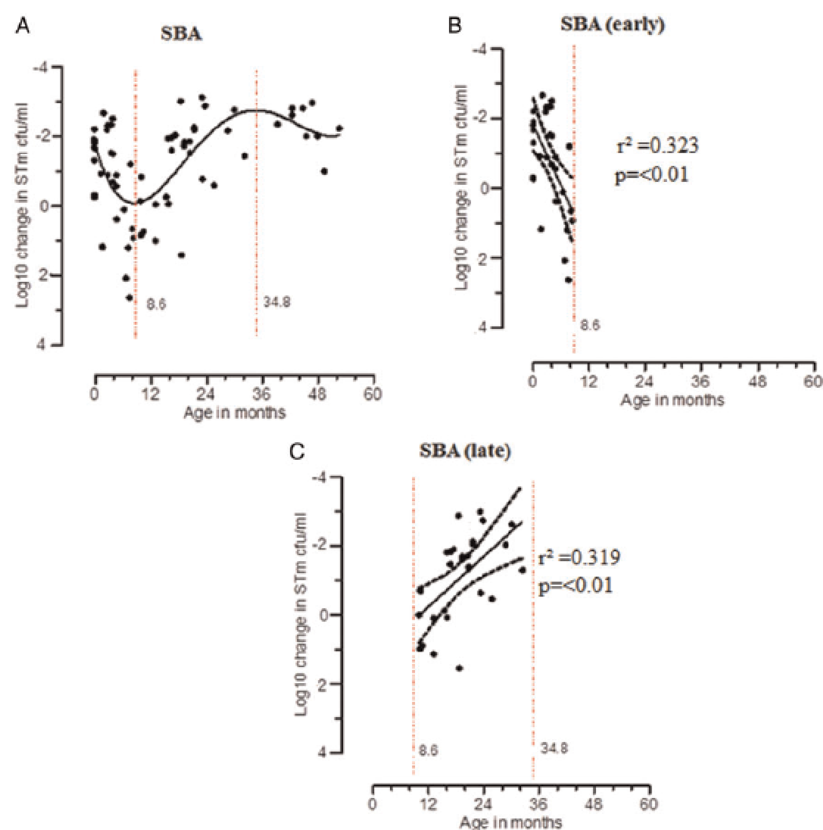

Figure 4. Acquisition of *Salmonella* Typhimurium (STm)–specific serum bactericidal activity (SBA) among children. The $\log_{10}$ change in STm (in colony-forming units [CFU] per milliliter) relative to the control condition was plotted against age. The y-axis was inverted. Nonlinear regression polynomial model is represented by solid lines (*A*; n = 65). SBA responses within early (*B*; n = 29) and late (*C*; n = 27) periods was determined by linear regression, represented by solid central lines; dashed lines represent 95% confidence intervals.

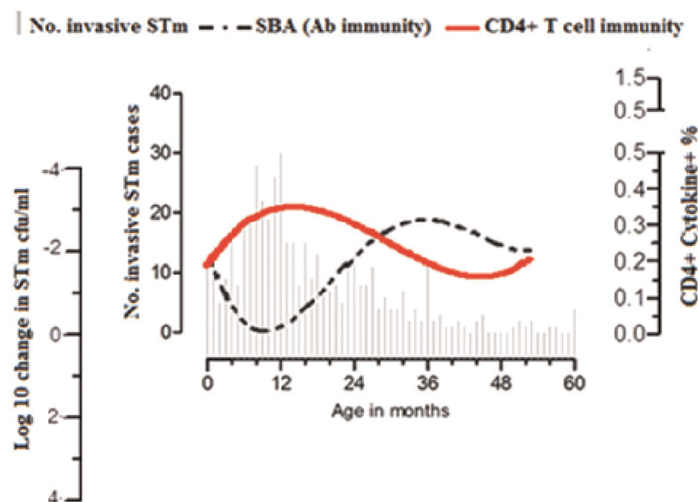

Figure 5. Sequential acquisition of T cells and antibodies to *Salmonella* Typhimurium (STm) in children. Age distribution of STm bloodstream infection in children <5 years old at Queen Elizabeth Central Hospital (Blantyre, Malawi; 2006–2011) was superimposed with kinetics of STm-specific $CD4^+$ T-cell immune responses and STm-specific serum bactericidal activity (y-axis was inverted) in children aged 0–60 months. Abbreviations: CFU, colony-forming units; SBA, serum bactericidal activity.

