UNIVERSITY OF LIVERPOOL

Mucosal and peripheral blood responses to vaccination with the licensed live oral typhoid vaccine Ty21a

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Master of Philosophy

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DECLARATION

This thesis is the result of my own work except where indicated. My contributions for the reported work were as follows:

Activity	**Responsibility**
Ethics application	Shared
Recruitment of volunteers	None
Collection of clinical specimens	None
Sample processing	Sole
Intracellular cytokine staining assay	Sole
CFSE proliferation assay	Sole
Flow cytometry data analysis	Sole
Data presentation	Sole
Statistical data analysis	Shared
Thesis preparation	Sole

Due acknowledgement has been made within the text to all other material used.

The material contained in this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification elsewhere.

Ameeka Louise Thompson

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APC	Antigen-presenting cells
BAL	Bronchoalveolar lavage
BALT	Bronchus-associated lymphoid tissue
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CI	Confidence interval
CLN	Cervical lymph nodes
CMI	Cell-mediated immune
CSP	Circumsporozoite protein
CTL	Cytotoxic T lymphocyte
CVD	Centre for vaccine development
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FDC	Follicular dendritic cell
GALT	Gut-associated lymphoid tissue
ICS	Intracellular cytokine staining
IL-2	Interleukin 2
ILF	Isolated lymphoid follicles
IFN-γ	Interferon-γ
LPS	Lipopolysaccharide
LSTM	Liverpool School of Tropical Medicine
MAdCAM1	Mucosal vascular addressin cell adhesion molecule 1
MALT	Mucosa-associated lymphoid tissue
MHC	Major histocompatibility complex
MIS	Mucosal immune system
MLN	Mesenteric lymph nodes
MMC	Mucosal mononuclear cell
NALT	Nasopharynx-associated lymphoid tissue
NS	Non-stimulated, stained
OPV	Oral polio vaccine
P. aeruginosa	Pseudomonas aeruginosa
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
pIgR	Polymeric Ig receptor
RLUH	Royal Liverpool University Hospital
SIgA	Secretory immunoglobulin A
SIgM	Secretory immunoglobulin M
SEB	Staphylococcal enterotoxin B
S. Typhi	*Salmonella enterica* serovar Typhi
S. Typhimurium	*Salmonella enterica* serovar Typhimurium
TCR	T cell receptor
Tcm	Central memory T cell
Tem	Effector memory T cell
Th1	T helper 1 cell
Th2	T helper 2 cell

Th17	T helper 17 cell
TNF-α	Tumour necrosis factor-α
Treg	Regulatory T cell
TTSS	Type three secretion system
UDP	Uridine diphosphate
UoL	University of Liverpool

ABSTRACT

The mucosal membranes represent the most common portal of entry for human pathogens and are protected by a specialised mucosal immune system which is distinct from the systemic immune system. *Salmonella enterica* serovar Typhi (*S.* Typhi) penetrates the body through the intestinal mucosa and causes both mucosal and systemic illness. Mucosal vaccination against *S.* Typhi with the licensed live oral typhoid vaccine Ty21a should elicit protective cellular responses both systemically and at the mucosal surface. However, nothing is known about the cell-mediated immune responses that are induced locally in the mucosa of the small intestine. It is also unclear whether Ty21a can elicit cellular mucosal immune responses at other mucosal surfaces, a property that would make it a candidate vaccine vector. This study aimed to answer these questions and to determine whether there is a correlation between the immune response elicited at different mucosal sites or between the intestinal mucosa and blood.

Healthy adults were either vaccinated with Ty21a (n=10) or allocated to the unvaccinated control group (n=7). Blood was collected from both groups on day 0 (pre-vaccination sample) and day 18 (post-vaccination sample) by venesection. Cells were separated by differential centrifugation and analysed by flow cytometry. Mucosal samples (duodenum ± sigmoid colon) were collected by endoscopy and mucosal pinch-biopsy on day 18. Cells were separated by collagenase digestion and mechanical disruption and analysed by flow cytometry. Two functional T cell assays, an intracellular cytokine staining assay (interferon-γ, tumour necrosis factor-α and interleukin-2) and a lymphoproliferation assay, were used to assess antigen-specific cellular immune responses.

The results demonstrated that in blood, there was no statistically significant difference in the mean percentage of antigen-specific CD4+ T cells in the intracellular cytokine staining assay between the control and vaccinated groups at day 0 but there was at day 18. This was due to an unexpected fall in the mean percentage of antigen-specific CD4+ T cells in the control group between day 0 and day 18. In the blood lymphoproliferation assay, there was no statistically significant difference between the control and vaccinated groups at day 0 or day 18. There was, however, a statistically significant increase in the mean percentage of antigen-specific cytokine-producing CD4+ T cells in the vaccinated group compared to the control group in duodenal tissue, but not in the colon at day 18. In the vaccinated

group, there was not a strong correlation between duodenum and colon or between duodenum and blood in terms of the percentage of antigen-specific cytokine-producing CD4+ T cells.

This is the first study to report antigen-specific mucosal T cell responses to an oral vaccine, and we were able to demonstrate a significant increase in intracellular cytokine responses to Ty21a antigens in T cells from the mucosal surface. The results from this study support the theory that local immune responses against pathogens such as *S.* Typhi which gain access to the body through mucosal membranes are important. In addition, they suggest that the cellular immune response measured in peripheral blood may not correlate with that measured at the mucosal surface, emphasising the potential value of obtaining mucosal tissue for direct study. This has implications for the evaluation of future vaccine candidates which often relies on systemic immune responses to assess vaccine immunogenicity. This study will now be extended beyond this interim analysis by recruiting more individuals to confirm the findings.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 MUCOSAL IMMUNE SYSTEM

Mucous membranes cover an area of several hundred square metres in an adult, lining the gastrointestinal, respiratory and urogenital tracts as well as the ducts of all the exocrine glands in the body. The physiological functions of mucous membranes, for example gas exchange, food absorption and reproduction, require them to be thin and permeable and, consequently, they are the major portal of entry for human pathogens, especially bacteria and viruses (Murphy et al., 2008). These mucosal membranes are protected by a highly specialised innate and adaptive mucosal immune system. This local immune system contributes approximately 80% of all immunocytes in a healthy adult. These immunocytes are found in the mucosa-associated lymphoid tissues (MALT), which together form the largest mammalian lymphoid organ system (Holmgren and Czerkinsky, 2005).

1.1.1 Organisation of the mucosal immune system

The mucosal immune system can be divided into inductive sites and effector sites (Figure 1.1). At inductive sites, antigens that have been sampled from the mucosal surface are presented to cognate naïve T and B cells in order to stimulate them to differentiate into effector cells. At effector sites, these effector cells perform their effector functions after extravasation and differentiation (Brandtzaeg et al., 2008).

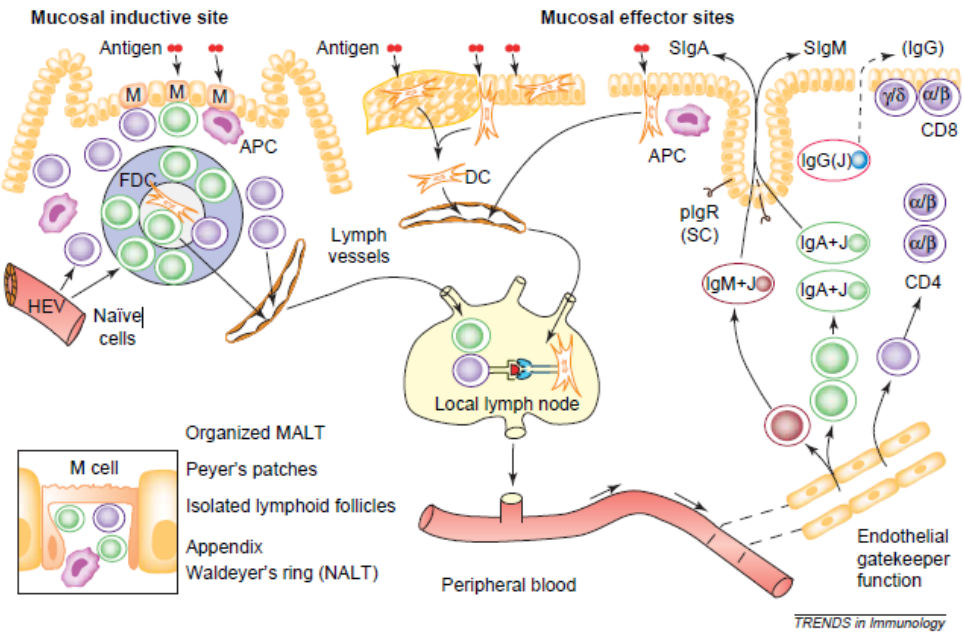

Figure 1.1 Schematic depiction of the mucosal immune system from (Brandtzaeg and Pabst, 2004). Mucosal immune inductive sites are constituted by regional mucosa-associated lymphoid tissue (MALT) such as Peyer's patches and isolated lymphoid follicles. MALT has an M cell (M)-containing follicle-associated epithelium through which antigen can be transported to reach antigen-presenting cells (APCs) such as dendritic cells (DCs), macrophages and follicular dendritic cells (FDCs). Exogenous antigens can also be captured by subepithelial DCs. APCs migrate to local and regional lymph nodes where they prime naïve B and T cells to become memory-effector B and T cells. These primed cells migrate into the peripheral blood and are transported to mucosal effector sites where extravasation occurs. Extravasation is controlled by the endothelial cells. The gut mucosal effector site, the lamina propria, contains IgA, IgM and IgG plasma cells and T lymphocytes. The IgA and IgM plasma cells produce secretory IgA (SIgA) and secretory IgM (SIgM), respectively, via polymeric Ig receptor (pIgR)-mediated epithelial transport.

1.1.1.1 Immune inductive sites of mucosae

Inductive sites consist of organised MALT and local and regional mucosa-draining lymph nodes. MALT is sub-divided according to anatomical regions, for example gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT) and nasopharynx-associated lymphoid tissue (NALT). Mucosa-draining lymph nodes include the mesenteric lymph nodes (MLN) and the cervical lymph nodes (CLN). The structure of all MALT resembles that of lymph nodes, with T cell zones, B cell follicles and antigen-presenting cells such as dendritic cells and macrophages. In contrast to lymph nodes, MALT lacks afferent lymphatics because antigens are sampled directly from the mucosal surface (Brandtzaeg et al., 2008).

GALT, the largest and best-defined MALT structure, includes Peyer's patches, the appendix and isolated lymphoid follicles (ILFs). Peyer's patches contain between five and 200 aggregated lymphoid follicles and in humans, are found mainly in the distal ileum (Brandtzaeg et al., 2008). The number of macroscopically visible Peyer's patches varies with age, increasing from around 50 before 30 weeks gestation to 100 at birth and 250 in the mid-teens before decreasing to approximately 100 over 70 years of age (Brandtzaeg et al., 2008; Cornes, 1965). There are over 30,000 ILFs in the human gut. In both the small and large intestine, ILFs increase in density distally (Brandtzaeg et al., 2008).

Lymphocytes encounter antigens derived from the intestinal lumen in GALT and MLNs. GALT structures such as Peyer's patches acquire antigens directly from the intestinal lumen in different ways. Soluble protein antigens can be passively absorbed across the intestinal epithelium. Alternatively, antigens can be acquired through a follicle-associated epithelium

containing specialised M cells (M for microvilli or microfold). M cells overlie approximately 10% of Peyer's patches (Lefrancois and Puddington, 2006). Antigens are transported through the apical membrane of the M cell and then through its extensively folded basement membrane, which is closely apposed to underlying antigen-presenting cells (APCs) and T cells (Murphy et al., 2008). MLNs receive antigen or antigen-bearing APCs in afferent lymph from the intestine and efferent lymph drainage from Peyer's patches (Lefrancois and Puddington, 2006).

1.1.1.2 Effector sites of mucosae

After activation in secondary lymphoid tissues, lymphocytes are able to leave the immune inductive sites and migrate to all non-lymphoid tissues (Lefrancois and Puddington, 2006). These effector sites include the lamina propria of various mucous membranes, the stroma of exocrine glands and surface epithelia (Brandtzaeg and Pabst, 2004). The lamina propria of the intestine is defined as a 'loosely organised connective tissue beneath the basement membrane supporting the overlying epithelial cells of the small and large intestine' (Lefrancois and Puddington, 2006). It contains cells of both the innate and adaptive immune system, including IgA-producing plasma cells, dendritic cells, macrophages and lymphocytes. In the lung, effector cells are found in the lung parenchyma, which is analogous to the intestinal lamina propria, and the airways (Lefrancois and Puddington, 2006).

1.1.2 Compartmentalisation of immunity

1.1.2.1 Mucosal versus systemic immunity

The mucosal immune system is distinct from the systemic immune system. The concept that mucosal humoral immunity is distinct from systemic humoral immunity was conceived by Besredka in 1919. He orally immunised rabbits with *Shigella dysenteriae* and showed that they were protected against fatal dysentery, irrespective of their serum antibody titre. This implied that an adaptive immune response was generated in the gut that was independent of the systemic immune response (Brandtzaeg, 1996). The distinctive features of the mucosal immune system are summarised in Table 1.1.

Anatomical features	Intimate interactions between mucosal epithelia and lymphoid tissues
	Discrete compartments of diffuse lymphoid tissue and more organised structures such as Peyer's patches, isolated lymphoid follicles and tonsils
	Specialised antigen-uptake mechanisms, e.g. M cells in Peyer's patches, adenoids and tonsils
Effector mechanisms	Activated/memory T cells predominate even in the absence of infection
	Nonspecifically activated 'natural' effector/regulatory T cell present
Immunoregulatory environment	Active downregulation of immune responses (e.g. to food and other innocuous antigens) predominates
	Inhibitory macrophages and tolerance-inducing dendritic cells

Table 1.1 Distinctive features of the mucosal immune system, from (Murphy et al., 2008).

1.1.2.2 Compartmentalisation of the mucosal immune system

The concept of a common mucosal immune system (MIS) whereby activation of immunocytes at one site could induce an immune response at remote mucosal effector sites has support (Czerkinsky et al., 1987; McDermott and Bienenstock, 1979; Mestecky et al., 1978). For example, Mestecky *et al.* orally immunised humans with capsules which contained killed *Streptococcus mutans* and found specific antibodies in saliva and lacrimal secretions (Mestecky et al., 1978). More recently, however, the concept of a common MIS has been superceded by that of a compartmentalised MIS, in which specific mucosal inductive sites are linked with particular effector sites (Eriksson et al., 1998; Haneberg et al., 1994; Kozlowski et al., 1997). For example, Haneberg *et al.* immunised mice with cholera toxin via different routes (oral cavity, stomach, colon-rectum or vagina) and measured the specific IgA antibody response at these different sites. They found that the concentration of specific IgA in secretions at the different mucosal sites varied greatly, depending on the route of immunisation (Haneberg et al., 1994). Such compartmentalisation within the MIS restricts the choice of vaccination route for any given disease. For example, giving an oral vaccine for a disease of the female genital tract would

not necessarily induce an optimally effective immune response in the female genital tract mucosa (Figure 1.2).

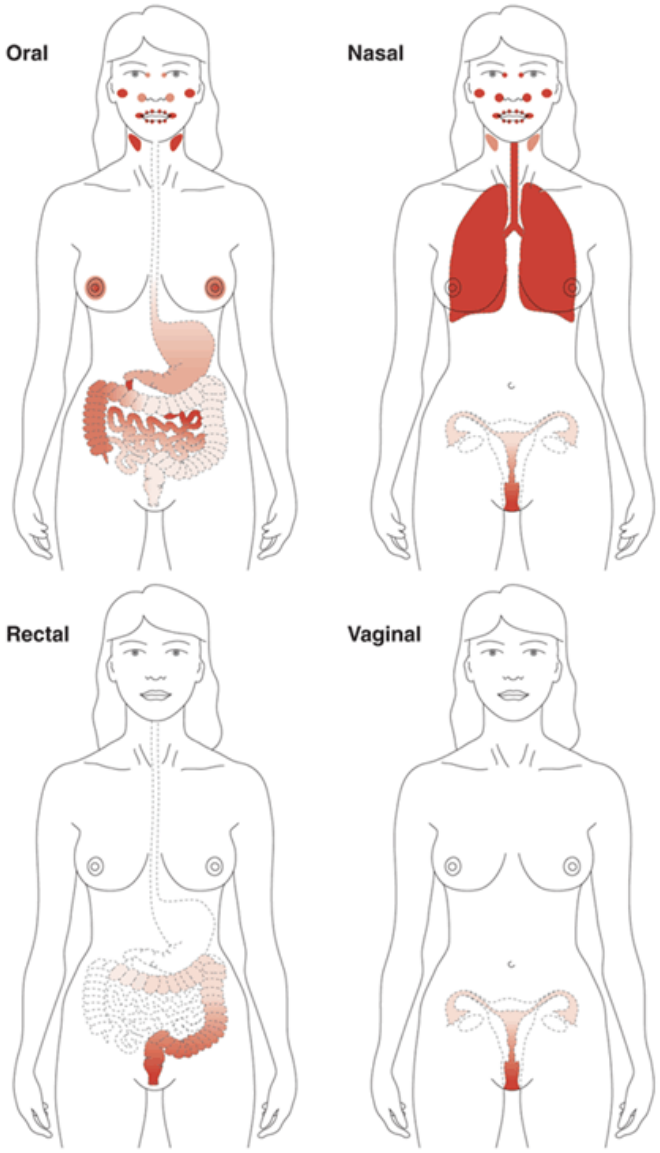

Figure 1.2 Expression of mucosal IgA immune responses after different routes of vaccination, from (Holmgren and Czerkinsky, 2005). Shading indicates the strength of the immune response.

1.1.3 Mucosal vaccination

Arguably the greatest advantage of mucosal vaccination over parenteral vaccination is that mucosal vaccination establishes humoral and cellular protective immune responses both systemically and at mucosal surfaces, whereas parenteral vaccination is only effective in clearing systemic infections and fails to protect at the mucosal surface (Mann et al., 2009).

Given that mucosal surfaces represent the most common portal of entry or colonisation for human pathogens, there is a clear advantage of a vaccination strategy that induces both layers of antigen-specific immunity (Mann et al., 2009). In addition, mucosal vaccination targets immune inductive sites and protects against infection and disease, whereas parenteral vaccination does not target inductive sites and offers protection predominantly against disease (Medina and Guzman, 2001).

The oral route of vaccination offers other significant advantages over systemic delivery. Whereas the injection of a vaccine with a needle and syringe requires medical professionals, oral vaccines are non-invasive and involve a relatively simple administration technique, offering the potential for self-administration. This makes them more suitable for mass-vaccination campaigns (Baumann, 2008). Furthermore, oral vaccines would remove the risk of injury or cross infection through contaminated needles. Finally, mucosal vaccines have a much smaller impact on the environment; there are no needles and syringes which need to be decontaminated and disposed of (Mann et al., 2009).

Successful oral vaccines include the live-attenuated polio vaccine (Oral Polio Vaccine®) and the live-attenuated monovalent human rotavirus vaccine (RotaRix®). The Oral Polio Vaccine® (OPV) was developed by Albert Sabin in the 1960s and was the first approved mucosal vaccine (Yuki and Kiyono, 2009). A global polio eradication campaign using OPV reduced the number of cases from an estimated 350,000 worldwide in 1988 to 2,971 worldwide in 2000 (Chumakov et al., 2007). The RotaRix® vaccine protects against rotavirus which is a major cause of acute gastroenteritis worldwide in children less than five years old. Vaccine efficacy ranges from 41% to 92% depending on the serotype involved (Yuki and Kiyono, 2009).

Live mucosal vaccines have obvious strengths but there are problems and potential hazards associated with them. These include the possibility that they will revert to virulence and cause disease in recipients, or that they will develop new mutations. The OPV, for example, has been associated with the development of paralytic poliomyelitis in vaccinees and their contacts. The incidence of this vaccine-associated paralytic poliomyelitis is highest in immunosuppressed patients, suggesting that immunosuppression should be a contraindication for live mucosal vaccines (Ogra et al., 1980). Other concerns include interference by microorganisms at the mucosal surface and, in young children, interference by pre-existing mucosal antibody passed on via breast-feeding (Ogra et al., 1980). There is also the need for cold chain storage and distribution.

1.1.4 Cellular immunity

T cell precursors are derived from pluripotent haematopoietic stem cells in the bone marrow. They migrate through the blood to the thymus where further differentiation to mature naïve T cells occurs (Picker and Butcher, 1992). Bone marrow and the thymus are both central lymphoid organs. When T cell precursors first enter the thymus from the bone marrow, they are known as CD4-CD8- double-negative thymocytes, expressing neither of these T cell surface markers. They then become CD4+CD8+ double-positive thymocytes. Double-positive thymocytes undergo positive selection, whereby cells that are potentially reactive to antigens presented by self-MHC molecules are selected for, and negative selection, whereby self-reactive T cells are eliminated. Cells that do not receive T cell receptor (TCR) signals are also eliminated. Double-positive thymocytes are then induced to differentiate into single-positive thymocytes before their eventual export from the thymus (Takahama, 2006).

On leaving the thymus, naïve T cells enter the bloodstream. When a peripheral lymphoid organ is reached, they leave the blood and migrate through the lymphoid tissue. Entry into lymphoid tissue is a four-step process involving rolling, activation by chemokines, adhesion by integrins and diapedesis (Eksteen et al., 2008). If naïve T cells do not encounter their cognate antigen, they return to the bloodstream via the lymphatic system and continue to re-circulate between blood and peripheral lymphoid tissues. If a naïve T cell encounters its cognate antigen presented by an APC, T cell activation occurs. Naïve CD8+ T cells recognise antigen presented by major histocompatibility complex (MHC) class I molecules on APCs whereas naïve CD4+ T cells recognise antigen presented by MHC class II molecules on APCs. Activation requires three signals (Jambo et al., 2010):

1. Recognition of the foreign-peptide: self-MHC complex on APCs by the TCR on T cells and ligation of the co-receptor CD4 or CD8
2. Interaction of co-stimulatory molecules on T cells (CD40L and CD28) with their receptors on APCs (CD40 and CD80/CD86, respectively)
3. Secretion of polarising cytokines by APCs. The particular cytokines secreted determine whether CD4+ T cells differentiate into effector T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) or regulatory T cells (Tregs)

Upon activation, naïve T cells become antigen-specific effector T cells. Naïve CD4+ T cells differentiate into Th1, Th2, Th17 or Treg cells. These T cell subsets have different

functions. Th1 cells secrete inflammatory cytokines such as interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and interleukin 2 (IL-2) which are necessary for the clearance of intracellular bacteria and viruses. They mediate cellular immunity by activating macrophages and CD8+ effector cells. Th2 cells secrete cytokines such as IL-4, IL-5 and IL-13 and mediate immunity against parasitic infections. They also play a role in allergic responses. Th17 cells protect against extracellular bacterial infections and play a role in autoimmunity (MacLeod et al., 2009). Tregs maintain peripheral tolerance (Vignali et al., 2008) and they have suppressive properties, preventing a protective immune response becoming a destructive immunopathology (Thompson and Powrie, 2004). Naïve CD8+ T cells become cytotoxic T lymphocytes (CTL) on activation. CTLs release perforin and granzymes leading to the induction of apoptosis of target cells (Seder et al., 2008).

Antigen-specific effector T cells that remain in the body during the contraction phase of an immune response become long-lived memory T cells (Woodland and Kohlmeier, 2009). Memory T cells can also be formed directly from the differentiation of naïve T cells, without first passing through an effector T cell phase (Moulton and Farber, 2006). There are two types of memory cell, central memory T cells (Tcm) and effector memory T cells (Tem), which can be distinguished on the basis of their phenotypic and functional properties. Tcm are $CD62L^{+}CCR7^{+}$ (as are naïve T cells) whereas Tem are $CD62L^{-}CCR7^{-}$. Tcm preferentially localise to lymphoid tissues (as do naïve T cells) whereas Tem preferentially localise to peripheral tissues (Woodland and Kohlmeier, 2009). In contrast to effector T cells, memory T cells require antigen stimulation to function. In response to a secondary infection and antigen stimulation, Tcm lack immediate effector function but can develop the phenotype and function of effector memory T cells rapidly whereas Tem can exert immediate effector functions and do not need to undergo further differentiation. After stimulation, both subsets produce IFN-γ and TNF-α but Tcm produce more IL-2, which is thought to increase their proliferative ability (Woodland and Kohlmeier, 2009).

Table 1.2 summarises the phenotypic properties that differentiate naïve, effector and memory T cells.

	Naïve	Effector	Memory
	Small	Large	Small
CD25: IL-2 receptor α chain	$CD25^{lo}$	$CD25^{hi}$	$CD25^{lo}$
CD44: adhesion marker	$CD44^{lo}$	$CD44^{hi}$	$CD44^{hi}$
CD62L: leukocyte adhesion molecule	$CD62L^{+}$	$CD62L^{-}$	$CD62L^{-}$ (Tem) or $CD62L^{+}$ (Tcm)
CCR7: chemokine receptor	$CCR7^{+}$	$CCR7^{-}$	$CCR7^{-}$ (Tem) or $CCR7^{+}$ (Tcm)
CD127: IL-7 receptor α chain	$CD127^{hi}$	$CD127^{lo}$	$CD127^{hi}$

Table 1.2 Distinguishing phenotypic properties of naïve, effector and memory CD4+ T cells, adapted from (Moulton and Farber, 2006; Sallusto et al., 1999).