than nonspecific LPS antibodies (Table 2). A lack of a correlation with anti–STm-OMP and anti–STm-FliC suggest that these targets do not substantially contribute to SBA in these children.

STm-Specific $CD4^+$ T-Cell Immune Responses in Early Childhood Associated With Generation of Anti-STm Protein Antibodies

Having shown that STm-specific $CD4^+$ T cells peak in early life (Figure 5), we investigated whether this immune memory was linked to the generation of anti–STm-OMP and anti–STm-FliC IgG antibodies. We found that STm-specific $CD4^+$ T-cell immune responses correlate with anti–STm-OMP and anti–STm-FliC IgG antibodies in early childhood ($r = 0.405$ [95% CI, .088–.647; $P = .01$] and $r = 0.394$ [95% CI, .080–.637; $P = .01$], respectively) and not anti–STm-LPS IgG antibodies ($r = -0.257$ [95% CI, −.547 to .087]; $P = .129$; Table 2). This contemporaneous development of antibodies to STm OMP and T-cell immunity is in line with the conventional paradigm of the T-cell–dependent immune response to a protein antigen [23, 24].

DISCUSSION

NTS infection in African children is associated with life-threatening bacteremia. Here we extend previous observations to show that STm-specific $CD4^+$ T-cell immunity is acquired early in childhood in parallel with antibody to STm protein antigens but precedes the development of complement-fixing antibody immunity. These findings suggest exposure to STm or cross-reactive protein antigens induces STm-specific $CD4^+$ T-cell immune responses early in life, presumably within the gut-associated lymphoid tissues [25]. Enteric pathogens colonize the gastrointestinal tract soon after birth, even in exclusively breastfed babies [26]. However, the incidence and frequency of *Salmonella* colonization of the gastrointestinal tract in this population, and whether repeated *Salmonella* infections are required to generate this natural immunity, is not known. Most Malawian children are initially exclusively breastfed but are then weaned onto mixed feeding after 3 months of age [27, 28]. This switch in food seem s to coincide with the observed emergence of STm-specific T cells and the appearance of anti–STm-OMP and anti–STm-FliC IgG antibodies. It is uncertain why STm-specific T-cell immunity declines in older children following evidence of immune maturation, but this could be due to decreased exposure of the immune system to STm and homing of residual specific CM $CD4^+$ T-cell memory to lymphoid tissues. To what extent this T-cell and B-cell immunity protects against NTS then becomes a key question.

Both previous [10] and current surveillance in Malawi show that 80% of STm bacteremia cases occur in children <24 months among under five children, with a peak at 13 months. Acquisition of STm-specific $CD4^+$ T cells in early childhood parallels the age-related increase in incidence of STm bacteremia, suggesting that the early acquisition of T-cell immunity to NTS

alone is insufficient to protect against iNTS disease and that additional immune modalities are required. The association of an age-related decline in incidence of STm bacteremia with increasing levels of STm-specific complement-fixing antibodies is suggestive of protective immunity [10]. Indeed, both a previous study [10] and the current one found that STm-specific SBA is detectable in newborns (consistent with passively acquired maternal antibody) and that the natural decline in this antibody with age coincides with an increase in the incidence of iNTS.