1.1.4.1 Tissue-specific T cell homing

As mentioned above (section 1.1.4), naïve T cells and Tcm preferentially migrate to secondary lymphoid organs. Effector T cells and Tem, in contrast, migrate to non-lymphoid tissues such as the gut lamina propria, lungs, skin, liver and sites of inflammation (Mora, 2008). They preferentially migrate to the non-lymphoid tissues that are connected to the secondary lymphoid organs in which antigen was first encountered. For example, oral antigens, which are first encountered by GALT, induce effector T cells and Tem that express the cell-surface molecules needed for homing to mucosal surfaces such as the small intestine (Mora et al., 2003). These cell-surface molecules are the integrin α4β7 which binds to mucosal vascular addressin cell adhesion molecule 1 (MAdCAM1) and the chemokine receptor CCR9 which interacts with the gut-associated chemokine CCL25 (Woodland and Kohlmeier, 2009). This gut-specific homing is imprinted on T cells by dendritic cells in GALT (Mora et al., 2003). These dendritic cells produce the vitamin A (retinol) metabolite, retinoic acid, which induces the expression of α4β7 and CCR9 on T cells (Iwata et al., 2004).

The integrin α4β7 is also important for T cell homing to the colon. However, there are no CCR9+ T cells in the colon and CCL25 is not expressed in this compartment. The chemokine CCL28 is expressed by colonic epithelial cells, but its receptor, CCR10, has not yet been reported on colonic T cells; it has only been found on mucosal antibody-secreting cells (Mora, 2008).

1.1.5 Assays for measuring cellular immune responses

The activation of antigen-specific T cells by antigen-presenting cells results in a number of biological events including the production and secretion of cytokines and the proliferation of antigen-specific clones (Godoy-Ramirez et al., 2004). Numerous techniques are available

for the detection and measurement of these biological events and these techniques fall into one of two categories: bulk assays or single-cell assays.

In a bulk assay, immune responses are analysed by averaging data from large groups of cells rather than by looking at individual cells. Consequently, such assays make the assumption that cells of a given phenotype show identical behaviour. Bulk assays cannot distinguish between whether a certain effect results from a small, homogenous response from all cells or a large response from a subset of cells, for example (Lindstrom and Andersson-Svahn, 2010). Bulk assays used to measure T cell function include the enzyme-linked immunosorbent assay (ELISA) and the [^{3}H] thymidine proliferation assay (Gauduin, 2006).

Single-cell assays measure immune responses from individual cells. A commonly used method for single-cell analysis is flow cytometry, a method which allows for the analysis of hundreds of thousands of individual cells per minute based on cellular properties such as size, granularity and fluorescence (Lindstrom and Andersson-Svahn, 2010). Flow cytometric T cell assays include the intracellular cytokine staining (ICS) which measures cytokine production and secretion and the carboxyfluorescein succinimidyl ester (CFSE) proliferation assay which measures cell division.

The ICS assay is based on the principle that T cells respond to antigen stimulation by releasing cytokines. Cytokines play an essential role in the initiation and maintenance of the immune response and different cytokines lead to the selection and propagation of different effector mechanisms (Godoy-Ramirez et al., 2004). In this assay, T cells are incubated with an antigen of interest. After 1-2 hours, brefeldin A is added. Brefeldin A blocks secretory pathways and therefore causes cytokine accumulation within the cell. After 6-24 hours, cells are harvested and stained extracellularly for phenotypic markers. Cells are then permeabilised and fixed, allowing intracellular staining by fluorochrome-conjugated antibodies that are specific to the cytokines of interest. Acquisition on a flow cytometer can then take place (Letsch and Scheibenbogen, 2003).

The CFSE assay is based on the principle that T cells respond to antigen stimulation by undergoing mitotic cell division and rapidly increasing in number. In this assay, cells are labelled with CFSE and then incubated with an antigen of interest. CFSE is a dye that is taken up by T cells and partitioned equally between daughter cells following cell division. The intracellular CFSE is therefore diluted in each subsequent generation (Lyons, 2000).

After a week the cells are harvested, stained extracellularly for phenotype and the sample is acquired on a flow cytometer.

The major advantage of flow cytometry is that it enables the analysis of multiple parameters per cell. In the ICS assay, for example, cytokine and cell-surface markers can be measured simultaneously meaning that more information is obtained from a single specimen. This is especially advantageous when the amount of specimen material is limited. In addition, ICS allows for the simultaneous measurement of multiple cytokines from the same cell meaning that the chance of detecting antigen-specific cells is increased (Bolton and Roederer, 2009).

As with all techniques, flow cytometry has some weaknesses. First, the number of antigen-specific cytokine-producing T cells is low and, consequently, difficult to detect (Godoy-Ramirez et al., 2004). This raises the question of how many single cells should be investigated and acquired on the flow cytometer in order to be confident in the conclusions drawn from the data. Second, the method by which cells are processed prior to acquisition on a flow cytometer can alter their phenotype. Third, T cell activation can lead to the down-regulation of surface markers such as CD4 and flow cytometric phenotyping relies on the expression of such surface markers. For example, if CD4 is down-regulated on peripheral blood mononuclear cells (PBMCs) that are expressing IFN-γ, one might arrive at the conclusion that IFN-γ is produced by CD4- T cells, such as natural killer cells or CD8+ T cells (Pala et al., 2000). Lastly, even though in principal flow cytometry allows for the measurement of multiple cytokines simultaneously, the time course and therefore the optimal detection time for different cytokines varies, and this limits investigation of co-expression of different cytokines in single cells (Pala et al., 2000).

1.2 TYPHOID FEVER

Typhoid fever is a systemic infection which is caused by the bacterium *Salmonella enterica* serovar Typhi (*S.* Typhi). Annually, there are an estimated 16-33 million new typhoid fever cases and 500,000-600,000 deaths worldwide (Ivanoff et al., 1994; Plotkin and Orenstein, 2004). *S.* Typhi is a human-specific pathogen and is transmitted via the ingestion of food and water contaminated by faecal or urinary carriers excreting *S.* Typhi. Other sources of infection include vegetables fertilised by night soil and eaten raw, and shellfish taken from sewage-contaminated beds (Girard et al., 2006). In the 19th century, typhoid fever was an important cause of illness and death in the United States and Europe due to overcrowding

and unsanitary urban conditions. However, the provision of clean water and good sewage systems at the turn of the 20th century resulted in a dramatic decrease in the incidence of typhoid in these regions (Parry et al., 2002). Today, typhoid remains endemic in most of the less developed areas of the world, including many countries in Africa, Asia and South America (Crump et al., 2004; Crump and Mintz, 2010).

1.2.1 Molecular and biological features

Isolates of *S.* Typhi often share common antigenic determinants: the protein flagellar antigen, H, the lipopolysaccharide antigens O9 and O12, and the polysaccharide capsular Vi (virulence) antigen (Girard et al., 2006).

The complete genome sequence of a multidrug-resistant strain of *S.* Typhi (CT18) was determined in 2001 and CT18 was found to contain 4,809,037 base pairs. Over two hundred pseudogenes are present in the genome of *S.* Typhi, a feature which is thought to contribute to its human-restricted host range (Parkhill et al., 2001).

In the genome of *S.* Typhi, the genes for virulence factors cluster into pathogenicity islands. SPI-1 and SPI-2 are the two major pathogenicity islands and they encode the type three secretion system (TTSS) which is responsible for the translocation of bacterial virulence proteins into host cells during infection (Zhang et al., 2008). SPI-1 encodes the TTSS and its effectors which are required for the invasion of epithelial cells. It is thought to be activated under conditions present in the intestinal lumen before host cell invasion such as high osmolarity and low aeration (Faucher et al., 2009). SPI-2 encodes a second TTSS, effector proteins, molecular chaperones and a regulatory system that activates SPI-2 promoters, and is essential for intracellular survival and replication at systemic sites of infection (Zhang et al., 2008). It is activated intracellularly and SPI-2 TTSS genes are induced by an acidic pH and low concentrations of magnesium and phosphate (Faucher et al., 2009).

S. Typhi has many other pathogenicity islands, most of which are of great importance to the virulence and survival of the bacterium. SPI-7 encodes the genes for Vi polysaccharide capsule production. This capsule is thought to be important in the survival of the bacterium within macrophages (Zhang et al., 2008). SPI-7 is a mobile and potentially unstable genetic region and this could have important implications for the Vi-based polysaccharide typhoid vaccine; capsule replacement events could occur as a result of selection driven by Vi-based mass-vaccination campaigns (Baker and Dougan, 2007).

1.2.2 Pathogenesis

S. Typhi is ingested in contaminated water or food and must pass through the acid stomach in order to reach the small intestine. Once in the small intestine, typhoid bacilli must adhere to mucosal cells and then penetrate the gut mucosa (Parry et al., 2002). Active attachment to mucosal cells is promoted by the bacilli and is thought to involve adhesion molecules on the bacteria interacting with receptors on the host cell, an interaction which may involve fimbriae (House et al., 2001). Invasion of mucosal cells is mediated by SPI-1 and SPI-2. SPI-1 encodes regulator proteins, a TTSS which delivers bacterial proteins into the host cell, and effector proteins which bring about changes in the host cell and promote bacterial uptake (House et al., 2001).

There are four different ways in which typhoid bacilli can penetrate the mucosal epithelium to reach the lamina propria: (1) bacilli are actively taken up by M cells, the specialised epithelial cells which are found overlying Peyer's patches, (2) bacilli are taken up by normally non-phagocytic enterocytes as a result of *Salmonella*'s SPI-1 and other invasive mechanisms, (3) *Salmonella* can cross the epithelium paracellularly and (4) direct uptake of *Salmonella* from the gut lumen by dendritic cells in the lamina propria which are able to extend dendrites through the basement membrane and between the epithelial cells to enter the gut lumen (Biedzka-Sarek and El Skurnik, 2006). This latter route involves the transport of *Salmonella* from the gastrointestinal tract to the bloodstream. Consequently, it does not induce mucosal immunity but is thought to be important for the induction of systemic immunity to gastrointestinal pathogens (Vazquez-Torres et al., 1999).

Following penetration of the mucosal epithelium, typhoid bacilli elicit an influx of macrophages which ingest the organisms but do not kill them. *Salmonella* reside within macrophages in specialised compartments known as *Salmonella*-containing vacuoles (Alpuche-Aranda et al., 1994). Some bacilli remain within macrophages in the small intestine lymphoid tissue whilst others are drained into MLNs where further ingestion by macrophages occurs. Shortly after invasion of the intestinal mucosa, primary bacteraemia occurs (Plotkin and Orenstein, 2004). Some bacilli then pass to the reticuloendothelial tissue of the liver and spleen where they are able to survive and multiply.

After about 8 to 14 days, *S.* Typhi are released from their intracellular location, resulting in secondary bacteraemia, fever and systemic infection (Gordon, 2008). Haematogenous

spread means that *S.* Typhi is widely disseminated and able to infect a number of sites (Figure 1.3). The most common sites of secondary infection are the liver, spleen, bone marrow, gallbladder and Peyer's patches. Gallbladder invasion occurs directly from the blood or by retrograde spread from the bile (Parry et al., 2002). In 2% to 5% of cases, gallbladder infection becomes chronic (Plotkin and Orenstein, 2004). Bacilli which are excreted in the bile either reinvade the intestinal wall or are shed in the faeces, an essential step in the transmission of typhoid fever (Parry et al., 2002).

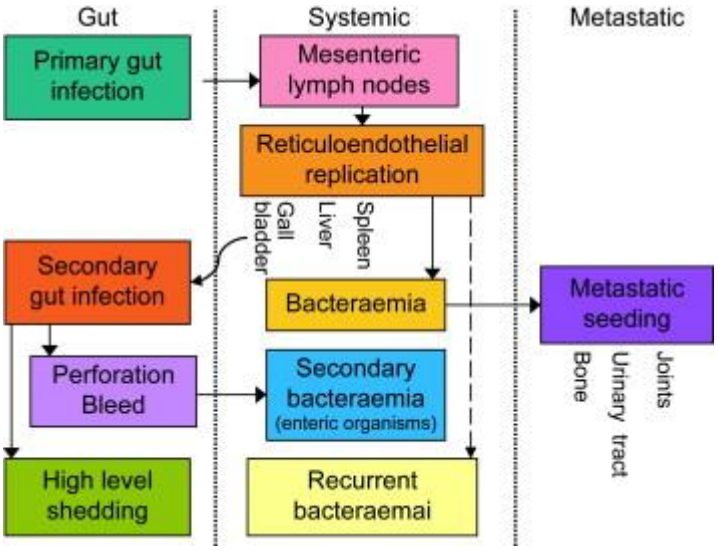

Figure 1.3 The pathogenesis of typhoid fever from (Gordon, 2008).

1.2.3 Clinical features

Typhoid fever is characterised by fever and abdominal symptoms. Nonspecific symptoms are often present before the onset of fever. Such symptoms include cough, sore throat, headache, dizziness, loss of appetite, chills, perspiration, diarrhoea or constipation, and muscle pains. Other symptoms include splenomegaly, hepatomegaly, bradycardia and rose spots (Fraser et al., 2007). Complications occur in 10% to 15% of patients and are more likely in patients who have been ill for more than a fortnight. The most important complications are gastrointestinal bleeding, intestinal perforation and typhoid encephalopathy (Parry et al., 2002). Gastrointestinal bleeding is the most common complication, occurring in up to 10% of patients. It is the result of erosion of a necrotic Peyer's patch through the wall of an enteric vessel. In the majority of cases the bleeding is not severe but in 2% of cases, bleeding is clinically significant and can be rapidly fatal (Parry et al., 2002).

In the pre-antibiotic era, the case-fatality rate for typhoid fever was 10-20%. The use of antibiotics such as chloramphenicol to treat typhoid fever saw case fatality drop to below 1%. However, the emergence of antibiotic-resistant strains of *S.* Typhi in several parts of the world during the last decade of the twentieth century has led to an increase in the incidence of severe cases and an increase in typhoid mortality (Plotkin and Orenstein, 2004).

1.2.4 Vaccination

There are three currently licensed typhoid vaccines available: inactivated phenol-preserved whole-cell parenteral vaccine, purified Vi polysaccharide parenteral vaccine and attenuated *S.* Typhi strain Ty21a which is used as a live oral vaccine.

1.2.4.1 Parenteral inactivated whole-cell vaccine

The first parenteral whole-cell typhoid vaccine was introduced in 1896 but it was not until the 1960s that its efficacy was established in field trials (Parry et al., 2002). Although these vaccines are efficacious, high rates of systemic and local adverse reactions make them unsuitable for use as public health tools and they have generally been abandoned (Garmory et al., 2002).

1.2.4.2 Purified Vi polysaccharide parenteral vaccine

The subunit Vi vaccine was developed in the 1980s (Girard et al., 2006). The Vi capsular polysaccharide is an important virulence factor. As well as preventing antibodies from binding to the O antigen, which allows S. Typhi to survive in the blood, it is associated with both the inhibition of complement activation and resistance to complement-mediated lysis and phagocytosis (Garmory et al., 2002; Looney and Steigbigel, 1986). This vaccine is administered as a single subcutaneous or intramuscular injection and is both safe and effective (Fraser et al., 2007).

Vi-based subunit vaccines have three main drawbacks: they are unable to stimulate mucosal immunity, revaccination does not elicit any booster effect, and they do not protect young children. Absence of a booster effect occurs as immune responses against polysaccharides do not involve T cells and so immunological memory cannot be established. A lack of effective protection in children younger than two years occurs as

young children cannot mount effective immune responses to bacterial polysaccharides (Guzman et al., 2006).

These limitations could be overcome by using a conjugate vaccine in which the T-independent Vi polysaccharide antigen was linked to a T-dependent protein carrier. Such a vaccine, in which Vi polysaccharide is covalently bound to recombinant *Pseudomonas aeruginosa* exotoxin A, is under development but is not yet licensed (Guzman et al., 2006). This vaccine, known as Vi-rEPA, was evaluated in a randomised control trial in children two to five years old in Vietnam where its efficacy was found to be 91.5% (Lin et al., 2001). It has recently been evaluated in infants (two to twelve months) and was found to be safe, compatible with the Expanded Program on Immunisation vaccines and able to induce protective anti-Vi antibodies (Thiem et al., 2011). Currently, conjugate vaccines are licensed for use against pathogens such as *Haemophilus influenzae* type b, *Neisseria meningitidis* and *Streptococcus pneumoniae* (Guzman et al., 2006).

1.2.4.3 Ty21a live oral vaccine

Ty21a was developed in the 1970s by chemical mutagenesis of the wild-type strain Ty2 and has a GalE- and Vi-negative phenotype (Germanier and Fuer, 1975). The *galE* gene encodes the enzyme uridine diphosphate (UDP)-galactose-4-epimerase, an enzyme which is responsible for the conversion of UDP-glucose to UDP-galactose and vice versa. A GalE-negative phenotype therefore results in the absence of this enzyme, with the result that UDP-galactose cannot be metabolised. When galactose is present in the growth medium, UDP-galactose therefore accumulates in the cytoplasm, causing cell lysis and attenuation (Guzman et al., 2006). When galactose is not present in the growth medium, Ty21a does not express smooth O antigen in its lipopolysaccharide (LPS) because it has no source of galactose and in this state it is not virulent or immunogenic. Ty21a is therefore supplied with exogenous galactose during vaccine production (Guzman et al., 2006). As a result of nonspecific chemical mutagenesis, other mutations are also present within strain Ty21a but not all of these are characterised (Dietrich et al., 2003).

Ty21a (Vivotif®) is manufactured by Crucell and contains lyophilised *S.* Typhi Ty21a, with not less than 2×10^9 viable cells per capsule. It is recommended for adults and children from six years of age. Throughout the world (with the exception of the USA and Canada) a

three-dose oral immunisation schedule is recommended, with one capsule being taken on days one, three and five, with lukewarm water at least one hour before meals. Protection becomes effective about seven to ten days after the last dose (Plotkin and Orenstein, 2004) (Appendix 1). Contraindications to immunisation with Ty21a include hypersensitivity to any components (e.g. gelatine), immune deficiency, concomitant treatment with immunosuppressive drugs, pregnancy, certain antibiotics and acute febrile or gastrointestinal illness (Plotkin and Orenstein, 2004)(Appendix 1).

The efficacy of the Ty21a vaccine has been evaluated in many clinical trials. These have shown variable protective efficacy, ranging from 96% in Egypt after three years (Wahdan et al., 1982) to 67% in Chile after three years (Levine et al., 1999) to 42% in Indonesia after two and a half years (Simanjuntak et al., 1991). In a meta-analysis, the three year cumulative efficacy of Ty21a was found to be 51% (Engels et al., 1998).

Ty21a is an extremely safe and well-tolerated vaccine, a fact which has been confirmed in more than 200 million vaccinees during over 20 years of use worldwide. Between 1990 and 2000, over 38 million people were vaccinated with Ty21a and there were only 743 reports of adverse effects, an incidence of 0.002% (Guzman et al., 2006). The most common adverse effects reported were mild gastrointestinal disturbances and general symptoms, such as fever (Guzman et al., 2006). Upon administration of the vaccine, there is either a limited and transient level or a complete lack of faecal excretion of Ty21a (Levine et al., 2001). Genetic attenuation combined with low shedding rate significantly reduces the environmental risks posed by the use of Ty21a (Guzman et al., 2006).

1.2.4.4 Newer generation unlicensed oral vaccines

Drawbacks of the Ty21a vaccine, such as the requirement for multiple doses to elicit a protective response and incomplete protection, led to the development of a number of novel live attenuated S. Typhi strains during the last three decades (Guzman et al., 2006), although none of these strains are currently licensed as typhoid vaccines. Novel strains include Ty800, Centre for Vaccine Development (CVD) 908, CVD 908-htrA, CVD 909 and M01ZH09. Ty800 is a derivative of S. Typhi strain Ty2 which is mutated in phoP/phoQ, two virulence-regulating genes. In clinical trials this strain induced vigorous immune responses without adverse side effects, apart from diarrhoea in a small number of individuals (Hohmann et al., 1996).

Strains CVD 908, CVD 908-*htrA* and CVD 909 are all based on mutations in *aroC* and *aroD*, genes whose function is the biosynthesis of para-aminobenzoic acid, 2,3-dihydroxybenzoate and aromatic amino acids (Mastroeni et al., 2001). *Salmonella* with mutations in *aro* genes have impaired ability to proliferate within mammalian cells, but they grow intracellularly for long enough to stimulate immune responses (Tacket and Levine, 2007). CVD-908 is an *aroC/aroD* deleted derivative of *S.* Typhi strain Ty2. This vaccine was well tolerated after a single dose and six out of twelve volunteers developed serum IgG anti-lipopolysaccharide antibodies whilst IgA anti-LPS antibody-secreting cells were detected in nine of eleven volunteers (Tacket et al., 1992). However, subsequent clinical studies found that clinically silent, self-limited vaccine bacteraemia occurred in between 50-100% of volunteers depending of the dose of organism given and this was thought to be an indicator of unacceptable reactogenicity in future studies which could involve larger numbers of volunteers, not all of who would be healthy adults (Tacket and Levine, 2007).

A deletion in the *htrA* gene was introduced into CVD 908 to yield the strain CVD 908-*htrA*. The *htrA* gene encodes a heat-shock protein and when this gene is deleted, the resulting mutant is less virulent due to a reduced ability to survive and replicate in host tissues (Tacket and Levine, 2007). CVD 908-*htrA* retained the immunogenicity of CVD 908 but no vaccine bacteraemia was detected. A single dose of CVD 908-*htrA* stimulated strong mucosal, humoral and cellular immune responses that were equal or better than those measured after multiple doses of Ty21a (Tacket et al., 2000b).

A drawback of both CVD 908 and CVD 908-*htrA* was an inability to consistently stimulate serum Vi antibody. The *viaB* locus of *S.* Typhi encodes the genes needed for the synthesis, surface transport and anchoring of Vi. Strain CVD 909 was produced by replacing the promoter P_{tviA} (the most upstream gene in the *viaB* locus of CVD 908-*htrA*) with the strong constitutive promoter P_{tac} (Wang et al., 2000). CVD 909 was found to be safe in a phase I clinical trial but while strong gut-derived IgA antibody-secreting cell responses were induced against Vi-antigen, only two out of 32 vaccinees exhibited anti-Vi IgG in serum (Tacket et al., 2004).

M01ZH09 is a single-dose candidate vaccine with two well-defined attenuating mutations. There is a mutation in a structural protein (SsaV) of SPI-2 which destroys the function of SPI-2, preventing the systemic spread of *S.* Typhi, and an aromatic mutation (*aroC*-) which deprives the bacterium of essential nutrients that it obtains from the human host

(Kirkpatrick et al., 2005). This vaccine has been showed to be safe and immunogenic in Western adult volunteers (Hindle et al., 2002). In 2010, the results of a phase II trial of M01ZH09 in children in Vietnam, an endemic country, were published (Tran et al., 2010). The vaccine had an appropriate safety profile and a positive immune response was detected in 98 out of 101 vaccinated children.

1.3 ADAPTIVE IMMUNE RESPONSES TO SALMONELLA TYPHI

1.3.1 Immune response to natural *S.* Typhi infection

Natural infection with *S.* Typhi induces humoral and cellular immune responses. Humoral responses include serum antibodies against bacterial antigens such as O-antigen (lipopolysaccharide), H-antigen (flagellar component) and Vi polysaccharide capsular antigen, and secretory IgA (sIgA) against bacterial antigens such as O-antigen. sIgA is found in intestinal fluids and bile. Cell-mediated responses in the blood include T cell proliferation and increased levels of Th1-type cytokines such as IFN-γ, TNF-α, IL-1 and IL-6 (Pasetti et al., 2011). Little is known about the mucosal cell-mediated immune (CMI) response to natural infection.

1.3.2 Immune response to vaccination with the Vi polysaccharide vaccine

Vaccination with the parenteral Vi polysaccharide vaccine stimulates the production of systemic antibodies (mainly IgG and IgA) against the Vi capsular antigen only (Pasetti et al., 2011). No cell-mediated immune response is induced by this vaccine because polysaccharides are T-cell-independent antigens.

1.3.3 Immune response to vaccination with Ty21a

Vaccination with Ty21a differs from natural infection because Ty21a does not express the Vi polysaccharide capsular antigen and is consequently unable to stimulate Vi antibody (Levine et al., 2001). Additionally, Ty21a differs from its wildtype parent, the pathogenic *S.* Typhi strain Ty2, by more than two dozen mutations (Pasetti et al., 2011). However, as with natural infection, oral vaccination can activate every effector arm of the immune system and both humoral and cell-mediated systemic and mucosal immune responses are elicited. Serum IgG against O-antigen is induced (D'Amelio et al., 1988; Viret et al., 1999) as well as antibodies against a number of proteins including H-antigen, porins, outer membrane proteins, heat-shock proteins, flagella and fimbriae (Mastroeni et al., 2001). Locally, intestinal secretory antibodies are induced against O-antigen (Ivanoff et al., 1994;

Levine et al., 2001). The Ty21a vaccine stimulates strong cell-mediated CD4+ and CD8+ immune responses. In the blood, there is T cell proliferation and the production and secretion of Th1-type cytokines such as IFN-γ (Table 1.3). Nothing is known about the CMI responses that are induced locally in the gut mucosa (Sztein, 2007).

Study	Methods	Systemic cellular response	Mucosal cellular response
(Salerno-Goncalves et al., 2002)	Flow cytometry, ELISPOT	Significant increase in IFN-γ production in 4/5 individuals. IFN-γ secreted predominantly from $CD8^+$ T cells	-
(Lundin et al., 2002)	ELISA, [^{3}H]thymidine	Significant (>2-fold) increase in rate of proliferation and IFN-γ production in 6/8 individuals. IFN- γ secreted predominantly from CD8+ T cells	-
(Kilhamn et al., 2003)	ELISA, [^{3}H]thymidine	$CD4^+$: significant (> 2-fold) proliferative response and IFN-γ production in 3/10 individuals $CD8^+$: significant (> 2-fold) proliferative response and IFN-γ production in 6/10 individuals	-
(Lundgren et al., 2009)	ELISA, Pelikine compact human granzyme A and B kits, [^{3}H]thymidine	Vaccinees responded with significantly (>2-fold) increased levels of IFN-γ (8/11) and granzyme A (7/11) but not granzyme B. Significantly increased proliferative response (7/15)	-
(Viret et al., 1999)	ELISA, [^{3}H]thymidine	$CD4^+$: Significantly increased proliferative responses and IFN-γ production in 5/5 individuals	-

Table 1.3 A summary of studies measuring cellular immunological responses to the live oral typhoid vaccine Ty21a in humans. In all studies, individuals were vaccinated with three doses of Ty21a at two day intervals except (Salerno-Goncalves et al., 2002) in which individuals were vaccinated with four doses of Ty21a at two day intervals

1.4 VECTOR VACCINES

The majority of pathogens enter the body through the mucous membranes (Czerkinsky and Holmgren, 2009). For this reason, mucosally-delivered vaccines, which have the potential to elicit both systemic and mucosal immune responses, have an advantage over parenteral vaccines, which induce a systemic response but have limited capacity to induce a mucosal response. Antigens delivered by the oral mucosal route are exposed to the acidity of the gastrointestinal tract and can be degraded by enzymes at mucosal surfaces; for these reasons, such antigens are often poorly immunogenic. This issue can be overcome by using live bacteria as vectors. Bacteria can be engineered to express antigens from unrelated

pathogens and deliver these heterologous antigens to the immune system, leading to the generation of mucosal and systemic immune responses (Garmory et al., 2003). If the concept of a common mucosal immune system is taken as fact, the generated immune response would be present at both the immune inductive site in which antigen presentation occurred and at remote mucosal sites (Medina and Guzman, 2001).

Attenuated strains of *S.* Typhi and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) can be used as live vector vaccines. *Salmonella* strains are suited to this role because they can be administered orally (natural route of infection), they are transported through M cells to GALT (immune inductive site), they are readily taken up by antigen-presenting cells such as dendritic cells and macrophages and they stimulate a wide array of systemic and mucosal humoral and cellular immune responses (Galen et al., 2009). *Salmonella* are also easy to genetically manipulate (Garmory et al., 2002). Potential problems of live bacterial carriers include reversion to virulence or reactogenicity, stability of the recombinant phenotype and horizontal gene transfer between the vaccine strain and mucosal or environmental microorganisms (Medina and Guzman, 2001). There is also the issue of pre-existing immunity. Prior immunity to *S.* Typhi, for example, may adversely affect the magnitude of the immune response against the heterologous antigens.

S. Typhimurium infection of rodents closely resembles *S.* Typhi infection of humans (Pasetti et al., 2003). Preclinical work using a Typhimurium mouse model has highlighted the potential of *Salmonella* vector vaccines. For example, DiGiandomenico *et al.* (2004) orally vaccinated BALB/c mice with attenuated *S*. Typhimurium expressing *Pseudomonas aeruginosa* serogroup O11 O antigen. Vaccination stimulated the production of O11-specific IgG and IgA antibodies in serum and remotely in bronchoalveolar lavage fluid (BAL). When orally vaccinated mice were challenged with an O11 strain of *P. aeruginosa* at six and twelve times the 50% lethal dose, survival was increased compared to the control group. Vaccinated mice also demonstrated an ability to clear the bacteria from their lungs (DiGiandomenico et al., 2004). Live recombinant *S.* Typhimurium vaccines expressing pneumococcal proteins such as pneumococcal surface protein A have also been developed. Vaccination of mice with these vaccines elicited serum IgG and mucosal IgA responses. Furthermore, vaccinated mice were protected against intraperitoneal, intravenous and intranasal pneumococcal challenge (Xin et al., 2009).

S. Typhi is a human-restricted pathogen and, consequently, it is not known to be virulent in any animal model after oral vaccination (Garmory et al., 2002). However a mouse model of

intranasal immunisation exists. Mice were immunised via the intranasal route with the live attenuated *S.* Typhi strain CVD 908-*htrA* expressing fragment C of tetanus toxin. In addition to the detection of humoral and cell-mediated systemic responses against fragment C, a mucosal response was also seen; both IgG and IgA fragment C-specific antibody-secreting cells were demonstrated in the lung (Capozzo et al., 2004). Recently, a humanised mouse has been developed for the study of typhoid fever. Nonobese diabetic-*scid IL2rγ*^{null} mice were engrafted with human haematopoietic stem cells (hu-SRC-SCID mice). *S.* Typhi was able to replicate in these mice, causing a lethal infection with pathological and inflammatory features that resembled human typhoid fever (Libby et al., 2010). This humanised mouse model will be invaluable for understanding more about the pathogenesis of *S.* Typhi and for identifying vaccine candidates that could lead to the prevention of typhoid fever.

There have only been a small number of human clinical trials of *S.* Typhi vector vaccines. Gonzalez *et al.* (1994) vaccinated individuals with the *S.* Typhi strain CVD 908 expressing the circumsporozoite protein (CSP) of *Plasmodium falciparum* and they demonstrated a serologic and mucosal IgA response to CSP (Gonzalez et al., 1994). This was the first clinical trial of its kind. More recent clinical trials have used antigens from *Helicobacter pylori* (Metzger et al., 2004) and *Clostridium tetani* (Tacket et al., 2000a). In general, the immune responses detected in such trials have been weak. Galen *et al.* conclude that this is because 'no trial has tested a robust live vector strain that expresses an antigen of predictable immunogenicity (based on other vaccine or natural infection experience) from a stabilized gene (chromosomal or state-of-the-art plasmid maintenance system), under a suitable promoter and (critically!) with either surface expression or export of the antigen out of the bacteria. Thus, to date, no clinical trial has been carried out with an optimum construct' (Galen et al., 2009).

1.5 RATIONALE AND AIMS

Ty21a is the only licensed live oral typhoid vaccine and it has been licensed for many decades. Despite this, very little is known about the cellular immune responses elicited locally in the intestinal mucosa after vaccination. A better understanding of these local immune responses and how well they correlate with systemic immune responses would inform the development of future vaccines, which relies on the measurement of the systemic immune response to evaluate vaccine candidates.

The concept of a common mucosal immune system means that oral vaccines could be developed which could elicit immune responses at remote mucosal sites. The use of *Salmonella* as vector vaccines has shown great promise in animal models but more work needs to be done in humans to see how effective such vaccines are.

Aims:

- To determine whether vaccination with Ty21a induces antigen-specific CD4+ T cells in peripheral blood, duodenum and colon
- To determine whether there is a relationship between the magnitude of the antigen-specific response in duodenum compared to colon or blood

CHAPTER 2: STUDY DESIGN, MATERIALS AND METHODS

2.1 STUDY LOCATION

This study was conducted in Liverpool and was sponsored by the Royal Liverpool University Hospital (RLUH) and the Liverpool School of Tropical Medicine (LSTM). The clinical work was carried out at the RLUH and the laboratory work was carried out at the LSTM.

2.2 STUDY POPULATION

Study participants were recruited by poster advertisement (Appendix 2) around the University of Liverpool (UoL) campus, RLUH and LSTM and by electronic advertisement of the intranet of UoL. Seventeen healthy adult study participants (median age 26 years, age range 21-58 years, 9 females) were enrolled in the study after they had given informed consent.

2.2.1 Inclusion and exclusion criteria

The inclusion and exclusion criteria for participation in the study were as follows:

- Inclusion criteria for control and vaccinated groups:
    - Adults aged between eighteen and sixty years
    - Adults in full health (to minimise the risk of the vaccination or the procedures)
    - Fluent spoken English (to ensure that volunteers understand the purpose of the research project and their role within it and are able to give informed consent)
    - Capacity to give informed consent
- Exclusion criteria for control and vaccinated groups:
    - Pregnancy
    - Chronic illness or immunocompromise
    - Anaemia
    - Platelet count below 30,000
    - Individuals who have received the parenteral typhoid vaccine within the past three years or the oral typhoid vaccine within the past 12 months (the recommended booster intervals)
    - Smoker or ex-smoker who has smoked more than 20 cigarettes a day for ten years

    - Individuals already involved in another clinical trial
- Additional exclusion criteria for vaccinated group:
    - Previous adverse reaction to vaccination

Individuals who had previously been vaccinated against typhoid outside the recommended booster intervals were accepted for the study as this was thought to be likely to yield useful information about the magnitude and character of primary compared to boosted responses to vaccination. Such individuals were allocated to the vaccinated arm of the study.

2.2.2 Sample size calculation

A sample size of 21 in each group (42 in total) will have 80% power to detect a two-fold difference in the mean percentage of T cells showing antigen-specific intracellular IFN-γ production at a 0.05 two-sided significance level between unvaccinated and vaccinated individuals. This calculation was based on PBMC responses to mucosally presented antigens such as flu and pneumococcus as the best available data are for PBMC responses to these antigens as opposed to mucosal responses to typhoid. The assumption was made that this sample size would also be adequate for mucosal responses as one would anticipate that mucosal T cell responses to typhoid should be of a greater magnitude than PBMC responses, as typhoid is presented through the mucosa. A 15% drop-out during the study was anticipated so the plan was to recruit 48 individuals in total, 24 in each group.

2.2.3 Ethical approval

Ethical approval for this study was obtained from the National Research Ethics Service (10/H1005/20) (Appendix 3) and the Liverpool School of Tropical Medicine ethics committee (SG10.01) (Appendix 4).

The statistician that reviewed the protocol for the ethics application suggested that, given the invasive nature of the study, an interim analysis should be carried out after 16 subjects had completed the study in order to check the assumptions made in the power calculation. This thesis reports the results of this interim analysis.

2.3 STUDY DESIGN

Venous blood (30-50ml) was collected from both groups on day 0 (pre-immunisation sample) and day 18 (post-immunisation sample collected 14 days after the last dose of the

vaccine). D2-D3 duodenal pinch biopsies were collected on day 18 from 10 vaccinees and seven controls. In addition, sigmoid colonic biopsies were collected on day 18 from seven vaccinees and four controls (Figure 2.1).

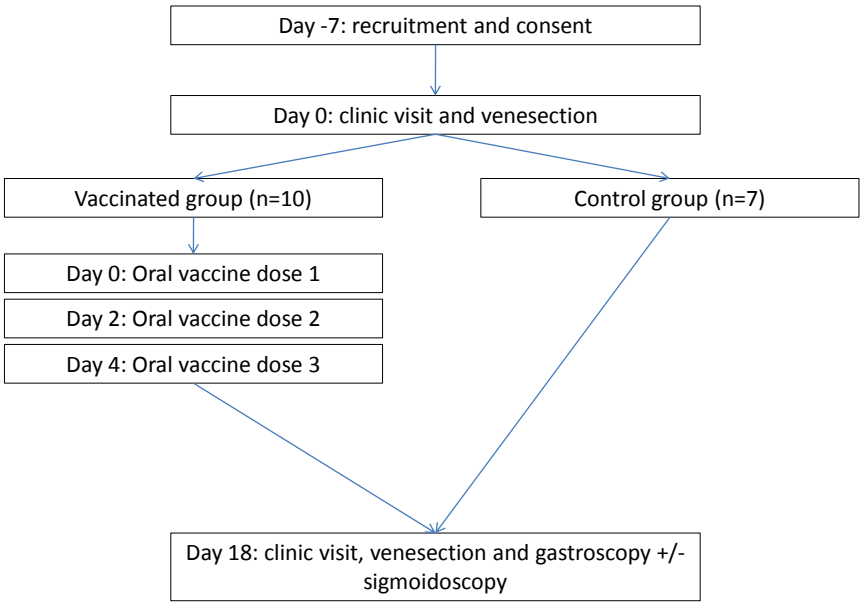

Figure 2.1 Timeline showing the collection of clinical samples from volunteers.

2.3.1 Vaccine and vaccination

Participants were allocated to the vaccinated group if they had previously received a typhoid vaccine outside of the recommended booster interval. Those who were thought to be totally naïve were randomly allocated to either group.

The live attenuated oral vaccine Vivotif® was used in this study. Vivotif® contains lyophilised *Salmonella enterica* serovar Typhi Ty21a, with not less than 2 x 10^9 viable cells per capsule (Appendix 1).

Study participants were given three 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. Participants were not observed taking doses two and three of the vaccine, but were reminded to take them via text message. Verbal confirmation was received on the day of the endoscopic procedure.

2.3.2 Venepuncture

30-50ml of blood was collected in a syringe containing sodium heparin.

2.3.3 Endoscopy

Individuals fasted from midnight before going to the hospital at 8am, where consent for endoscopic procedures was confirmed by the endoscopist. If the individual was having a flexible sigmoidoscopy in addition to a gastroscopy, a Fleet(TM) phosphate liquid enema (133mls) was given per rectum 20 minutes before the procedure. Sedation was optional; individuals who requested sedation were given up to 5mg maximum of midazolam intravenously. Nasal oxygen was administered during the procedures, and pulse oximetry monitoring was conducted throughout the procedures. 12-15 D2-D3 duodenal single-bite cold biopsies and 12-15 single-bite cold biopsies from the sigmoid colon at 20-25cm insertion were collected using Boston Scientific large capacity 'jumbo' forceps which passed through a standard 2.8mm endoscopic biopsy channel. Biopsies were placed in MR15 medium[1] containing antibiotics, and transported on ice prior to processing in the laboratory. Individuals were observed for two hours post-procedure, given a drink and snack, and then discharged home.

2.4 SAMPLE PROCESSING

2.4.1 Isolation of mononuclear cells

2.4.1.1 Isolation of peripheral blood mononuclear cells (PBMCs)

Heparinised venous blood (30-50ml) was diluted with an equal volume of PBS. The diluted blood was overlayed onto Lymphoprep™ at a ratio of 2:1 and centrifuged at 836xg for 25 minutes at 4˚C with the centrifuge brake disabled. PBMCs formed a floccular band between the plasma and the Lymphoprep™ and this band was transferred to a new tube with a Pasteur pipette. The PBMCs were washed in 50ml phosphate buffered saline (PBS) and spun at 470xg with the brake disabled for ten minutes at 4˚C. They were then washed in 30(50)ml PBS and spun at 209xg with the brake disabled for five minutes at 4˚C. Finally, the

[1] MR15 medium was made by adding 50ml foetal bovine serum (FBS), 10ml 200mM L-glutamine, 1ml Tazocin (piperacillin 250mg/ml, tazobactam 31.25mg/ml) and 2.5ml amphotericin B (250µg/ml) to a 500ml bottle of RPMI-1640

PBMCs were resuspended in 15(20)ml complete medium[2] and counted. After counting, the PBMCs were used for downstream applications on the same day.

2.4.1.2 Isolation of mucosal mononuclear cells (MMCs)

Biopsies were collected in 40ml MR15 medium on ice and transferred to the laboratory where they were centrifuged at 400xg for ten minutes at room temperature with the brake disabled. The biopsy pellet was then resuspended in 12.5ml CII-S medium[3] and incubated at a 45˚ angle in a shaking incubator for 30 minutes (37˚C, 220rpm). After this time, the tissue suspension was decanted into a syringe that was attached to a 16-gauge blunt-ended needle and rested on an open 50ml Falcon tube. The syringe barrel was then introduced and the suspension was forced through the syringe and into the Falcon tube. This procedure was done to disrupt the tissue and was repeated five times. A 70µm cell strainer was then placed on the trim of a Falcon tube and the disrupted cell suspension supernatant was passed through it. Tissue fragments and clumps left in the cell strainer were transferred into the original tube by rinsing the cell strainer with 12.5ml of CII-S medium, ensuring that all the CII-S medium also entered the original tube. The original tube was then placed back into the shaking incubator and the procedure of incubating the cells with collagenase and mechanically disrupting them was repeated twice more. Whilst the tube containing tissue fragments was shaking, the tube containing the free cells was washed with 30ml MR15 medium and centrifuged at 400xg for ten minutes. The pellet was resuspended in 15ml MR15 and the tube was then left on ice; this procedure was repeated with the free cells obtained from passages two and three. The free cells from all three passages were then pooled and the tube topped up to 50ml with MR15 before being spun for ten minutes at 400g. The pellet was then resuspended in 5ml MR15 and the cells were counted. After counting, the cell were seeded on a 24-well plate at a concentration of one million cells per ml and rested overnight by incubating the plate at 37˚C, 5% CO_2. The cells were then used for downstream applications.

This method was a modified version of that developed by the International AIDS Vaccine Initiative (Kaltsidis et al., 2011).

[2] Complete medium was made by adding 50ml FBS and 10ml 200mM L-glutamine to a 500ml bottle of RPMI-1640

[3] CII-S medium was made by adding 50mg collagenase II-S to 100ml MR15

2.4.2 Cell counting

PBMCs and MMCs were counted using an Improved Neubauer haemocytometer. 20µl of the cell suspension was added to 20µl of trypan blue and mixed well. 10µl of this mixture was then pipetted under the coverslip on one side of the haemocytometer and the number of viable lymphocytes or macrophages (cells excluding the trypan blue stain) were counted in the green squares shown on the diagram below (Figure 2.2).

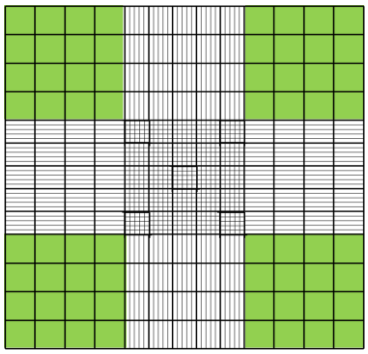

Figure 2.2 The grid of an improved Neubauer haemocytometer. The cells than were counted are shown in green.

The number of cells per ml was calculated as follows:

$$\frac{\text{Total number of counted cells}}{4} * 2(\text{dilution factor}) * 10^4(\text{multiplication factor})$$

2.5 FUNCTIONAL T CELL ASSAYS

Flow cytometry was used to assess antigen-specific cellular immune responses. Two functional T cell assays were used; an intracellular cytokine staining (ICS) assay and a CFSE lymphoproliferation assay.

2.5.1. Antigens used for stimulation

The antigens used for stimulation in the functional T cell assays were:

- Staphylococcal enterotoxin B (SEB) - SEB is an exotoxin excreted by the bacterium *Staphylococcus aureus* and it is commonly associated with food poisoning in humans (Bergdoll et al., 1974). SEB is a superantigen, meaning that it possesses extremely potent stimulatory activity on T lymphocytes (Liu et al., 2006), causing the release on massive amounts of cytokine. This therefore makes it ideal as a positive control for the intracellular cytokine staining assay.

- Phytohaemagglutinin (PHA) - PHA is a plant extract and a T cell mitogen (Nowell, 1960); its ability to trigger cell division in T lymphocytes makes it ideal as a positive control for the lymphoproliferation assay.
- Influvac® sub-unit - Influvac® is an inactivated influenza vaccine. The active substances in the vaccine are haemagglutinin and neuraminidase antigens of three different Influenza viruses.
- Vivotif® - The original vaccine strain, Ty21a, was used for stimulation. A stock of heat-killed Ty21a was made by dissolving two vaccine capsules in 4ml of PBS. The solution was divided into four Eppendorf tubes and these were placed in a heating block at 95˚C for 30 minutes. Pilot work showed that this was the minimum amount of time needed to kill all of the bacteria in the vaccine. After this time, some of the suspension from each Eppendorf was plated onto Columbia blood agar plates and incubated overnight at 37˚C. The plates were clear the following morning, indicating that the heat treatment had killed the bacteria. The solution in the four Eppendorf tubes was then pooled, shaken and distributed among a number of Eppendorf tubes which were stored as stock at -70˚C.

These four antigens were titrated using the CFSE lymphoproliferation assay to determine the optimal volume to use for cell stimulation.

2.5.2 Intracellular cytokine staining assay

Antigen-specific cytokine-producing T cells can be detected using an ICS assay. Briefly, this assay involves incubating T cells with antigen, adding the protein transport inhibitor brefeldin A to cause cytokine accumulation within cells, and permeabilising the cells to allow anti-cytokine fluorescent antibody conjugates to enter them and bind to any accumulated cytokine (Letsch and Scheibenbogen, 2003). Cytokine production is then analysed using a flow cytometer. Flow cytometry allows for the simultaneous measurement of multiple cytokines from the same cell meaning that the chance of detecting antigen-specific cells is increased (Bolton and Roederer, 2009).

2.5.2.1 Intracellular cytokine staining method

Cell stimulation: After the isolation of mononuclear cells from blood or mucosal samples, the cells were stimulated with antigen. For cell stimulation, cells were adjusted to the following concentrations:

- PBMCs: 5 million cells per ml
- MMCs: Between 1.5 million and 2.5 million cells per ml depending on the number of cells available

200µl of the cell suspension was then added to each of five wells and antigen added as appropriate. The wells were:

- Non-stimulated, non-stained
- Non-stimulated, stained (NS)
- SEB (added 1µl of 100µg/ml stock)
- Flu (added 0.5µl of 0.225µg/ml stock)
- Ty21a (added 1µl 0.0632µg/µl stock)

The cells were incubated at 37˚C for two hours, after which time 1µl of brefeldin A was added to each well. The cells were then incubated for a further 16 hours before they were harvested.

Harvesting cells: After 18 hours, the cells from each well in the plate were transferred to separate FACS (fluorescence activated cell sorting) tubes. 200µl of RPMI-1640 medium was added to each well on the plate and pipetted up and down to remove any remaining non-adherent cells. These cells were then transferred to the corresponding FACS tubes and the tubes were vortexed and spun (800g, 7 minutes, 4˚C). The supernatant was poured off and the cells resuspended in 1ml PBS containing 1µl ViViD (violet fluorescent reactive dye). This viability dye is taken up by dead cells and is used to exclude them from analysis (Mahnke and Roederer, 2007). The tubes were vortexed and then incubated for 20 minutes at 4˚C in the dark. After this time, 1ml cold PBS was added to each tube and the tubes were spun (800g, 7 minutes, 4˚C).

Surface staining: The supernatant was poured off and the cells resuspended in a cocktail of surface staining antibodies. A saturating amount of antibody was used:

- CD3-APC - 10µl
- CD4-APCH7 - 5µl
- CD8-PECy7 - 1µl
- CD14-Pacific Blue - 2µl
- CD19-Pacific Blue - 2µl
- PBS - 5µl

This cocktail was added to all tubes except the non-stimulated, non-stained tube. 30µl PBS was then added to the NS and SEB tubes. The tubes were vortexed and 30µl of solution transferred from each of these tubes into new FACS tubes which contained 70µl PBS. 2µl of beta7-PeCy5 was then added to the four original tubes. The two 'new' tubes did not contain any beta7 antibody. It was important to have these 'fluorescence minus one controls' in order to be able to accurately place gates on the beta7 negative and positive populations; the separation between these populations is poor.

The cells were then incubated for 15 minutes at 4˚C in the dark. After this time, the cells were washed with 1ml PBS and resuspended in 250µl Cytofix/Cytoperm to permeabilise the cells for intracellular staining. They were then incubated for 20 minutes at 4˚C in the dark before being washed with 1ml Perm/Wash.

Intracellular staining: The cells were then resuspended in a cocktail of intracellular cytokine staining antibodies. A saturating amount of antibody was used:

- IFNγ-AF700 - 1µl
- TNFα – AF488 – 2.5µl
- IL2-PE - 10µl
- PermWash – 1.5µl

Next, the cells were incubated for 30 minutes at 4˚C in the dark. After this time, the cells were washed with 1ml PermWash and resuspended in 350µl FACS flow before acquisition. Acquisition was carried out on a BD LSR II flow cytometer (BD Biosciences UK) and analysis was performed using FlowJo (Tree Star Inc., San Carlos, CA).