The strong relationship between anti–STm-IgG antibodies targeting STm-LPS and SBA, and a lack of correlation with STm-OMP, STm-FliC, or *E. coli*–LPS support previous evidence that anti–STm-LPS IgG antibodies mediate this SBA [29–31] and suggest that these antibodies recognize the variable component of LPS (O-antigen). In some HIV-infected adults with dysregulated humoral immunity and hypergammaglobulinemia, excess IgG antibody to STm-LPS prevents killing of NTS [17], but this is at levels much higher than those found in healthy HIV-uninfected children and adults and was not apparent in our studies.

Our findings do not preclude an important role for T cells in elimination of salmonellae from the intracellular niche. Clearance of disseminated *Salmonella* infection is thought to require a specific Th1 response [32]. Mastroeni [33] hypothesized, based on murine models, that protective immunity to *Salmonella* infection is acquired in a stepwise fashion constituting innate cells, T cells, and then antibody. Preexisting antibodies against *Salmonella* reduce murine bacteremia by preventing early infection [34]. Protection induced by heat-killed salmonellae correlates with anti-*Salmonella* antibody titers [35], with SBA attributable to anti-LPS antibodies [29] and with binding of *Salmonella*-specific antibodies. These facilitate the development of T-cell immunity by enhancing bacterial uptake through opsonization and also antigen presentation by macrophages [36].

Based on our human studies, it is likely that the early development of T cells specific for STm protein antigens and subsequent cognate interactions with B cells leads to antibody production against these antigens, class-switching, affinity maturation and memory formation [23]. We speculate that, in view of the complex nature of the *Salmonella* antigens presented during natural exposure, these STm protein-specific T cells may also provide bystander (hapten-carrier) help to B cells specific for STm-LPS. LPS alone is a T-cell–independent type 2 antigen, but when taken up by antigen presenting cells in combination with STm proteins, has potential to act in the same way that polysaccharide-conjugate vaccines generate isotype-switched memory B-cell immunity [37].

In conclusion, STm-specific $CD4^+$ T cells seem insufficient to protect against iNTS disease, but sequential acquisition of SBA to STm LPS is associated with a decline in incidence of iNTS. STm-specific $CD4^+$ T cells may drive the development of protective antibody responses through bystander interactions with B cells. Given the burden of iNTS in sub-Saharan Africa [2], a vaccine is urgently required. STm LPS O-antigen has considerable potential as a vaccine target, and there are currently several groups developing conjugate vaccines for this purpose to overcome the short-lived T-independent antibody response generated by polysaccharide alone [38]. Immunization with STm-OMP and STm-FliC induce both T cells and antibodies in animal models and are therefore also being investigated as vaccine candidates, either separately [39] or covalently linked to O-antigen as glycoconjugates [40].

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Contributions. T. S. N., C. A. M., W. L. M., and R. S. H. conceived and designed the experiments. T. S. N. and J. J. G. performed the experiments. T. S. N., J. J. G., N. A. F., S. J. G., N. B., M. A. G., C. A. M., W. L. M., R. S. H. analyzed the data. All authors contributed to and have approved the final manuscript.

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Potential conflict of interests. C. A. M. is an employee of the Novartis Vaccines Institute for Global Health and recipient of a Clinical Research Fellowship from GlaxoSmithKline.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Feasey NA, Dougan G, Kingsley RA, Heyderman RS, Gordon MA. Invasive non-typhoidal *Salmonella* disease: an emerging and neglected tropical disease in Africa. Lancet **2012**; 379:2489–99.
2. Reddy EA, Shaw AV, Crump JA. Community-acquired bloodstream infections in Africa: a systematic review and meta-analysis. Lancet Infect Dis **2010**; 10:417–32.
3. Mackenzie G, Ceesay SJ, Hill PC, et al. A decline in the incidence of invasive non-typhoidal *Salmonella* infection in The Gambia temporally associated with a decline in malaria infection. PloS One **2010**; 5:e10568.
4. Kariuki S, Revathi G, Kiiru J, Lowe B, Berkley JA, Hart CA. Decreasing prevalence of antimicrobial resistance in non-typhoidal *Salmonella*