2.5.3 Lymphoproliferation assay

This assay is also used for the detection of antigen-specific T cells. Antigen-specific T cells must be able to proliferate rapidly in response to infection in order for the infection to be deal with effectively. Cell proliferation can be measured using CFSE. CFSE is a fluorescent dye which becomes incorporated in the cytoplasm of cells (Last'ovicka et al., 2009). When the cells divide, CFSE is partitioned equally between the daughter cells and therefore diluted, allowing for the identification of up to ten discrete generations (Lyons, 2000). This assay was only conducted using PBMCs; the number of MMCs collected was insufficient to run this assay in addition to the ICS assay.

2.5.3.1 Lymphoproliferation method

Cell stimulation: After the isolation of PBMCs from blood, they were adjusted to a concentration of 1 million cells per ml. The cells were then spun (800g, 7 minutes, 4˚C) and resuspended in 10ml PBS containing 1.25µM/ml of CellTrace™ CFSE. The cells were incubated in the dark at 37˚C for 8 minutes, after which time 10ml PBS was added. Next, the cells were spun (800g, 7 minutes, 4˚C) and resuspended in 5ml RPMI. A cell count was conducted to make sure that the concentration of lymphocytes was between 0.8-1.2 million cells per ml. 1ml of the cell suspension was then added to each of five wells and antigen added as appropriate. The five wells were:

- Non-stimulated, non-stained
- Non-stimulated, stained
- PHA (added 1µl of 0.5µg/ml stock)
- Flu (added 2.5µl of 0.225µg/ml stock)
- Ty21a (added 5µl of 0.0632µg/µl stock)

The cells were incubated at 37˚C for seven days, after which time they were harvested.

Harvesting cells: After seven days, the cells from each well in the plate were transferred to separate FACS tubes. 1ml of RPMI was added to each well on the plate and pipetted up and down to remove any remaining non-adherent cells. These cells were then transferred to the corresponding FACS tubes and the tubes were vortexed and spun (800g, 7 minutes, 4˚C). The supernatant was poured off and the cells resuspended in 1ml PBS. The tubes were vortexed and then spun (800g, 7 minutes, 4˚C).

Surface staining: The supernatant was poured off and the cells resuspended in a cocktail of surface staining antibodies. A saturating amount of antibody was used:

- CD3-APC - 10µl
- CD4-APCH7 - 5µl
- PBS - 5µl

The cells were then incubated for 15 minutes at 4˚C in the dark. After this time, the cells were washed with 0.5ml PBS and resuspended in 350µl FACS flow before acquisition. Acquisition was carried out on a BD LSR II flow cytometer and data analysis was performed using FlowJo.

2.6 REAGENTS

Reagents from a number of manufacturers were used in this study (Table 2.1).

Item	Product number	Manufacturer
Lymphoprep	1114545	Axis-Shield
Phosphate buffered saline	14190-169	Invitrogen
RPMI-1640	R0883	Sigma-Aldrich
L-glutamine	G7513	Sigma-Aldrich
Foetal bovine serum	10500-064	Invitrogen
Tazocin	-	Wyeth Pharmaceuticals
Amphotericin B	A2942	Sigma-Aldrich
Collagenase II-S	C1764	Sigma-Aldrich
Blunt-end needles	130-091-558	Miltenyi Biotec
Trypan Blue	T8154	Sigma-Aldrich
SEB	S4881-1MG	Sigma-Aldrich
Influvac sub-unit	-	Solvay Biologicals B.V.
Ty21a	-	Berna Biotech
BD GolgiPlug™	555029	BD Biosciences
ViViD viability dye	L34958	Invitrogen
Anti-human CD3 APC	555342	BD Biosciences
Anti-human CD4 APCH7	641398	BD Biosciences
Anti-human CD8PECy7	557746	BD Biosciences
Anti-human Beta7 PECy5	551059	BD Biosciences

Anti-human CD14 Pacific Blue	558121	BD Biosciences
Anti-human CD19 Pacific Blue	560353	BD Biosciences
BD Cytofix/Cytoperm™	554722	BD Biosciences
BD Perm/Wash	554723	BD Biosciences
Anti-human IFN-γ AF700	557995	BD Biosciences
Anti-human TNF-α AF488	557722	BD Biosciences
Anti-human IL2 PE	559334	BD Biosciences
BD FACSFlow™ Sheath Fluid	342003	BD Biosciences
Anti-mouse compensation particles set	552843	BD Biosciences
Anti-rat compensation particles set	552844	BD Biosciences
CFSE	C34554	Invitrogen
PHA	L2769	Sigma-Aldrich

Table 2.1 A list of the reagents used in this study along with product numbers and manufacturers.

2.7 STATISTICAL ANALYSIS

All graphs were made using GraphPad Prism 5 and show means and 95% confidence intervals. Statistical analyses were performed using Stata version 11 and SPSS version 18. Statistical comparisons were computed using maximum likelihood generalised linear modeling (regression) methods, assuming a Normal distribution. Because of the small sample size, bootstrapping was performed for each analysis, with 500 replicates per analysis, to provide more robust estimates of effect size (i.e. group/time differences). Comparisons were considered statistically significant if the bootstrapped confidence interval did not span zero. Correlations were analysed using the Pearson product moment correlation.

CHAPTER 3: ANTIGEN-SPECIFIC CD4+ T-CELL IMMUNE RESPONSES IN PERIPHERAL BLOOD

3.1 INTRODUCTION

Typhoid bacilli enter the body in contaminated food or water. They penetrate the gut mucosa and are ingested by macrophages, cells in which they are able to survive and multiply. After 8-14 days, systemic infection with bacteraemia and fever is established (Gordon, 2008). The nature of typhoid pathogenesis means that both humoral and cell-mediated immune responses are important. An antibody response is important in preventing mucosal invasion and protecting against bacteraemic organisms whereas a cell-mediated response is essential for the elimination of intracellular bacilli (Ivanoff et al., 1994). Antigen-specific cell-mediated responses include the production and secretion of cytokines and the proliferation of antigen-specific clones. Th1-type and pro-inflammatory cytokines such as IFNγ, IL-2, TNF-α and IL-6 are produced and secreted in response to immunisation with Ty21a (Pasetti et al., 2011). Cytokine production is a primary defence mechanism. Cytokines have a number of functions, including the activation of macrophages, Th1/Th2 differentiation, T-cell proliferation, acute phase protein production and the promotion of inflammation (Murphy et al., 2008). Lymphoproliferation, which is the ability of T cells to rapidly increase in number in response to infection, is a second important effector function of T cells. Both cytokine production and lymphoproliferation can be measured in laboratory assays.

3.2 RESEARCH QUESTIONS

The importance of the cell-mediated immune response against *S.* Typhi suggests that its magnitude should be markedly greater in vaccinated individuals compared to unvaccinated individuals. The testable questions were:

1) Is there a difference in the percentage of typhoid antigen-specific cytokine-producing CD4+ T cells in peripheral blood between unvaccinated and vaccinated individuals at day 0 (pre-vaccination) or day 18 (two weeks post-vaccination)?
2) Is there a difference in the percentage of typhoid antigen-specific proliferating CD4+ T cells in peripheral blood between unvaccinated and vaccinated individuals at day 0 (pre-vaccination) or day 18 (two weeks post-vaccination)?

3.3 MATERIALS AND METHODS

3.3.1 Study location and population

As described in sections 2.1 and 2.2.

3.3.2 Study design

Venous blood (30-50ml) was collected from both groups on day 0 (pre-immunisation sample) and day 18 (post-immunisation sample collected two weeks after the last dose of the vaccine). See section 2.3.1 for information about the vaccine and vaccination.

3.3.3 Sample processing

Peripheral blood was processed as described in section 2.4.1.1 and PBMCs were counted as described in section 2.4.2.

3.4.4 Flow cytometry phenotyping assays

3.4.4.1 Intracellular cytokine staining assay

As described in section 2.5.2.

The antigens used for stimulation are described in section 2.5.1.

Analysis of the data was performed on FlowJo (Treestar Inc., San Carlos, CA) using the gating strategy shown in Figure 3.1.

3.4.4.2 Lymphoproliferation assay

As described in section 2.5.3.

The antigens used for stimulation are described in section 2.5.1.

Analysis of the data was performed on FlowJo (Treestar Inc., San Carlos, CA) using the gating strategy shown in Figure 3.2.

3.4.5 Statistical analysis

Statistical analyses were performed using Stata version 11 and SPSS version 18. There were insufficient observations to make a definitive conclusion about the statistical distribution of the observations. Data were therefore analysed using a generalised linear (ANOVA) model assuming a Normal distribution with bootstrapping in order to obtain more reliable estimates of group differences. All graphs were produced using GraphPad Prism 5.

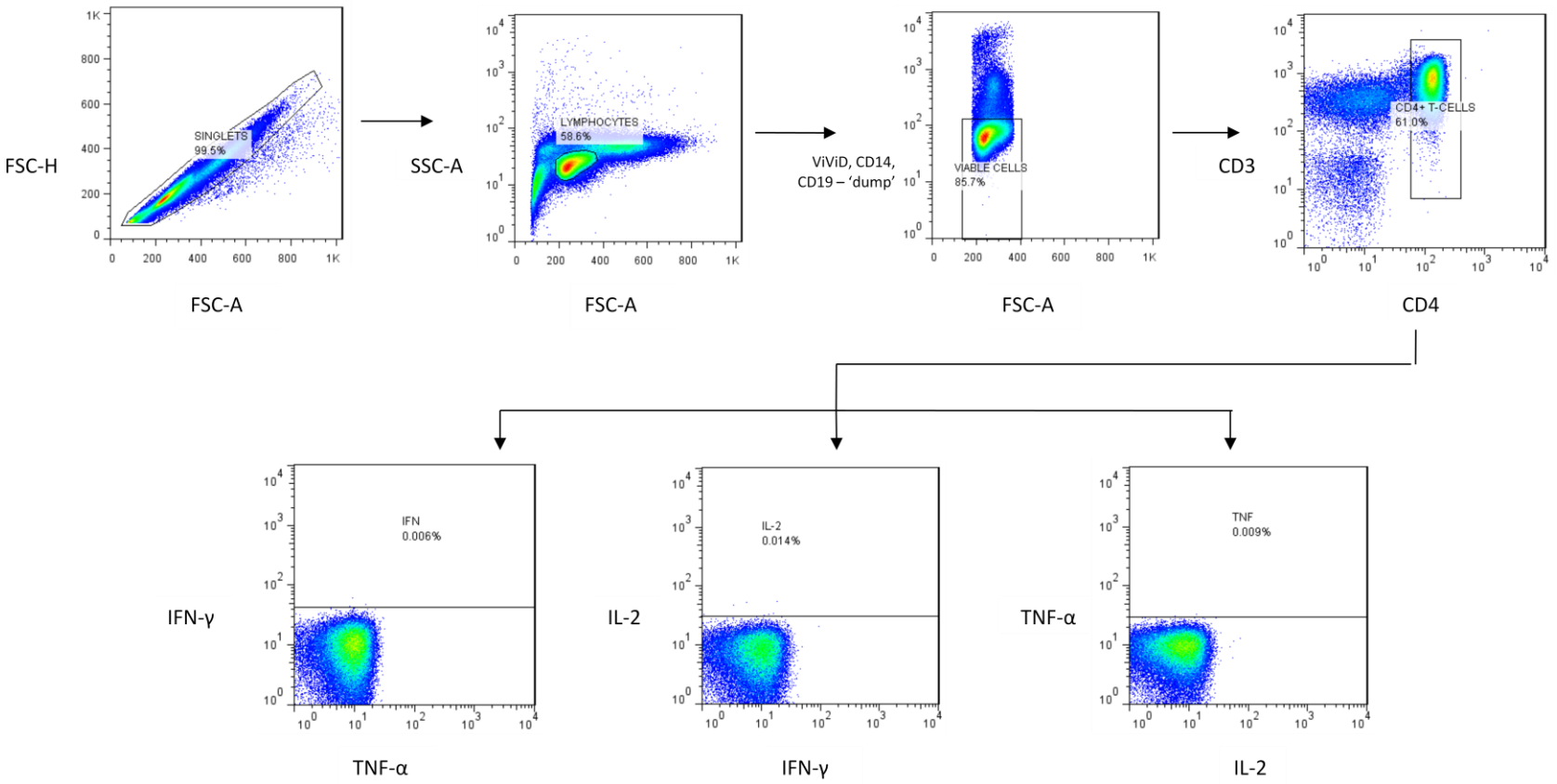

Figure 3.1 Intracellular cytokine staining flow cytometry gating strategy for peripheral blood. PBMCs were stained with monoclonal antibodies directed against CD3, CD4, CD8, IFN-γ, TNF-α, IL-2 and a combination (on the same colour) of CD14, CD19 and ViViD, a viability marker. Single cells were identified using forward scatter properties and lymphocytes were then identified using forward scatter and side scatter. The 'dump' channel was used to remove monocytes, B cells and dead lymphocytes. CD4+ T cells were then positively identified by CD3 and CD4. Within the CD4+ T cell population, cells producing IFN-γ, TNF-α or IL-2 were identified. Combination gating was used to identify single-, double- and triple-producing cells.

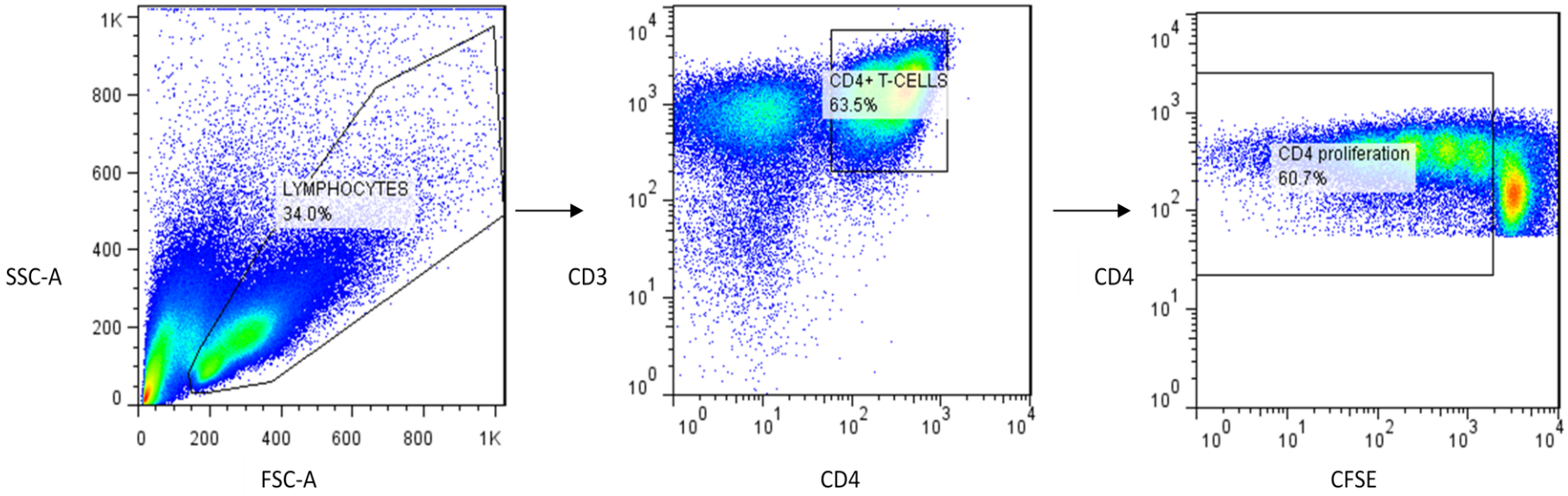

Figure 3.2 Lymphoproliferation assay flow cytometry gating strategy for peripheral blood. PBMCs were stained with monoclonal antibodies directed against CD3 and CD4. Lymphocytes were identified using forward scatter and side scatter properties. CD4+ T cells were then positively identified by CD3 and CD4. CD4 was then plotted against CFSE and proliferating CD4+ T cells were identified.

3.4 RESULTS

3.4.1 Characteristics of the study population

Study number	Female (F) or male (M)	Age	Vaccine (V) or control (C)	Assay
1	F	44	V*	ICS, CFSE
2	M	44	V*	-
3	F	22	C	ICS,CFSE
4	F	27	C	ICS, CFSE
5	M	38	V	ICS, CFSE
6	M	26	V	CFSE
7	M	58	V	ICS, CFSE
8	F	21	V	ICS, CFSE
9	M	24	V*	ICS
10	F	22	C	ICS
11	M	25	V*	ICS, CFSE
12	M	23	C	ICS, CFSE
13	F	27	V	ICS
14	F	21	V*	ICS
15	F	26	C	ICS, CFSE
16	F	26	C	ICS, CFSE
17	M	22	C	-

Table 3.1 Demographic characteristics of the study population for peripheral blood assays. * represents individuals who had received a parenteral typhoid vaccination, either whole cell or Vi polysaccharide, more than three years previously. Individuals who had received a typhoid vaccine within the last three years were excluded from the study.

The ICS assay was performed on samples from eight vaccinated individuals (median age 26, age range 21-58, four females) and six control individuals (median age 24.5, age range 22-27, five females). The CFSE assay was performed on samples from six vaccinated individuals (median age 32, age range 21-58, two females) and five control individuals (median age 26, age range 22-27, four females).

3.4.2 The proportion of CD4+ T cells is similar between control and vaccinated individuals at day 0 and day 18

The percentage of viable lymphocytes that were CD4+ was calculated for both groups at both time points for the PBMCs used in the ICS assay (Figure 3.3) and the proliferation assay (Figure 3.4). This was done in order to be confident that it was reasonable to then compare both the proportion of antigen-specific cytokine-producing CD4+ T cells and the proportion of antigen-specific proliferating CD4+ T cells between the two groups at the two time points. The percentage of viable lymphocytes that were CD4+ was similar between all groups in both cases.

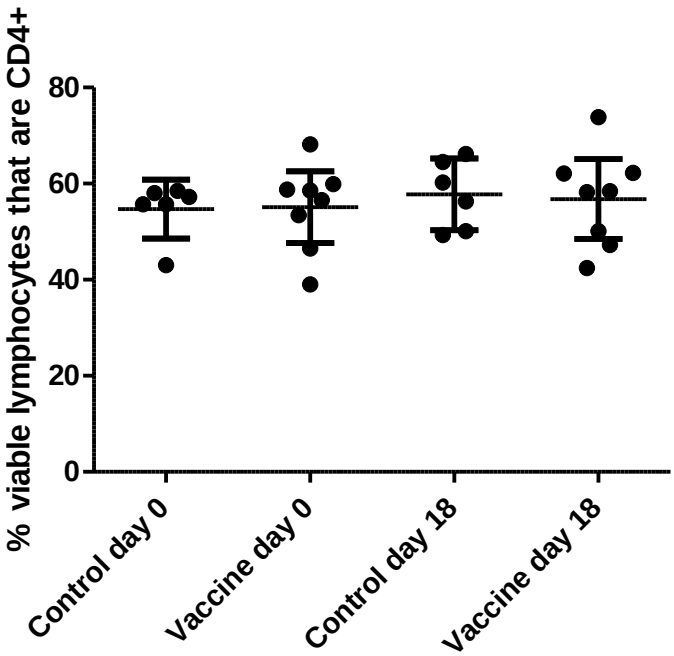

Figure 3.3 The percentage of viable lymphocytes in peripheral blood that express CD4 in the ICS assay. Lines represent the mean and 95% confidence interval.

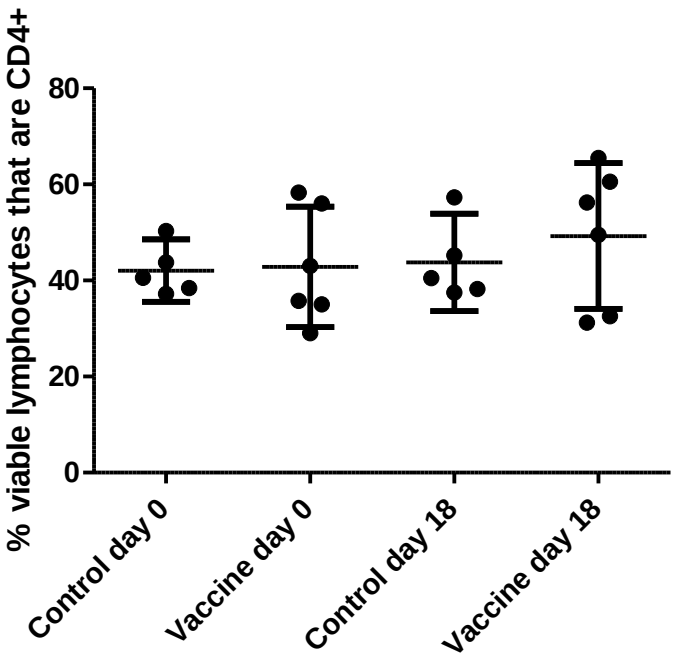

Figure 3.4 The percentage of viable lymphocytes in peripheral blood that express CD4 in the CFSE lymphoproliferation assay. Lines represent the mean and 95% confidence interval.

3.4.3 When cells are unstimulated, there is no statistically significant difference in the proportion of cytokine-producing CD4+ T cells between the control and vaccinated groups

Cytokine production was measured in CD4+ T cells that had not been stimulated by antigen (Figure 3.5). There was no statistically significant difference in the mean percentage of cytokine-producing CD4+ T cells between the control and vaccinated groups at day 0 (0.103% vs. 0.091%, bootstrapped 95% CI of -0.022 to +0.046) or day 18 (0.117% vs. 0.116%, bootstrapped 95% CI of -0.102 to +0.097). Furthermore, there was no statistically significant difference between day 0 and day 18 for the control group (0.103% vs. 0.117%, bootstrapped 95% CI of -0.058 to +0.086) or the vaccinated group (0.091% vs. 0.116%, bootstrapped 95% CI of -0.017 to +0.067).

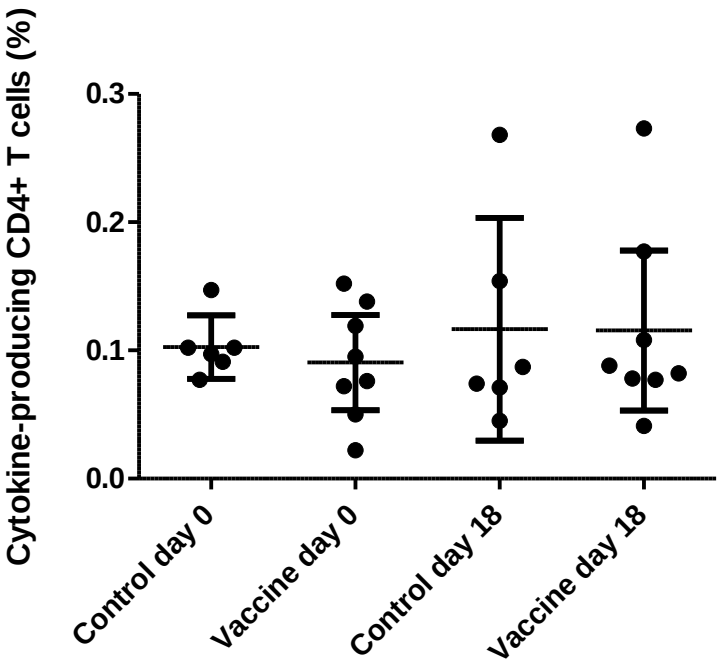

Figure 3.5 CD4+ T cell responses in unstimulated cells from peripheral blood. The percentage of unstimulated CD4+ T cells producing cytokine in the control and vaccinated groups at day 0 and day 18. Cytokine-producing cells are defined as those producing any combination of IFN-γ, TNF-α and IL-2. Lines represent the mean and the 95% confidence interval.

3.4.4 In vaccinated individuals, there is no statistically significant difference in the proportion of typhoid antigen-specific cytokine-producing CD4+ T cells between day 0 and day 18

To determine if there was evidence of a response to the oral typhoid vaccine Ty21a in peripheral blood, the magnitude of the CD4+ T cell response to Ty21a was measured by an ICS assay. SEB and flu antigen were used as positive controls. The magnitude of the response measured in unstimulated cells was subtracted from that measured in stimulated cells in order to make the response comparable between individuals. A positive response was measured when cells were stimulated with SEB, flu and Ty21a; the response was above that measured in unstimulated cells. Figure 3.6 shows representative flow cytometry dot plots from a vaccinated individual.

SEB

There was no statistically significant difference in the mean percentage of cytokine-producing CD4+ T cells between the control and vaccinated groups at day 0 (7.333% vs.

6.311%, bootstrapped 95% CI of -1.281 to +3.326) or day 18 (4.697% vs. 10.729%, bootstrapped 95% CI of -20.764 to +3.605) when cells were stimulated with SEB. There was a statistically significant difference between day 0 and day 18 for the control group (7.333% vs. 4.697%, bootstrapped 95% CI of -3.879 to -1.394) but not for the vaccinated group (6.311% vs. 10.729%, bootstrapped 95% CI of -3.788 to +12.623) (Figure 3.7A).