isolated from children with bacteraemia in a rural district hospital, Kenya. Int J Antimicrob Agents **2006**; 28:166–71.
5. Kingsley RA, Msefula CL, Thomson NR, et al. Epidemic multiple drug resistant *Salmonella* Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype. Genome Res **2009**; 19:2279–87.
6. Okoro CK, Kingsley RA, Connor TR, et al. Intracontinental spread of human invasive *Salmonella* Typhimurium pathovariants in sub-Saharan Africa. Nat Genet **2012**; 44:1215–21.
7. Msefula CL, Kingsley RA, Gordon MA, et al. Genotypic homogeneity of multidrug resistant *S.* Typhimurium infecting distinct adult and childhood susceptibility groups in Blantyre, Malawi. PloS One **2012**; 7: e42085.
8. Mastroeni P, Villarreal-Ramos B, Hormaeche CE. Adoptive transfer of immunity to oral challenge with virulent salmonellae in innately susceptible BALB/c mice requires both immune serum and T cells. Infect Immun **1993**; 61:3981–4.
9. Tam MA, Rydstrom A, Sundquist M, Wick MJ. Early cellular responses to *Salmonella* infection: dendritic cells, monocytes, and more. Immunol Rev **2008**; 225:140–62.
10. MacLennan CA, Gondwe EN, Msefula CL, et al. The neglected role of antibody in protection against bacteremia caused by nontyphoidal strains of *Salmonella* in African children. J Clin Invest **2008**; 118:1553–62.
11. Gondwe EN, Molyneux ME, Goodall M, et al. Importance of antibody and complement for oxidative burst and killing of invasive nontyphoidal *Salmonella* by blood cells in Africans. Proc Natl Acad Sci U S A **2010**; 107:3070–5.
12. Gordon MA, Jack DL, Dockrell DH, Lee ME, Read RC. Gamma interferon enhances internalization and early nonoxidative killing of *Salmonella enterica* serovar Typhimurium by human macrophages and modifies cytokine responses. Infect Immun **2005**; 73:3445–52.
13. Janssen R, Van Wengen A, Verhard E, et al. Divergent role for TNF-alpha in IFN-gamma-induced killing of *Toxoplasma gondii and Salmonella* typhimurium contributes to selective susceptibility of patients with partial IFN-gamma receptor 1 deficiency. J Immunol **2002**; 169:3900–7.
14. Gordon MA, Kankwatira AM, Mwafulirwa G, et al. Invasive non-typhoid salmonellae establish systemic intracellular infection in HIV-infected adults: an emerging disease pathogenesis. Clin Infect Dis **2010**; 50:953–62.
15. Gordon MA, Banda HT, Gondwe M, et al. Non-typhoidal *Salmonella* bacteraemia among HIV-infected Malawian adults: high mortality and frequent recrudescence. AIDS **2002**; 16:1633–41.
16. Nyirenda TS, Seeley AE, Mandala WL, Drayson MT, MacLennan CA. Early interferon-gamma production in human lymphocyte subsets in response to nontyphoidal *Salmonella* demonstrates inherent capacity in innate cells. PloS One **2010**; 5:e13667.
17. MacLennan CA, Gilchrist JJ, Gordon MA, et al. Dysregulated humoral immunity to nontyphoidal *Salmonella* in HIV-infected African adults. Science **2010**; 328:508–12.
18. Ygberg S, Nilsson A. The developing immune system: from foetus to toddler. Acta Paediatr **2012**; 101:120–7.
19. Bunce C, Bell EB. CD45RC isoforms define two types of CD4 memory T cells, one of which depends on persisting antigen. J Exp Med **1997**; 185:767–76.
20. Mackay CR, Marston WL, Dudler L. Naive and memory T cells show distinct pathways of lymphocyte recirculation. J Exp Med **1990**; 171:801–17.
21. Chipeta J, Komada Y, Zhang XL, et al. CD4+ and CD8+ cell cytokine profiles in neonates, older children, and adults: increasing T helper type 1 and T cytotoxic type 1 cell populations with age. Cell Immunol **1998**; 183:149–56.