Flu

Similarly, there was no statistically significant difference in the mean percentage of cytokine-producing CD4+ T cells between the control and vaccinated groups at day 0 (0.142% vs. 0.132%, bootstrapped 95% CI of -0.078 to +0.098) or day 18 (0.400% vs. 0.150%, bootstrapped 95% CI of -0.269 to +0.822) when cells were stimulated with flu. Furthermore, there was no statistically significant difference between day 0 and day 18 for the control group (0.142% vs. 0.400%, bootstrapped 95% CI of -0.254 to +0.769) or the vaccinated group (0.132% vs. 0.150%, bootstrapped 95% CI of -0.022 to +0.057) (Figure 3.7B).

Ty21a

When cells were stimulated with Ty21a, there was no statistically significant difference in the mean percentage of cytokine-producing CD4+ T cells between the control and vaccinated groups at day 0 (0.084% vs. 0.091%, bootstrapped 95% CI of -0.081 to +0.066) but there was a statistically significant difference between the two groups at day 18 (0.030% vs. 0.124%, bootstrapped 95% CI of -0.149 to -0.028). Unexpectedly, there was a statistically significant fall in the mean percentage of cytokine-producing CD4+ T cells in the control group between day 0 and day 18 (0.084% vs. 0.030%, bootstrapped 95% CI of -0.092 to -0.016), and this contributed markedly to the overall difference between controls and vaccinated groups at day 18. Although there was a rise in the mean percentage of cytokine-producing CD4+ T cells in the vaccinated group between day 0 and day 18, this rise was not statistically significant even after bootstrapping (0.091% vs. 0.124%, bootstrapped 95% CI of -0.005 to +0.070)(Figure 3.7C).

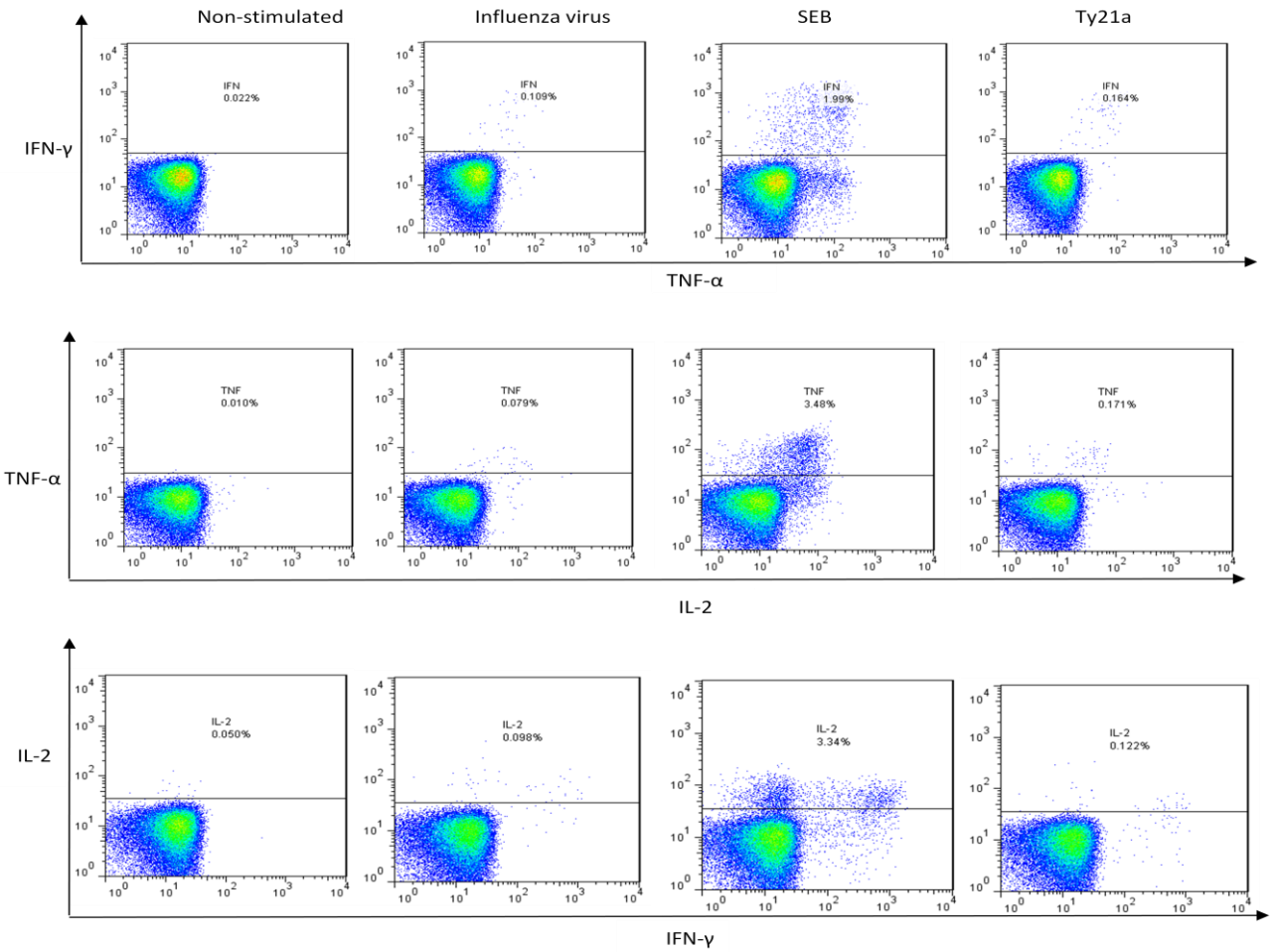

Figure 3.6 Representative flow cytometry dot plots from a vaccinated individual for the ICS assay. PBMCs were stimulated with antigen and antigen-specific CD4+ T cell responses were measured by intracellular cytokine staining. The production of the cytokines IFN-γ, TNF-α and IL-2 was determined. The antigens used for stimulation were influenza antigen, SEB and Ty21a, as described in section 2.5.1.

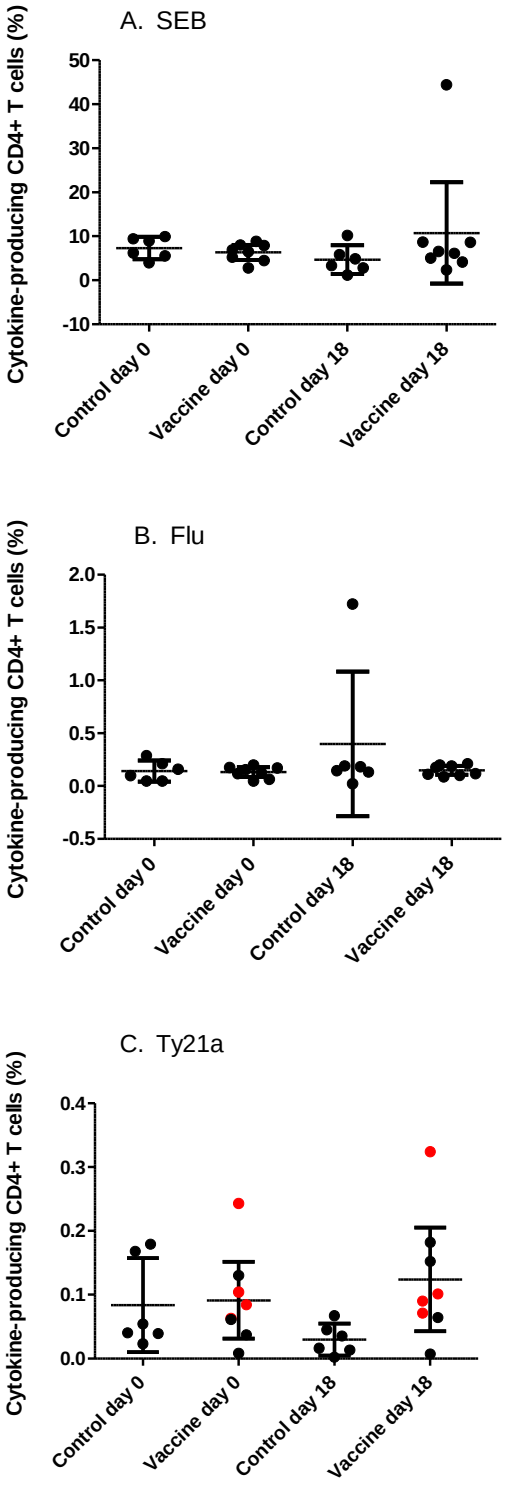

Figure 3.7 Antigen-specific CD4+ T cell responses in peripheral blood as measured by an ICS assay. A comparison of the percentage of CD4+ T cells producing cytokine in the control and vaccinated groups at day 0 (pre-vaccination) and day 18 (two weeks post-vaccination) in response to stimulation with A) SEB, B) Flu and C) Ty21a. Cytokine-producing CD4+ T cells are defined as those producing any combination of IFN-γ, TNF-α and IL-2. Red dots represent previously vaccinated individuals. Lines represent the mean and the 95% confidence interval.

3.4.5 When cells are unstimulated, there is no statistically significant difference in the proportion of proliferating CD4+ T cells between the control and vaccinated groups

Lymphoproliferation was measured in CD4+ T cells that had not been stimulated by antigen (Figure 3.8). There was no statistically significant difference in the mean percentage of cytokine-producing CD4+ T cells between the control and vaccinated groups at day 0 (0.904% vs. 0.916%, bootstrapped 95% CI of -1.375 to +1.351) or day 18 (2.221% vs. 4.209%, bootstrapped 95% CI of -8.977 to +5.027). There was a statistically significant difference between day 0 and day 18 for the control group (0.904% vs. 2.221%, bootstrapped 95% CI of +0.039 to +2.595) but not for the vaccinated group (0.916% vs. 4.209%, bootstrapped 95% CI of -1.924 to +8.510).

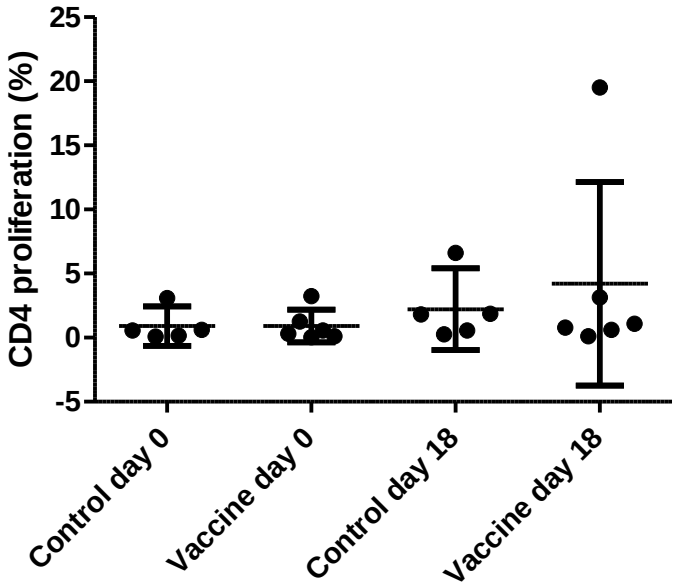

Figure 3.8 CD4+ T cell responses in unstimulated cells from peripheral blood. The percentage of unstimulated CD4+ T cells that proliferated in the control and vaccinated groups at day 0 and day 18. Lines represent the mean and the 95% confidence interval.

3.4.6 In vaccinated individuals, there is no statistically significant difference in the proportion of proliferating typhoid antigen-specific CD4+ T cells between day 0 and day 18

Evidence of a response to Ty21a in peripheral blood was also assessed by investigating the proliferative capacity of CD4+ T cells after stimulation with Ty21a. PHA and flu antigen were used as positive controls. The magnitude of the response measured in unstimulated

cells was subtracted from that measured in stimulated cells in order to make the response comparable between individuals. A positive response was measured when cells were stimulated with PHA, flu and Ty21a; the response was above that measured in unstimulated cells. Figure 3.9 shows representative dot plots from a vaccinated individual.

PHA

There was no statistically significant difference in the mean percentage of proliferating CD4+ T cells between the control and vaccinated groups at day 0 (54.316% vs. 48.717%, bootstrapped 95% CI of -14.767 to +25.964) or day 18 (38.879% vs. 38.891%, bootstrapped 95% CI of -21.362 to +23.859) when cells were stimulated with PHA. There was a statistically significant difference between day 0 and day 18 for the control group (54.316% vs. 38.879%, bootstrapped 95% CI of -27.111 to -3.763) but not for the vaccinated group (48.717% vs. 38.391%, bootstrapped 95% CI of -33.903 to +13.250)(Figure 3.10A).

Flu

Similarly, there was no statistically significant difference in the mean percentage of proliferating CD4+ T cells between the control and vaccinated groups at day 0 (29.370% vs. 14.211%, bootstrapped 95% CI of -6.872 to +37.189) or day 18 (23.779% vs. 21.474%, bootstrapped 95% CI of -24.711 to +39.161) when cells were stimulated with flu. Furthermore, there was no statistically significant difference between day 0 and day 18 for the control group (29.370% vs. 23.779%, bootstrapped 95% CI of -33.665 to +22.483) or the vaccinated group (14.211% vs. 21.474%, bootstrapped 95% CI of -12.931 to +27.457)(Figure 3.10B).

Ty21a

When cells were stimulated with Ty21a, there was no statistically significant difference in the mean percentage of proliferating CD4+ T cells between the control and vaccinated groups at day 0 (3.972% vs. 2.092%, bootstrapped 95% CI of -1.300 to +5.060) or day 18 (3.527% vs. 3.971%, bootstrapped 95% CI of -6.522 to +1.890). Furthermore, there was no statistically significant difference between day 0 and day 18 for the control group (3.972% vs. 3.527%, bootstrapped 95% CI of -1.815 to +0.925) or the vaccinated group (2.092% vs. 3.971%, bootstrapped 95% CI of -0.403 to +4.161)(Figure 3.10C).

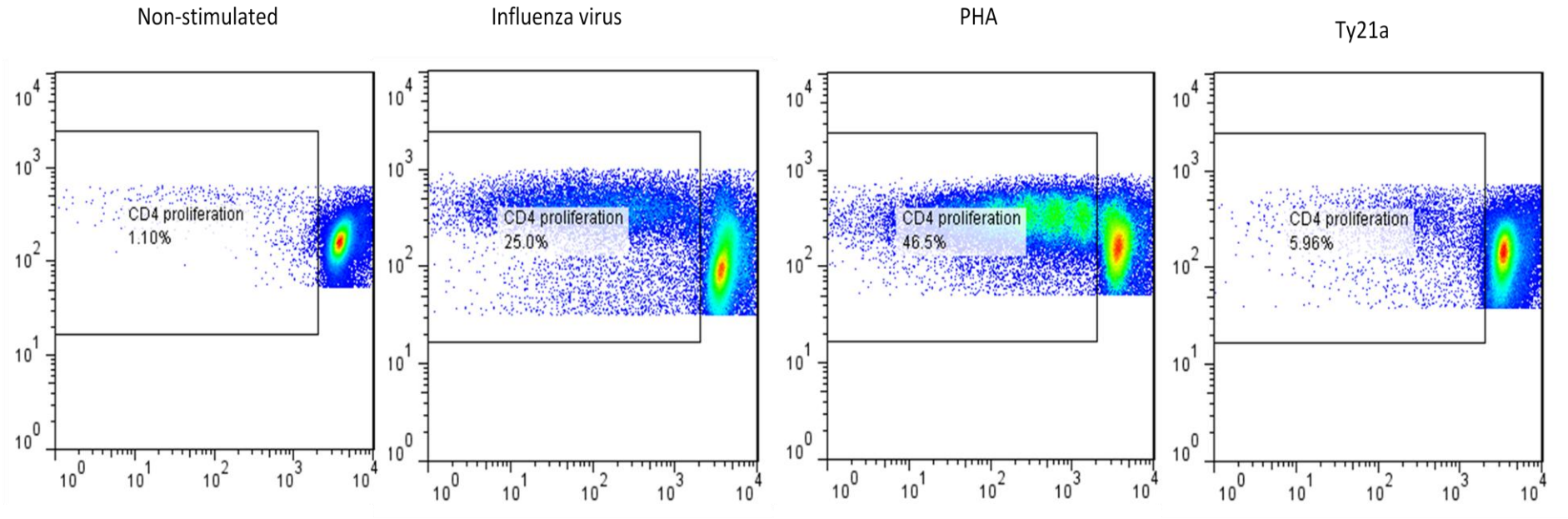

Figure 3.9 Representative flow cytometry dot plots from a vaccinated individual for the lymphoproliferation assay. PBMCs were stimulated with antigen and antigen-specific CD4+ T cell responses were measured using a lymphoproliferation assay. The antigens used for stimulation were influenza antigen, PHA and Ty21a, as described in section 2.5.1.

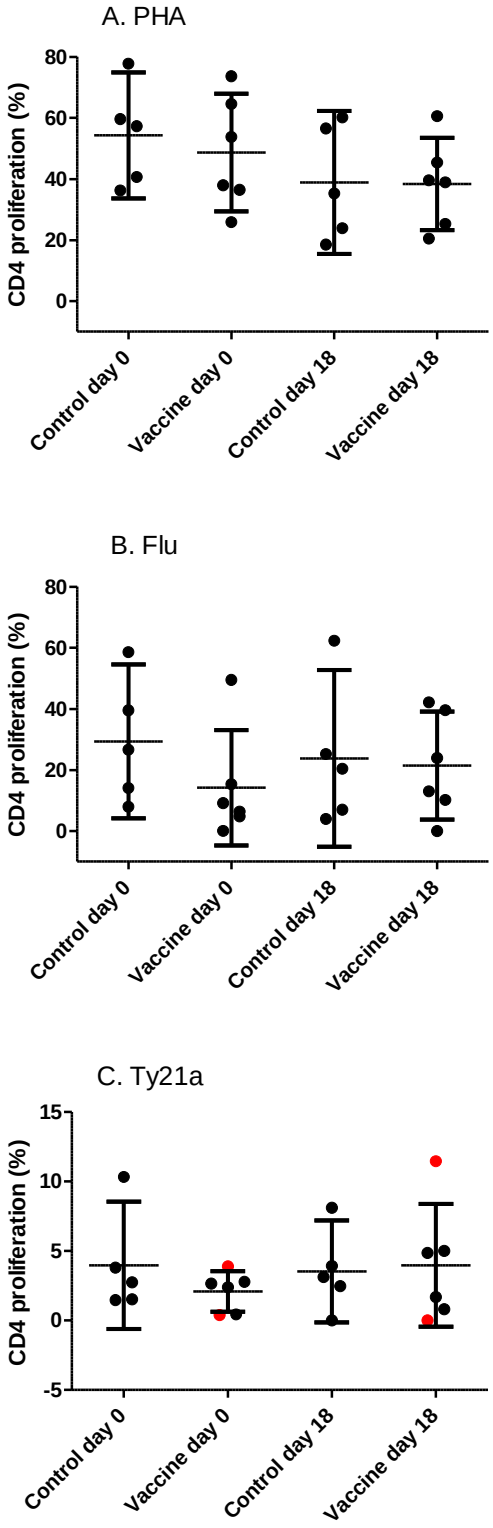

Figure 3.10 Antigen-specific proliferating CD4+ T cell responses in peripheral blood as measured by a lymphoproliferation assay. A comparison of the percentage of proliferating CD4+ T cells in the control and vaccinated groups at day 0 (pre-vaccination) and day 18 (two weeks post-vaccination) in response to stimulation with A) PHA, B) flu and C) Ty21a. Red dots represent previously vaccinated individuals. Lines represent the mean and the 95% confidence interval.

3.4.7 Comparison of the cytokine-producing T cell profile in unvaccinated and vaccinated individuals at day 0 and day 18

The cytokine-producing profile of unvaccinated (Figure 3.11A) and vaccinated (Figure 3.11B) individuals at day 0 and day 18 was described by calculating the proportions of single-, double- and triple-producing CD4+ T cells (IFN-γ, TNF-α, IL-2, IFN-γ + TNF-α, IFN-γ + IL-2, IL-2 + TNF-α, IFN-γ + TNF-α + IL-2) in the total typhoid antigen-specific cytokine-secreting CD4+ T cell population. In both unvaccinated and vaccinated individuals single-, double- and triple-producing effector T cells are all present. All three of the cytokines measured contribute to the antigen-specific CD4+ T cell cytokine response.

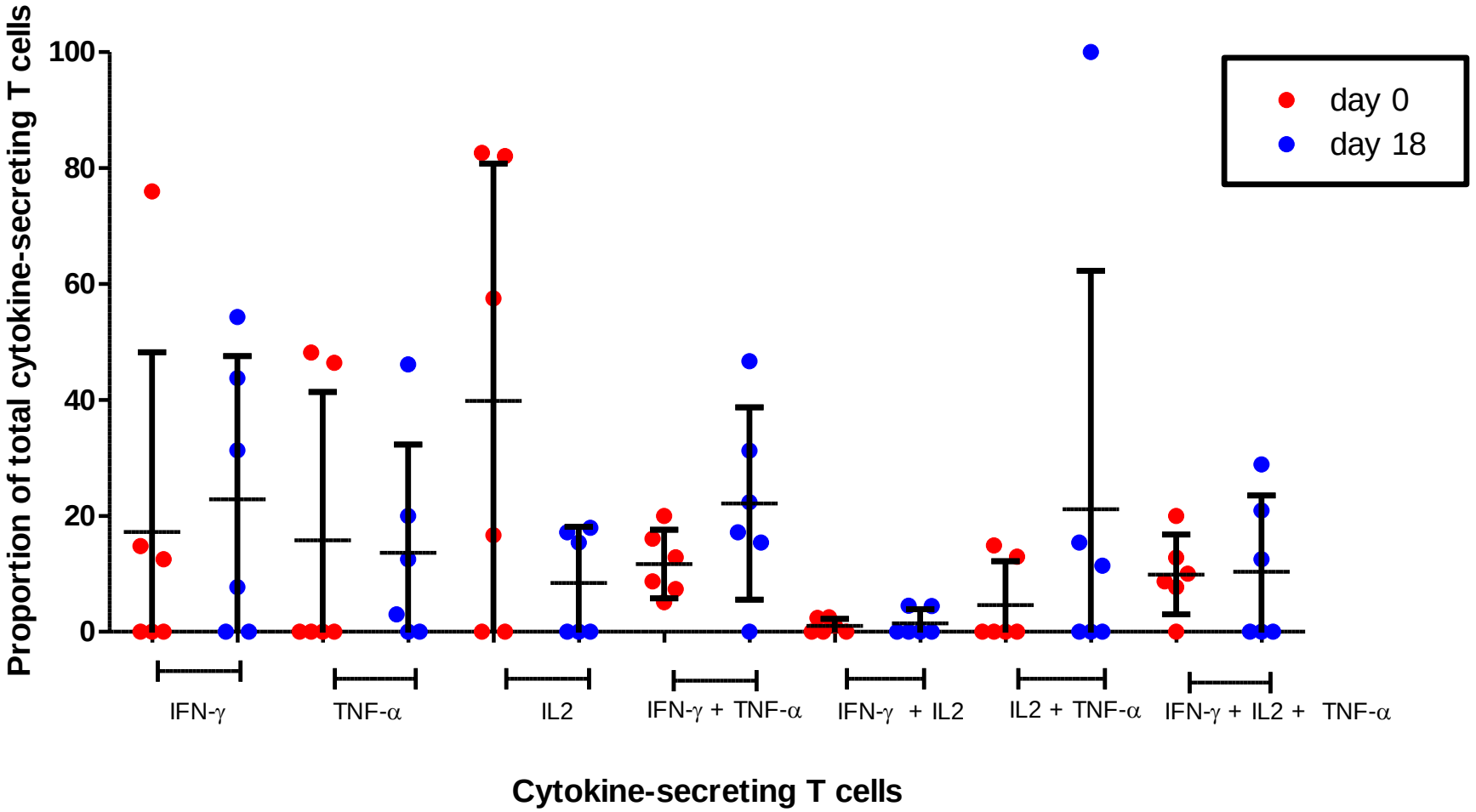

Figure 3.11A The proportions of single-, double- and triple- cytokine-producing typhoid antigen-specific CD4+ T cells in peripheral blood at day 0 and day 18 in the control group. Lines represent the mean and the 95% confidence interval.

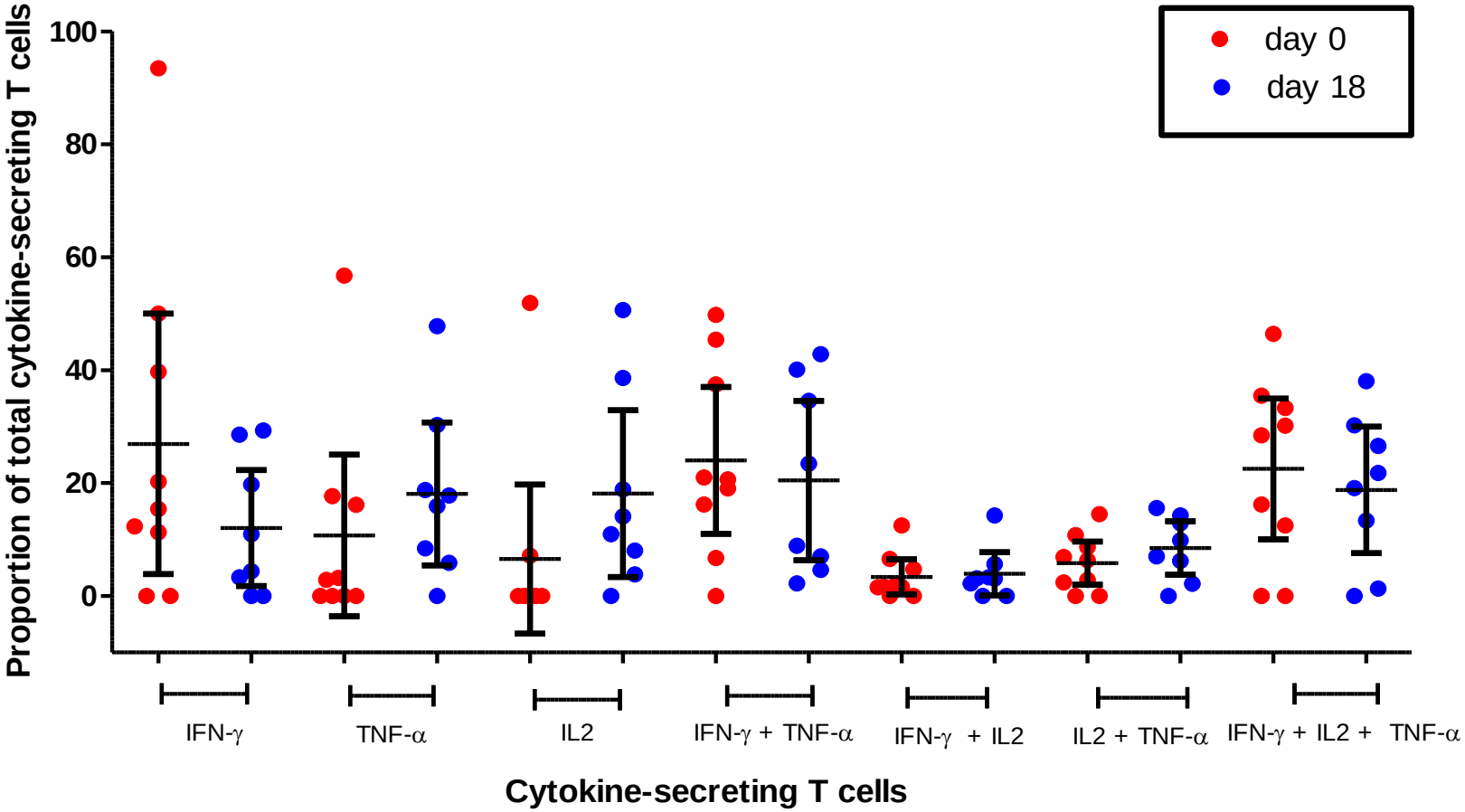

Figure 3.11B The proportions of single-, double- and triple- cytokine-producing typhoid antigen-specific CD4+ T cells in peripheral blood at day 0 and day 18 in the vaccinated group. Lines represent the mean and the 95% confidence interval.

3.5 DISCUSSION

This study set out to determine whether there was evidence of a response to the live oral typhoid vaccine Ty21a in peripheral blood using two assays, an intracellular cytokine staining assay and a lymphoproliferation assay. The ICS assay showed that when cells were stimulated with Ty21a, a statistically significant decrease in the mean percentage of typhoid antigen-specific cytokine-producing CD4+ T cells was observed in the control group between day 0 and day 18. There was an increase in the mean percentage of typhoid antigen-specific cytokine-producing CD4+ T cells in the vaccinated group between the two time points but this increase was not statistically significant. In the lymphoproliferation assay, an increase in the mean percentage of typhoid antigen-specific proliferating CD4+ T cells in the vaccinated group between day 0 and day 18 was observed, but this increase was not statistically significant.

In the ICS assay, the proportion of viable CD4+ T cells in the control and vaccinated groups at day 0 and day 18 were similar. This was also the case in the lymphoproliferation assay. In general, the proportion of viable CD4+ T cells was lower in the lymphoproliferation assay than the ICS assay. The most likely explanation for this is that cells for use in the ICS assay are incubated overnight whereas those for use in the lymphoproliferation assay are incubated for a week. After a week, the media in which the cells have been resting is likely to be nutrient scarce and unable to support all of the cells, resulting in cell death.

When cytokine production was measured in unstimulated cells in the ICS assay, there was no statistically significant difference between the two groups at either time point or between the two time points for either group. This was to be expected. When cytokine production was measured in unstimulated cells in the lymphoproliferation assay, there was also no difference between the two groups at either time point or between the two time points in the vaccinated group. There was a significant difference between the two time points in the control group. With a larger sample size, it is likely that this difference would become non-significant.

The production of cytokine-producing and proliferating CD4+ T cells in response to the positive controls is evidence that the cells that were isolated from peripheral blood were capable of responding to antigen stimulation. For both assays, the mean percentage of antigen-specific cells in response to stimulation with the positive controls was expected to be similar between the two groups and the two time points; this was not the case. For the

ICS assay, there was a statistically significant decrease in the mean percentage of antigen-specific cells in the control group between day 0 and day 18 when cells were stimulated with SEB and for the lymphoproliferation assay, there was a statistically significant decrease in the control group between day 0 and day 18 when cells were stimulated with PHA. These apparently anomalous results are most likely due to the relatively small sample size used.

The statistically significant decrease in the mean percentage of typhoid antigen-specific cytokine-producing CD4+ T cells between day 0 and day 18 in the control group in the ICS assay was an unexpected result for which there is no obvious biological explanation. Again, the most likely explanation for this result is that the sample size was very small (six individuals). Two individuals had high values at day 0 – natural variation in the population is wide and there may also be random day-to-day variation in laboratory conditions. It is possible that these two individuals had been exposed to *Salmonella* in the environment or that there was cross-reactivity with antigens from other bacteria (Lundgren et al., 2009), but this would not explain why the values had returned to 'normal' at day 18.

The increase in typhoid antigen-specific cytokine-producing CD4+ T cells and proliferating CD4+ T cells after vaccination with Ty21a was not statistically significant but it was expected. It is consistent with work carried out by others, in which vaccination with Ty21a induced systemic CD4+ T-helper type 1 responses and led to an increase in both the proliferation of and the IFN-γ production by blood T cells stimulated with the vaccine strain (Kilhamn et al., 2003; Lundin et al., 2002; Viret et al., 1999). Studies in which individuals were orally vaccinated with other live attenuated *S.* Typhi strains, such as CVD 908-htrA and CVD 909 have also produced similar results (Tacket et al., 2000b; Wahid et al., 2007). Methodological differences between these studies and this current study may explain why statistical significance was not obtained in this study. For example, cytokine release was measured with enzyme-linked immunosorbent assays as opposed to flow cytometry and proliferation was measured using ^{3}[H]-thymidine incorporation as opposed to flow cytometry (Kilhamn et al., 2003; Lundin et al., 2002; Viret et al., 1999).

There were also differences in the way in which Ty21a was prepared for cell stimulation. In some studies it was formalin-inactivated (Viret et al., 1999) whilst in others it was heat-killed in a water bath after subculture on horse blood agar plates (Kilhamn et al., 2003). In this study, the vaccine was dissolved in PBS and then heat-killed at 95°C for thirty minutes. Other ways of preparing Ty21a include killing the bacteria using phenol and making a whole

cell solubilised preparation. There is no consensus in the literature as to which is the best method of antigen preparation. The concentration of *S.* Typhi used in this study may also have affected the results obtained. However, the heat-killed Ty21a was titrated before use and higher concentrations than the one used in the assays were found to kill the majority of lymphocytes in lymphoproliferation assays. Instead of using killed whole cell *S.* Typhi for stimulation, purified antigens such as flagella H-antigen or the Vi polysaccharide capsular antigen could be used. However, the best protective correlate of any individual *S.* Typhi protein is unknown. It would be inappropriate to use Vi antigen in this study because a polysaccharide antigen would not elicit antigen-specific cellular immune responses, and in any case, Ty21a does not possess the Vi antigen.

Studies which were conducted using new generation vaccines such as CVD 909 are likely to have produced significant results because such vaccines are more immunogenic than Ty21a. Additionally, these studies used autologous PBMCs, which had been stimulated with *S.* Typhi and grown to be APCs, to act as stimulator cells in the preparation of effector cells for use in an IFN-γ enzyme-linked immunosorbent spot assay (Wahid et al., 2007). This method was also used by a study in which volunteers were immunised with Ty21a (Salerno-Goncalves et al., 2002) and is likely to increase the magnitude of the resulting immune response.

Other reasons why statistical significance may not have been achieved include the possibility that not all of the vaccinated individuals responded to the vaccine. This is to be expected because the efficacy of the vaccine is not 100% (Black et al., 1990; Engels et al., 1998). In addition, due to a small sample size and natural variation in the population, the confidence intervals around the data are wide. A larger sample size may resolve this issue. Including previously vaccinated individuals in the vaccinated group could have inflated the mean percentage of typhoid antigen-specific cytokine-producing CD4+ T cells at baseline because such individuals had some level of immunity to *S.* Typhi already. When these individuals were re-vaccinated, the magnitude of the resultant immune response to *S.* Typhi compared to baseline would not have been as great as it would have been in naïve individuals. Consequently, including these individuals in the vaccinated group may have decreased the mean percentage of typhoid antigen-specific cytokine-producing CD4+ T cells at day 18 compared to day 0. This may help to explain why a significant result was not achieved in the ICS assay, but it is unlikely since most of the previously vaccinated individuals had the Vi polysaccharide vaccine (rather than the older whole-cell killed

typhoid vaccine), and Ty21a does not possess the Vi antigen. The similarity at baseline between controls and vaccines also does not support this theory. Previously vaccinated individuals did not appear to behave differently to naïve individuals in the lymphoproliferation assay.

The cytokine-producing profiles of vaccinated and unvaccinated individuals show that single-, double- and triple- cytokine-producing T cells are present. All three cytokines that were measured, IFN-γ, TNF-α and IL-2, contributed to the antigen-specific immune response. Measuring three cytokines as opposed to one or two is preferable because it decreases the fraction of the antigen-specific population that is not picked up by the assay (Seder et al., 2008). Whereas effector CD4+ T cells predominantly produce IFN-γ, Tcm produces IFN-γ, TNF-α and IL-2 and Tem produces mainly IFN-γ and TNF-α (Seder et al., 2008; Woodland and Kohlmeier, 2009). However, surface phenotyping to distinguish between these T cell types was not carried out in this assay so it is not known what proportion of each T cell type was present in the blood.

The main limitation of this study was the small number of individuals from whom samples were collected. Under the terms of the ethics approval for this study, there was a requirement for a pause in recruitment in order to conduct an interim analysis after the data from a small number of individuals had been analysed. This study reports the results of this interim analysis. More individuals need to be recruited to increase the sample size and this is currently being conducted after a minor amendment to the ethics form was approved.

Another limitation concerns the batch of Ty21a that was used for vaccination and cell stimulation. After all of the laboratory work was completed, a letter was received from the vaccine company, Crucell, stating that they were withdrawing the batch of the vaccine that had been used in this study because their stability monitoring had revealed a lower than expected potency value for that particular batch. It is possible that this decrease in potency affected the results that were obtained. However, the letter did state that the vaccine was still safe and potent – there was just a possibility that it would not remain potent over the full period of its shelf life. As the vaccine expiry date was 04/11 and the lab work was conducted between 05/10 and 08/10, this may not have been an issue. In addition, the relationship between potency as measured by Crucell and cytokine-production as measured here by ICS is unknown. Future work should compare the reduced

potency batch of the vaccine with another batch in a number of individuals to see if the magnitude of the cell-mediated immune response is altered.

A third limitation of this work was that vaccinated individuals were not observed taking each dose of their vaccine under the study protocol. However, a text was sent to all individuals on the relevant days to remind them to take each dose of the vaccine. Many texted back to confirm that they had taken the vaccine and all gave verbal confirmation at their next visit to the hospital.

3.6 CONCLUSION

In conclusion, this study shows a trend towards increased antigen-specific CD4+ T cell immune responses in individuals after vaccination with the live oral typhoid vaccine Ty21a. This finding is consistent with other work in this field.

CHAPTER 4: ANTIGEN-SPECIFIC CD4+ T-CELL IMMUNE RESPONSES IN MUCOSAL TISSUE

4.1 INTRODUCTION

S. Typhi is a human-specific pathogen that invades the body through M cells overlying Peyer's patches in the small intestine and transport across M cells brings typhoid bacilli into contact with gut-associated lymphoid tissue(Parry et al., 2002). After 8-14 days, systemic infection is established (Gordon, 2008). Exposure to *S.* Typhi therefore induces both local mucosal and systemic immune responses. The production of mucosal IgA responses after ingestion of attenuated *S.* Typhi strains has been well documented (Nisini et al., 1993; Tacket et al., 1997) but very little information is known about cellular immune responses to attenuated *S.* Typhi strains in the gut mucosa. This is likely to be due to the difficulties in obtaining intestinal biopsies from humans. However, work carried out with *S.* Typhimurium in mice has shown the importance of cytokines such as IFN-γ and TNF-α in providing protection against this bacterium (Mastroeni et al., 1992; Ramarathinam et al., 1991). In humans, it is known that effector T cells that are activated in Peyer's patches (secondary lymphoid organs) migrate preferentially to intestinal tissue and not to other peripheral sites such as the lung or the skin. Gut-homing T cells express both the integrin α4β7 which binds to MAdCAM1 on the blood vessels of the intestine and other mucosal surfaces and the chemokine receptor CCR9 which interacts with CCL25, a gut-associated chemokine (Woodland and Kohlmeier, 2009).

4.2 RESEARCH QUESTIONS

The nature of typhoid pathogenesis means that the gut cell-mediated immune response is likely to play a key role in protecting the host against *S.* Typhi. It is therefore important to characterise this response. It is also important to identify any correlation between gut and systemic cell-mediated responses because peripheral blood is more accessible than mucosal tissue and can be used in studies to evaluate future vaccine candidates. The testable questions were:

1) Is there a difference in the percentage of typhoid antigen-specific cytokine-producing CD4+ T cells in duodenal biopsies between unvaccinated and vaccinated individuals?

2) Is there a difference in the percentage of typhoid antigen-specific cytokine-producing CD4+ T cells in colonic biopsies between unvaccinated and vaccinated individuals?
3) Is there a correlation between the percentage of typhoid antigen-specific cytokine-producing CD4+ T cells in the duodenum and the percentage in the colon?
4) Is there a correlation between systemic and mucosal antigen-specific T cell responses – do vaccinated individuals with a high percentage of typhoid antigen-specific cytokine-producing CD4+ T cells in blood also have a high percentage in the duodenum?
5) Does the percentage of typhoid antigen-specific cytokine-producing CD4+ T cells that are beta7+ differ between duodenal biopsies and peripheral blood?

4.3 MATERIALS AND METHODS

4.3.1 Study location and population

As described in sections 2.1 and 2.2.

4.3.2 Study design

Ten individuals were vaccinated on day 0. See section 2.3.1 for information about the vaccine and vaccination. D2-D3 duodenal pinch biopsies were collected on day 18 from 10 vaccinees and seven controls. Sigmoid colonic biopsies were collected on day 18 from seven vaccinees and four controls.

4.3.3 Sample processing

Duodenal and colonic biopsies were processed as described in section 2.4.1.2 and MMCs were counted as described in section 2.4.2.

4.4.4 Flow cytometry phenotyping assays

4.4.4.1 Intracellular cytokine staining assay

As described in section 2.5.2.

The antigens used for stimulation are described in section 2.5.1.

Analysis of the data was performed on FlowJo (Treestar Inc., San Carlos, CA) using the gating strategy shown in Figure 4.1. In order to determine the percentage of antigen-specific cytokine-producing CD4+ T cells that were also beta7+, the gating strategy shown

in Figure 4.2 was employed for duodenum and that shown in Figure 4.3 employed for peripheral blood.

Staining of duodenal and colonic tissue with the fluorochrome CD4-APC-Cy7 was unsuccessful. There is no obvious biological explanation for why this would be the case. Consequently, CD4+ T cells could not be positively gated on. CD8- T cells had to be gated on instead, on the assumption that the CD8- T cell population was the same as the CD4+ T cell population. Work carried out in this laboratory, in which gut tissue was successfully stained with CD4-Pacific Blue supports this assumption. The CD4+ T cell population was shown to be equivalent to the CD8- T cell population.

4.4.5 Statistical analysis

Statistical analyses were performed using Stata version 11 and SPSS version 18. There were insufficient observations to make a definitive conclusion about the statistical distribution of the observations. Data were therefore analysed using a generalised linear (ANOVA) model assuming a Normal distribution with bootstrapping in order to obtain more reliable estimates of group differences. All graphs were produced using GraphPad Prism 5.

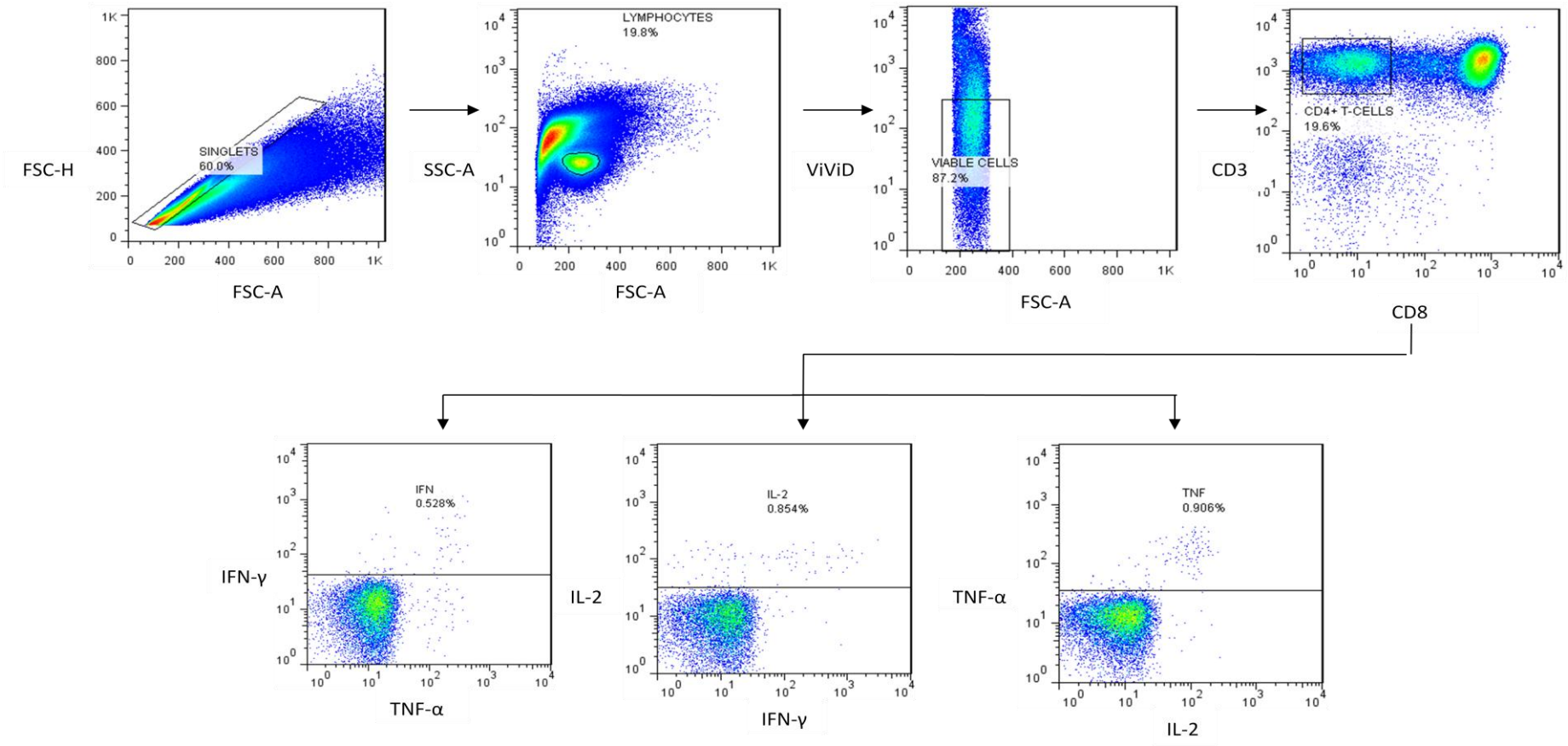

Figure 4.1 Intracellular cytokine staining flow cytometry gating strategy for mucosal samples. MMCs were stained with monoclonal antibodies directed against CD3, CD4, CD8, IFN-γ, TNF-α, IL-2 and a combination (on the same colour) of CD14, CD19 and ViViD, a viability marker. Single cells were identified using forward scatter properties and lymphocytes were then identified using forward scatter and side scatter. The 'dump' channel was used to remove monocytes, B cells and dead lymphocytes. CD4 staining was unsuccessful in mucosal samples so CD8 negative cells were gated on instead on the assumption that CD8 negative cells are CD4 positive. Within the CD4+ T cell population, cells producing IFN-γ, TNF-α or IL-2 were identified. Combination gating was used to identify single-, double- and triple-producing cells.

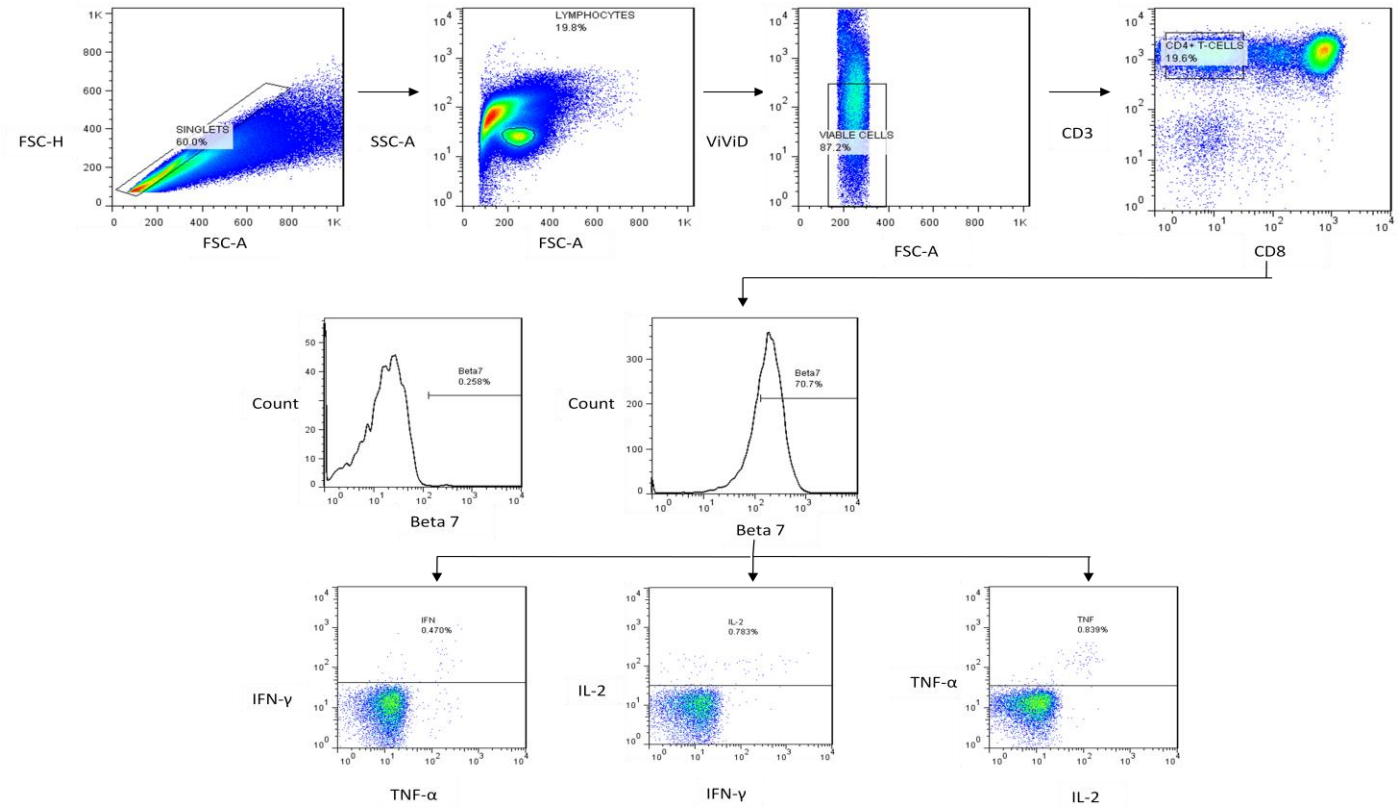

Figure 4.2 Intracellular cytokine staining flow cytometry gating strategy including beta7 for mucosal samples. MMCs were stained with monoclonal antibodies directed against CD3, CD4, CD8, IFN-γ, TNF-α, IL-2 and a combination (on the same colour) of CD14, CD19 and ViViD, a viability marker. Single cells were identified using forward scatter properties and lymphocytes were then identified using forward scatter and side scatter. The 'dump' channel was used to remove monocytes, B cells and dead lymphocytes. CD4 staining was unsuccessful in mucosal samples so CD8 negative cells were gated on instead on the assumption that CD8 negative cells are CD4 positive. Within the CD4+ T cell population, beta7+ cells were identified. This was done by gating on the beta 7 negative population in a tube of PBMCs to which all the fluorochromes except beta 7 had been added. Within the CD4+ beta7+ population, cells producing IFN-γ, TNF-α or IL-2 were identified. Combination gating was used to identify single-, double- and triple-producing cells.