22. Kannanganat S, Ibegbu C, Chennareddi L, Robinson HL, Amara RR. Multiple-cytokine-producing antiviral CD4 T cells are functionally superior to single-cytokine-producing cells. J Virol **2007**; 81:8468–76.
23. Mohr E, Cunningham AF, Toellner KM, et al. IFN-γ produced by CD8 T cells induces T-bet-dependent and -independent class switching in B cells in responses to alum-precipitated protein vaccine. Proc Natl Acad Sci U S A **2010**; 107:17292–7.
24. Sinha K, Mastroeni P, Harrison J, de Hormaeche RD, Hormaeche CE. *Salmonella* typhimurium aroA, htrA, and aroD htrA mutants cause progressive infections in athymic (nu/nu) BALB/c mice. Infect Immun **1997**; 65:1566–9.
25. Dougan G, John V, Palmer S, Mastroeni P. Immunity to salmonellosis. Infect Immun **2011**; 240:196–210.
26. Mata LJ, Urrutia JJ. Intestinal colonization of breast-fed children in rural area of low socioeconomic level. Ann N Y Acad Sci **1971**; 176:93–109.
27. Kalanda BF, Verhoeff FH, Brabin BJ. Breast and complementary feeding practices in relation to morbidity and growth in Malawian infants. Eur J Clin Nutr **2006**; 60:401–7.
28. Kerr RB, Berti PR, Chirwa M. Breastfeeding and mixed feeding practices in Malawi: timing, reasons, decision makers, and child health consequences. Food Nutr Bull **2007**; 28:90–9.
29. Rondini S, Lanzilao L, Necchi F, et al. Invasive African *Salmonella* Typhimurium induces bactericidal antibodies against O-antigens. Microb Pathog **2013**; 63:19–23.
30. Trebicka E, Jacob S, Pirzai W, Hurley BP, Cherayil BJ. Role of anti-lipopolysaccharide antibodies in serum bactericidal activity against *Salmonella* enterica serovar Typhimurium in healthy adults and children in the United States. Clin Vaccine Immunol **2013**; 20:1491–8.
31. MacLennan CA, Tennant SM. Comparing the role of antibodies to nontyphoidal *Salmonella* in high- and low-income countries and implications for vaccine development. Clin Vaccine Immunol **2013**; 20: 1487–90.
32. Ravindran R, McSorley SJ. Tracking the dynamics of T-cell activation in response to *Salmonella* infection. Immunology **2005**; 114:450–8.
33. Mastroeni P. Immunity to systemic *Salmonella* infections. Curr Mol Med **2002**; 2:393–406.
34. Mittrucker HW, Raupach B, Kohler A, Kaufmann SH. Cutting edge: role of B lymphocytes in protective immunity against *Salmonella* typhimurium infection. J Immunol **2000**; 164:1648–52.
35. Xu HR, Hsu HS, Moncure CW, King RA. Correlation of antibody titres induced by vaccination with protection in mouse typhoid. Vaccine **1993**; 11:725–9.
36. Eguchi M, Kikuchi Y. Binding of *Salmonella*-specific antibody facilitates specific T cell responses via augmentation of bacterial uptake and induction of apoptosis in macrophages. J Infect Dis **2010**; 201:62–70.
37. Mitchison NA. The carrier effect in the secondary response to hapten-protein conjugates. II. Cellular cooperation. Eur J Immunol **1971**; 1:18–27.
38. MacLennan CA. Vaccines for low-income countries. Semin Immunol **2013**; 25:114–23.
39. Gil-Cruz C, Bobat S, Marshall JL, et al. The porin OmpD from nontyphoidal *Salmonella* is a key target for a protective B1b cell antibody response. Proc Natl Acad Sci U S A **2009**; 106:9803–8.
40. Simon R, Levine MM. Glycoconjugate vaccine strategies for protection against invasive *Salmonella* infections. Hum Vaccin Immunother **2012**; 8:494–8.