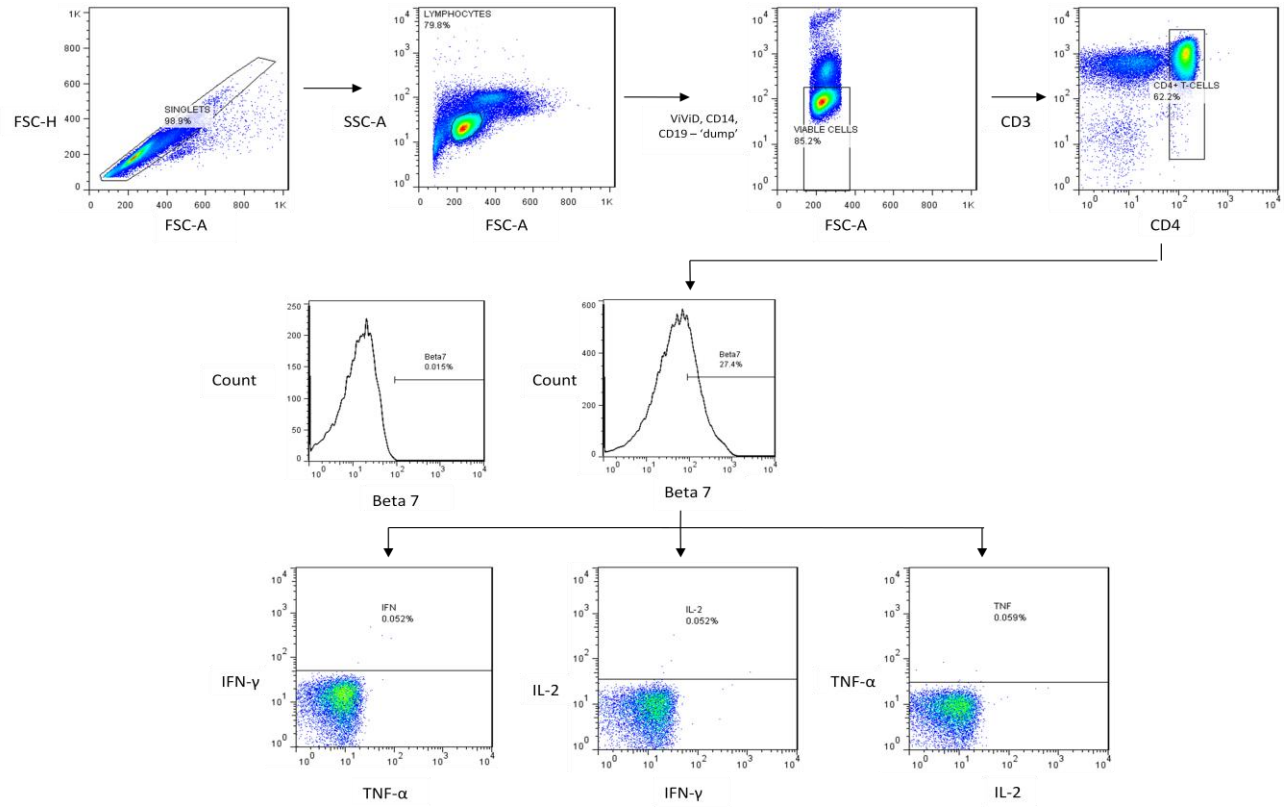

Figure 4.3 Intracellular cytokine staining flow cytometry gating strategy including beta7 for peripheral blood. PBMCs were stained with monoclonal antibodies directed against CD3, CD4, CD8, IFN-γ, TNF-α, IL-2 and a combination (on the same colour) of CD14, CD19 and ViViD, a viability marker. Single cells were identified using forward scatter properties and lymphocytes were then identified using forward scatter and side scatter. The 'dump' channel was used to remove monocytes, B cells and dead lymphocytes. CD4+ T cells were then positively identified by CD3 and CD4. Within the CD4+ T cell population, beta7+ cells were identified. This was done by gating on the beta7 negative population in a tube of PBMCs to which all the fluorochromes except beta7 had been added. Within the CD4+ beta7+ population, cells producing IFN-γ, TNF-α or IL-2 were identified. Combination gating was used to identify single-, double- and triple-producing cells.

4.4 RESULTS

4.4.1 Characteristics of the study population

Study number	Female (F) or male (M)	Age	Vaccine (V) or control (C)	Assay
1	F	44	V*	-
2	M	44	V*	-
3	F	22	C	Duo + Colon ICS
4	F	27	C	Duo ICS
5	M	38	V	Duo + Colon ICS
6	M	26	V	-
7	M	58	V	-
8	F	21	V	Duo + Colon ICS
9	M	24	V*	Duo + Colon ICS
10	F	22	C	Duo + Colon ICS
11	M	25	V*	Duo + Colon ICS
12	M	23	C	Duo ICS
13	F	27	V	Duo ICS
14	F	21	V*	Duo + Colon ICS
15	F	26	C	Duo ICS
16	F	26	C	Duo + Colon ICS
17	M	22	C	Duo + Colon ICS

Table 4.1 Demographic characteristics of the study population for mucosal assays. * represents individuals who had received a parenteral typhoid vaccination, either whole cell or Vi polysaccharide, more than three years previously. Individuals who had received a typhoid vaccine within the last three years were excluded from the study.

The duodenum ICS assay was performed on samples from six vaccinated individuals (median age 24.5, age range 21-38, three females) and seven control individuals (median age 23, age range 22-27, six females). The colon ICS assay was performed on samples from five vaccinated individuals (median age 24, age range 21-38, two females) and four control individuals (median age 22, age range 22-26, three females).

4.4.2 The proportion of CD4+ T cells is similar between controls and vaccinated individuals

The percentage of viable lymphocytes that were CD4+ was calculated for the control and vaccinated group for the MMCs used in the duodenal ICS assay (Figure 4.4) and the colonic ICS assay (Figure 4.5). This was done in order to be confident that it was reasonable to compare the proportion of antigen-specific cytokine-producing CD4+ T cells between the two groups. For both duodenal and colonic tissue, the percentage of viable lymphocytes that were CD4+ was similar between the control and the vaccinated group.

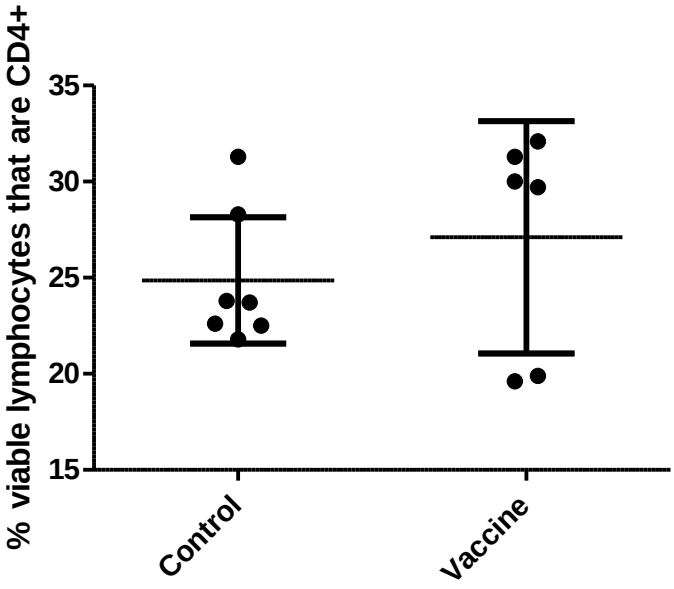

Figure 4.4 The percentage of viable lymphocytes in duodenal tissue that express CD4. Lines represent the mean and 95% confidence interval.

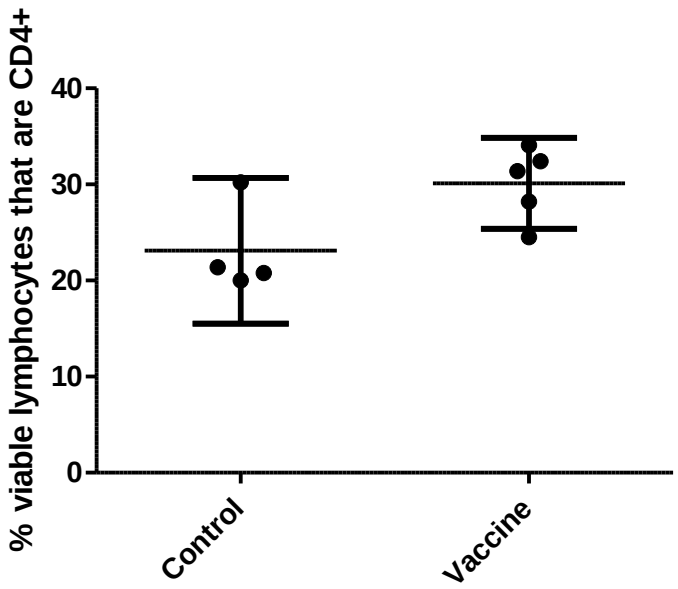

Figure 4.5 The percentage of viable lymphocytes in colonic tissue that express CD4. Lines represent the mean and 95% confidence interval.

4.4.3 In duodenal biopsies, when cells are unstimulated, there is no statistically significant difference in the proportion of cytokine-producing CD4+ T cells between the control and vaccinated groups

Cytokine production was measured in duodenal CD4+ T cells that had not been stimulated by antigen (Figure 4.6). There was no statistically significant difference in the mean percentage of cytokine-producing CD4+ T cells between the control and vaccinated groups (0.584% vs. 0.356%, bootstrapped 95% CI of -0.318 to +0.773).

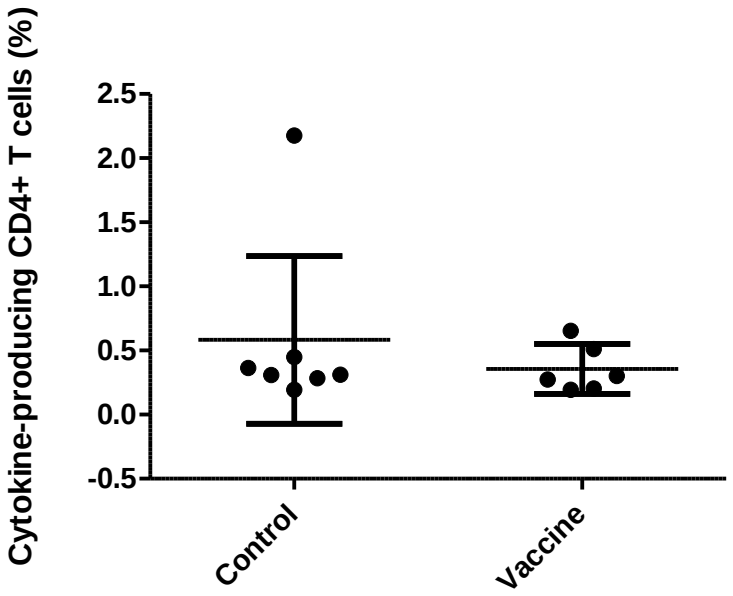

Figure 4.6 CD4+ T cell responses in unstimulated cells from the duodenum. The percentage of unstimulated CD4+ T cells producing cytokine in the control and vaccinated groups. Cytokine-producing cells are defined as those producing any combination of IFN-γ, TNF-α and IL-2. Lines represent the mean and the 95% confidence interval.

4.4.4 In duodenal biopsies, there is a higher proportion of typhoid antigen-specific cytokine-producing CD4+ T cells in the vaccinated group compared to the control group

To determine if there was evidence of a response to the oral typhoid vaccine Ty21a in duodenal biopsies, the magnitude of the CD4+ T cell response to Ty21a was determined using an intracellular cytokine staining assay. SEB and flu antigen were used as positive controls. The magnitude of the response measured in unstimulated cells was subtracted from that measured in stimulated cells in order to make the response comparable between individuals. A positive response was measured when cells were stimulated with SEB, flu and Ty21a; the response was above that measured in unstimulated cells. Figure 4.7 shows representative flow cytometry dot plots from a vaccinated individual.

There was no statistically significant difference in the mean percentage of cytokine-producing CD4+ T cells between the control and vaccinated groups when cells were stimulated with SEB (30.128% vs. 30.648%, bootstrapped 95% CI of -6.204 to +5.163)(Figure 4.8A). However, when cells were stimulated with flu (Figure 4.8B), there was a statistically significant increase in the mean percentage of antigen-specific cytokine-producing CD4+ T

cells in the vaccinated group compared to the control group (6.197% vs. 1.891%, bootstrapped 95% CI of -7.822 to -0.730).

When cells were stimulated with Ty21a (Figure 4.8C), there was a statistically significant increase in the mean percentage of typhoid antigen-specific cytokine-producing CD4+ T cells in the vaccinated group compared to the control group (1.041% vs. 0.604%, bootstrapped 95% CI of +0.005 to +0.807).

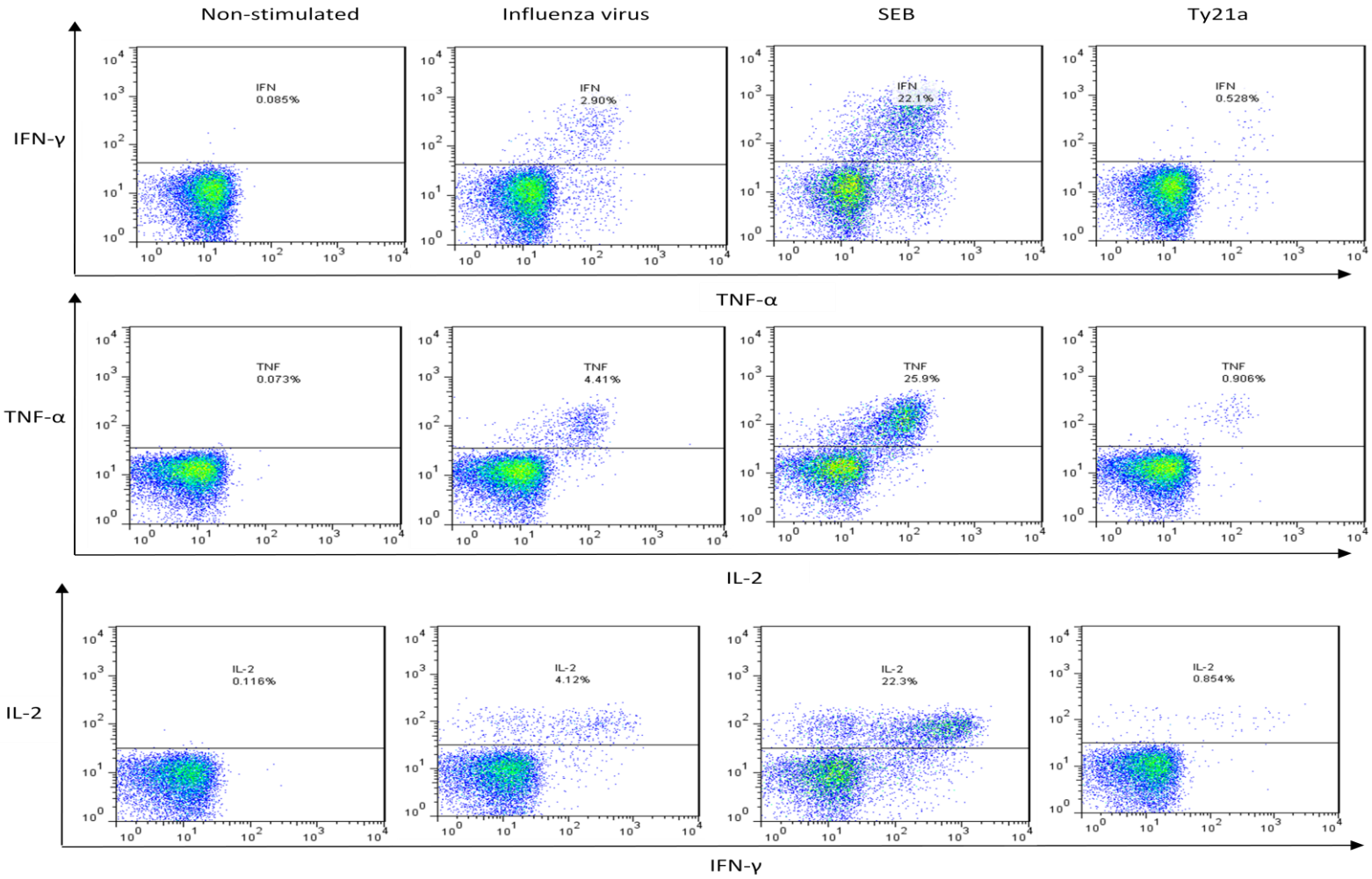

Figure 4.7 Representative flow cytometry dot plots from a vaccinated individual. MMCs were stimulated with antigen and antigen-specific CD4+ T cell responses were measured by intracellular cytokine staining. The production of the cytokines IFN-γ, TNF-α and IL-2 was determined. The antigens used for stimulation were influenza antigen, SEB and Ty21a, as described in section 2.5.1.

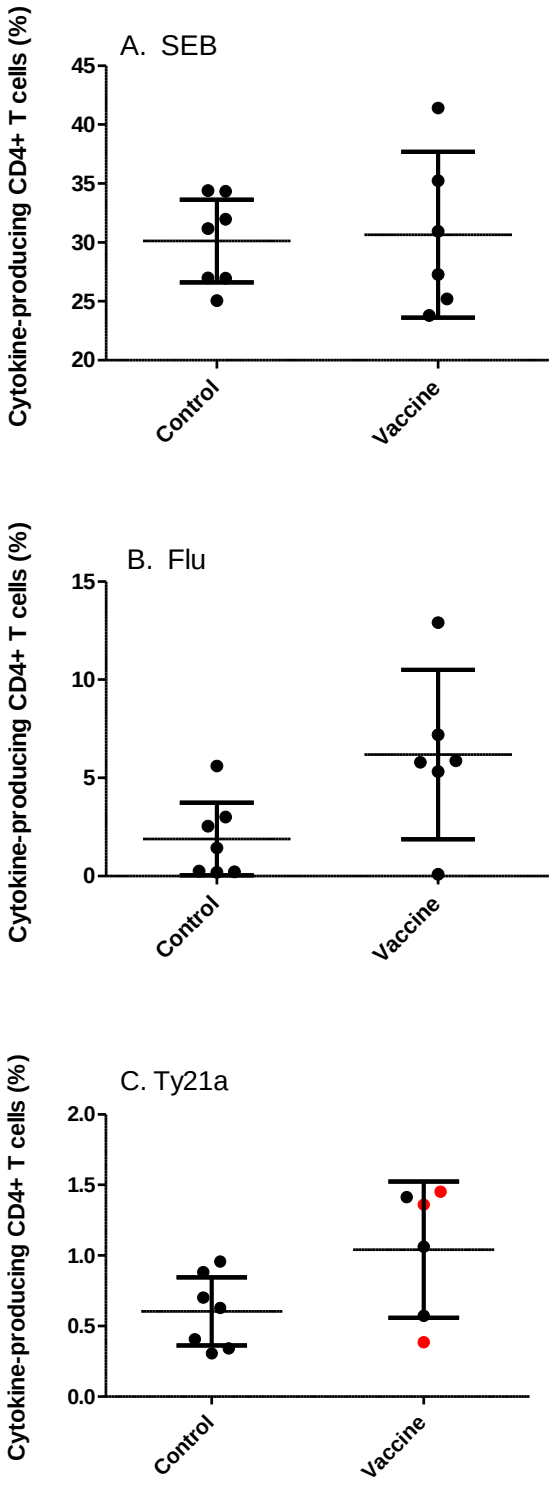

Figure 4.8 Antigen-specific CD4+ T cell responses in the duodenum as measured by an ICS assay. A comparison of the percentage of CD4+ T cells producing cytokine in the control and vaccinated groups in response to stimulation with A) SEB, B) Flu and C) Ty21a. Cytokine-producing CD4+ T cells are defined as those producing any combination of IFN-γ, TNF-α and IL-2. Red dots represent previously vaccinated individuals. Lines represent the mean and the 95% confidence interval.

4.4.5 In colonic biopsies, when cells are unstimulated, there is no statistically significant difference in the proportion of cytokine-producing CD4+ T cells between the control and vaccinated groups

Cytokine production was measured in CD4+ T cells from the colon that had not been stimulated by antigen (Figure 4.9). There was no statistically significant difference in the mean percentage of cytokine-producing CD4+ T cells between the control and vaccinated groups (6.657% vs. 1.729%, bootstrapped 95% CI of -1.141 to +11.298).

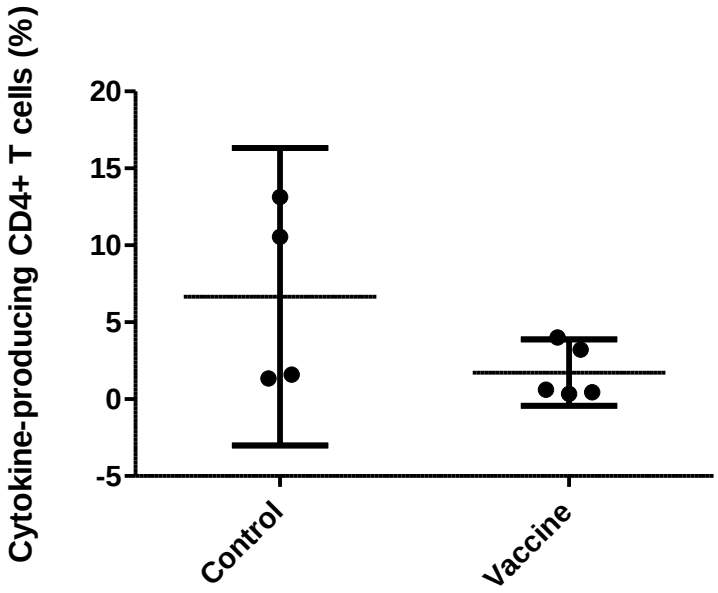

Figure 4.9 CD4+ T cell responses in unstimulated cells from the colon. The percentage of unstimulated CD4+ T cells producing cytokine in the control and vaccinated groups. Cytokine-producing cells are defined as those producing any combination of IFN-γ, TNF-α and IL-2. Lines represent the mean and the 95% confidence interval.

4.4.6 In colonic biopsies, there is no difference in the proportion of antigen-specific cytokine-producing CD4+ T cells in the vaccinated group compared to the control group

To determine if there was evidence of a response to the oral typhoid vaccine Ty21a in colonic biopsies, the magnitude of the CD4+ T cell response to Ty21a was determined using an intracellular cytokine staining assay. SEB and flu antigen were used as positive controls. The magnitude of the response measured in unstimulated cells was subtracted from that measured in stimulated cells in order to make the response comparable between

individuals. A positive response was measured when cells were stimulated with SEB, flu and Ty21a; the response was above that measured in unstimulated cells.

There was no statistically significant difference in the mean percentage of cytokine-producing CD4+ T cells between the control and vaccinated groups when cells were stimulated with SEB (21.959% vs. 19.037%, bootstrapped 95% CI of -4.107 to +9.952)(Figure 4.10A) or flu (1.820% vs. 2.255%, bootstrapped 95% CI of -2.300 to +1.429)(Figure 4.10B).

When cells were stimulated with Ty21a (Figure 4.10C), the difference in the mean percentage of antigen-specific cytokine-producing CD4+ T cells between the control group and the vaccinated group was not statistically significant (1.146% vs. 0.668%, bootstrapped 95% CI of -1.909 to +0.850).

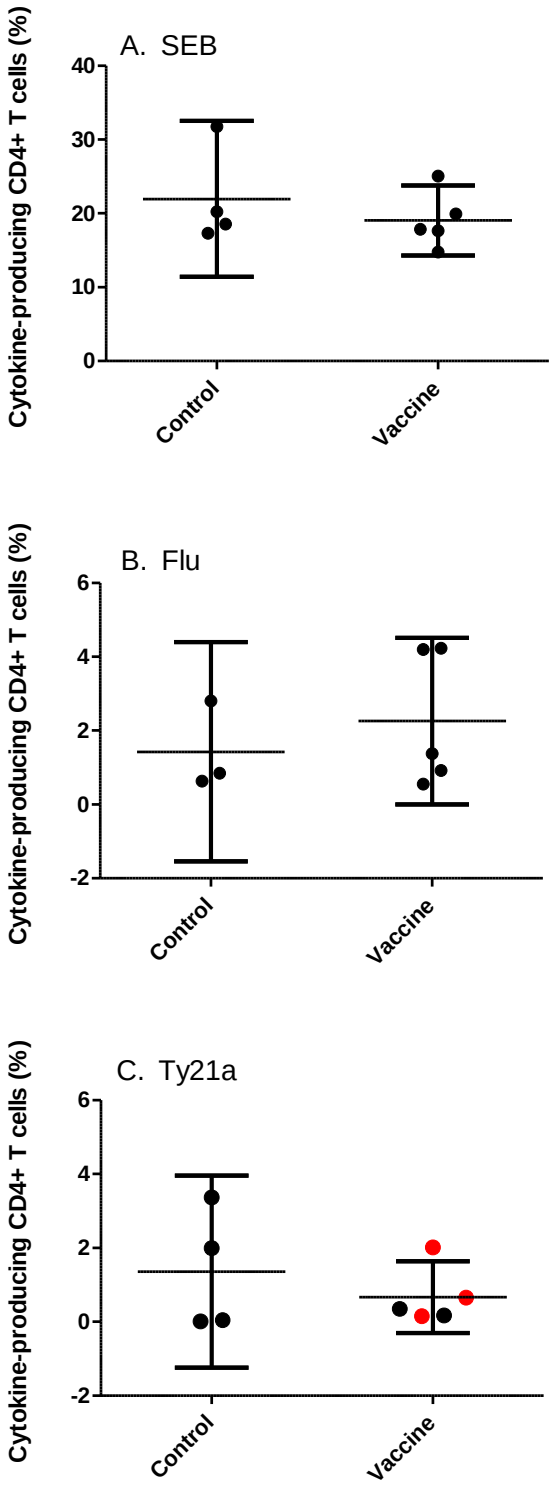

Figure 4.10 Antigen-specific CD4+ T cell responses in the colon as measured by an ICS assay. A comparison of the percentage of CD4+ T cells producing cytokine in the control and vaccinated groups in response to stimulation with A) SEB, B) Flu and C) Ty21a. Cytokine-producing CD4+ T cells are defined as those producing any combination of IFN-γ, TNF-α and IL-2. Red dots represent previously vaccinated individuals. Lines represent the mean and the 95% confidence interval.

4.4.7 Comparison of cytokine-secreting T cell subsets in unvaccinated and vaccinated individuals at day 18 in peripheral blood and duodenum

The cytokine-producing profile of unvaccinated (Figure 4.11A) and vaccinated (Figure 4.11B) individuals at day 18 in peripheral blood and duodenum was described by calculating the proportions of single-, double- and triple-producing CD4+ T cells (IFN-γ, TNF-α, IL-2, IFN-γ + TNF-α, IFN-γ + IL-2, IL-2 + TNF-α, IFN-γ + TNF-α + IL-2) in the total typhoid antigen-specific cytokine-secreting CD4+ T cell population. In both blood and duodenum in unvaccinated and vaccinated individuals, single-, double- and triple-producing effector T cells are all present. All three of the cytokines measured contribute to the antigen-specific CD4+ T cell cytokine response.

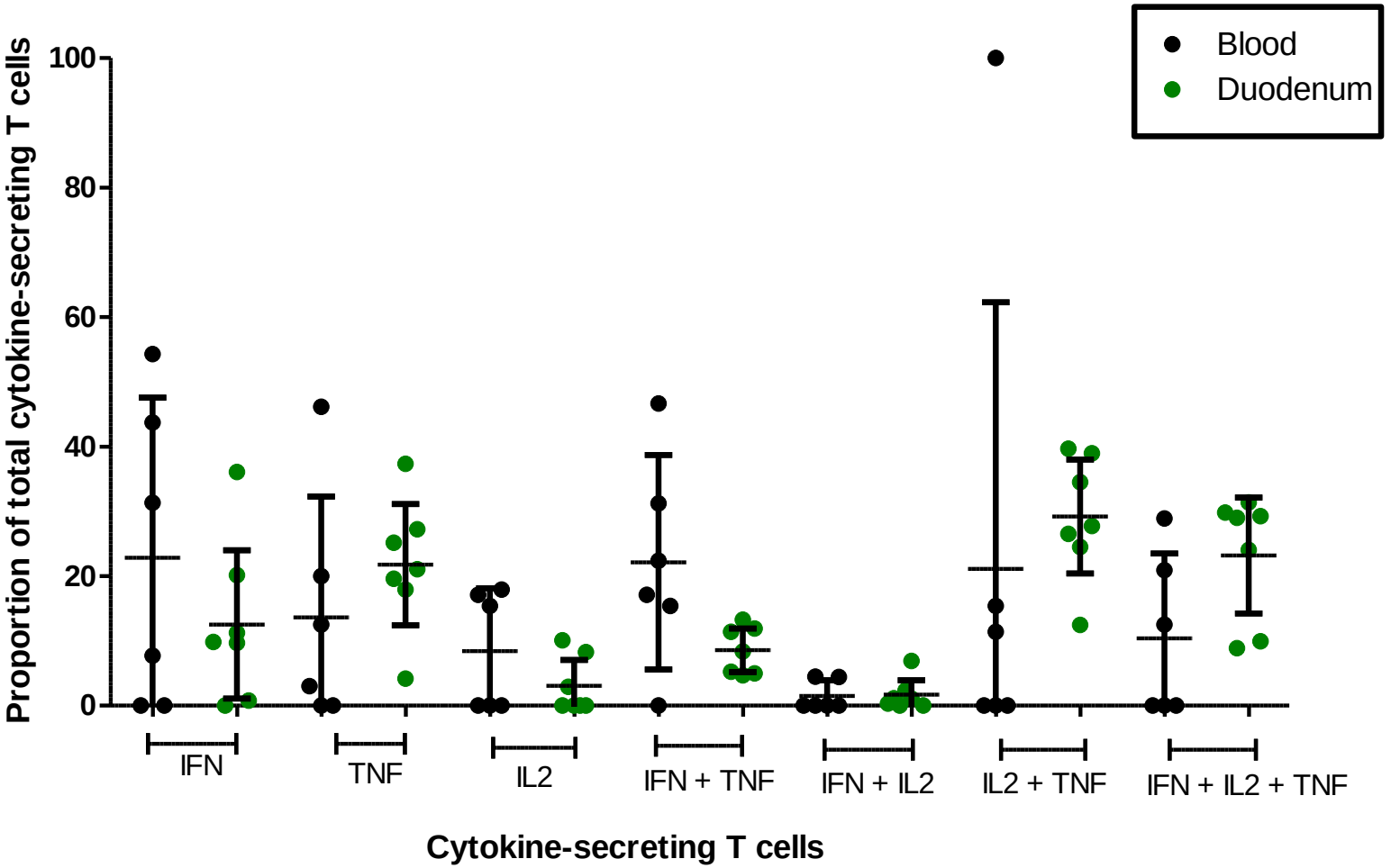

Figure 4.11A The proportions of single-, double- and triple- cytokine-producing typhoid antigen-specific CD4+ T cells in peripheral blood and duodenum in the control group. Lines represent the mean and the 95% confidence interval.

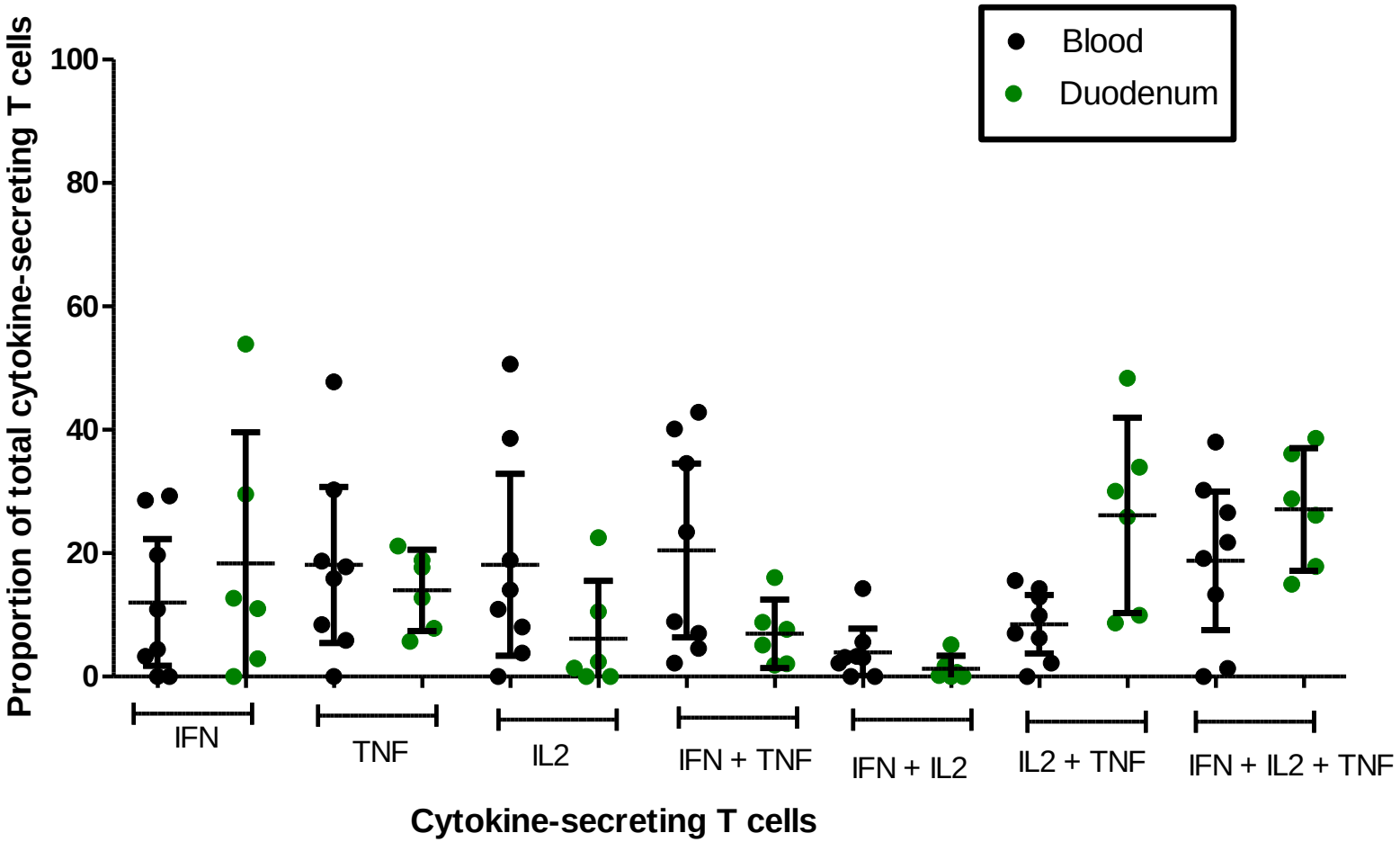

Figure 4.11B The proportions of single-, double- and triple- cytokine-producing typhoid antigen-specific CD4+ T cells in peripheral blood and duodenum in the vaccinated group. Lines represent the mean and the 95% confidence interval.

4.4.8 The relationship between antigen-specific CD4+ T cell responses in duodenum and those in the colon is not strong

In vaccinated individuals, there does not seem to be a strong correlation between the percentage of typhoid antigen-specific cytokine-producing cells in the duodenum and the percentage of typhoid antigen-specific cytokine-producing cells in the colon (Pearson correlation -0.621, p = 0.263)(Figure 4.12).

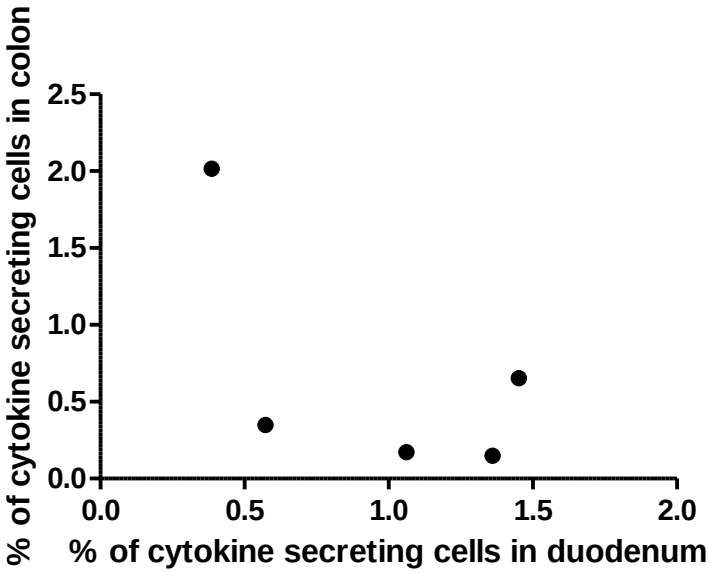

Figure 4.12 The relationship between the percentage of antigen-specific cytokine-producing CD4+ T cells in duodenal tissue compared to colonic tissue in vaccinated individuals

4.4.9 The relationship between antigen-specific CD4+ T cell responses in blood and those in duodenum is not strong

In vaccinated individuals, there does not seem to be a strong correlation between the percentage of typhoid antigen-specific cytokine-producing cells in peripheral blood and the percentage of typhoid antigen-specific cytokine-producing cells in the duodenum (Pearson correlation +0.068, p = 0.899)(Figure 4.13).

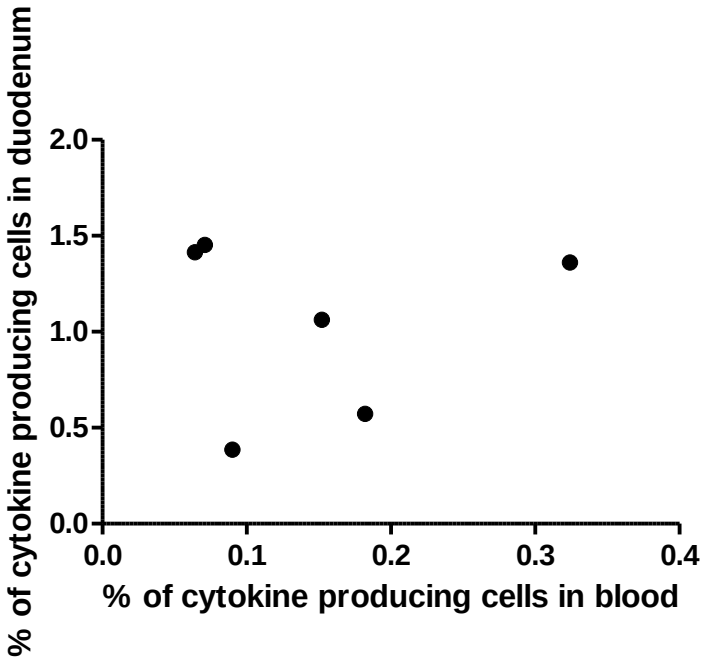

Figure 4.13 The relationship between the percentage of antigen-specific cytokine-producing CD4+ T cells in peripheral blood compared to duodenum in vaccinated individuals.

4.4.10 The proportion of antigen-specific cytokine-producing CD4+ T cells that express beta7 differs between peripheral blood and duodenal biopsies

The percentage of typhoid antigen-specific CD4+ T cells that expressed beta7 was significantly greater in duodenum than in blood (mean 75.05 vs. 51.92, bootstrapped 95% CI +7.72 to +37.97). The percentage of antigen-specific CD4+ T cells expressing beta7 was also significantly greater in duodenum than in blood following stimulation with the two positive controls, SEB and flu (mean 23.02 vs. 73.37, bootstrapped 95% CI +43.71 vs. +56.65 and mean 22.96 vs. 83.98, bootstrapped 95% CI +52.14 to +69.71, respectively)(Figure 4.14).

In blood, the percentage of typhoid antigen-specific CD4+ T cells that express beta7 was significantly greater than both the percentage of SEB antigen-specific CD4+ T cells (mean 51.92 vs. 23.02, bootstrapped 95% CI +15.16 to +42.80) and the percentage of flu antigen-specific CD4+ T cells (mean 51.92 vs. 22.96, bootstrapped 95% CI +14.58 to +45.19). No significant difference was observed in this percentage between SEB and flu stimulation (mean 23.02 vs. 22.96, bootstrapped 95% CI -7.86 to +7.32)(Figure 4.14).

In duodenum, the percentage of flu antigen-specific CD4+ T cells that express beta7 was significantly greater than both the percentage of SEB antigen-specific CD4+ T cells (mean 83.98 vs. 73.37, bootstrapped 95% CI -15.07 to -7.32) and the percentage of typhoid antigen-specific CD4+ T cells (mean 83.98 vs. 75.05, bootstrapped 95% CI -11.91 to -6.17)(Figure 4.14).

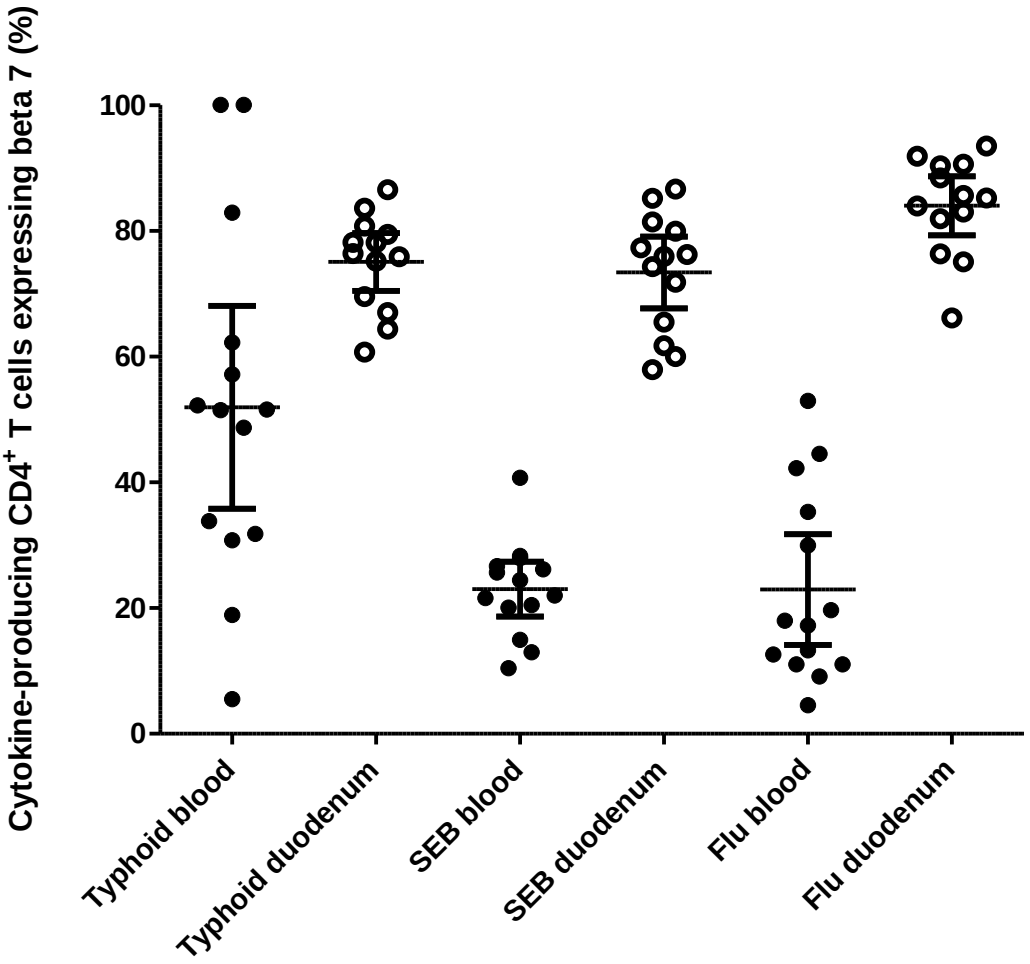

Figure 4.14 A comparison of the percentage of cytokine-producing CD4+ T cells expressing beta7 in peripheral blood and duodenal biopsies. Cytokine-producing cells are defined as those producing any combination of IFN-γ, TNF-α and IL-2. Beta7 is a mucosal homing marker. Cells were stimulated separately with heat-killed Ty21a, flu and SEB (flu and SEB are both positive controls). The graph includes participants in both the control and vaccinated groups as the key point is whether any antigen-specific cells express beta7, not whether vaccine-induced antigen-specific T cells express beta7. Lines represent the mean and the 95% confidence interval.

4.5 DISCUSSION

This study investigated whether there was evidence of an antigen-specific CD4+ T cell response to the licensed live oral typhoid vaccine Ty21a in mucosal tissue using an intracellular cytokine staining assay. In duodenal tissue, there was no statistically significant difference in the mean percentage of antigen-specific cytokine-producing CD4+ T cells between the vaccinated and control group at baseline or when the cells were stimulated with SEB, but there was a statistically significant difference when the cells were stimulated with flu. There was a statistically significant increase in the mean percentage of typhoid antigen-specific cytokine-producing CD4+ T cells in the vaccinated group compared to the control group. In colonic tissue, there was no statistically significant difference in the mean percentage of antigen-specific cytokine-producing CD4+ T cells between the vaccinated and control group at baseline or when the cells were stimulated with SEB or flu, the positive controls. There was also no difference in the mean percentage of typhoid antigen-specific cytokine-producing CD4+ T cells between the two groups. In the vaccinated group, there was not a strong relationship between duodenum and colon or between blood and duodenum in terms of the percentage of typhoid antigen-specific cytokine-producing CD4+ T cells. There was a statistically significant difference between blood and duodenum in the percentage of antigen-specific cytokine-producing CD4+ T cells that were beta7+, regardless of the antigen used for stimulation. In addition, a significantly higher proportion of blood CD4+ T cells were beta 7+ when stimulated with typhoid as opposed to SEB or flu and a significantly higher proportion of duodenal CD4+ T cells were beta 7+ when stimulated with flu as opposed to SEB or typhoid.

In the duodenum and colon ICS, the proportion of viable CD4+ T cells in the control group was similar to that in the vaccinated group, making it reasonable to then compare the antigen-specific CD4+ T cell responses between the two groups. The production of antigen-specific CD4+ T cell responses to the positive controls is evidence that the cells that were isolated from mucosal tissue were capable of responding to antigen stimulation. The statistically significant increase in the mean percentage of antigen-specific CD4+ T cells in the duodenum in the vaccinated group compared to the control group when stimulated with flu was unexpected. Possible explanations for this result include pure chance that more vaccinated individuals than control individuals had flu, but that this was not evident from the blood T cell responses. This is the most likely explanation, and might not be seen with a larger sample size. Alternatively, the vaccine may have led to non-specific

inflammation or priming of the gut, making cellular responses more likely in orally vaccinated individuals. Finally, a non-specific or cross-reactive element in the vaccine may have caused an antigen-specific response to flu, although this seems unlikely.

This study is the first in which duodenal and colonic biopsies were taken from individuals who were vaccinated with Ty21a to measure antigen-specific T cell responses. Others have measured the antigen-specific T cell responses in PBMCs expressing gut homing markers and inferred that the response in these cells is representative of the response in the gut microenvironment. For example, Wahid *et al.* found that immunisation with the oral attenuated CVD 909 typhoid vaccine elicited IFN-γ-secreting CD4+ memory T cells that were able to home to the gut (Wahid et al., 2008). This result is consistent with the results from this study, in which vaccinated individuals showed an increased percentage of antigen-specific cytokine-producing CD4+ T cells compared to the controls. In this study, vaccination had no effect on the cellular immune response in the colon. This result would be expected if there is distinct compartmentalisation of the mucosal immune system. If this was the case, the effector response would be stronger at the mucosal inductive site, i.e. the small intestine. The lack of response in the colon may be explained by the fact that the duodenum and the colon are derived from embryologically distinct compartments. The D3 segment of the duodenum from which biopsies were taken is derived from the embryological midgut and is supplied by the superior mesenteric artery, as is the terminal ileum. The terminal ileum is the site where *S.* Typhi is internalised. In contrast, the sigmoid colon (from which colonic biopsies were taken) is derived from the embryological hindgut which is supplied by the inferior mesenteric artery. However, the lack of response may have been due to our small sample size and if the immune response was measured in a bigger number of individuals, an antigen-specific response may have been seen in the colon.

Additionally, from looking at the literature, there is reason to believe that immunisation with oral vaccines could elicit effector responses at remote mucosal sites. Studies in mouse models have shown that oral vaccination can elicit immune responses at remote mucosal sites (DiGiandomenico et al., 2004; Nayak et al., 1998). DiGiandomenico *et al.* orally immunised mice with *Salmonella* Typhimurium expressing the antigen *Pseudomonas aeruginosa* O11 O antigen. They found O11-specific IgA and IgG in bronchoalveolar lavage fluid and that the vaccine was efficacious against pneumonia caused by *P. aeruginosa*. There have also been a small number of trials of *S.* Typhi live vector constructs in humans.

A trial in which individuals were vaccinated with the *S.* Typhi vaccine strain CVD 908 expressing the circumsporozoite protein of *Plasmodium falciparum* found that four out of seven individuals had a four-fold rise in jejunal secretory IgA antibodies against the circumsporzoite protein (Gonzalez et al., 1994). In another study, individuals were vaccinated with the live attenuated *S.* Typhi strain CVD 908-htrA which expressed fragment C of tetanus toxin. Systemic cellular immune responses were measured and 19 out of 20 subjects showed proliferative responses to tetanus toxoid (Tacket et al., 2000a). There is a need for trials in which mucosal cellular immune responses are measured in individuals who have been vaccinated with a *S.* Typhi vector vaccine.

In vaccinated individuals, there was not a strong correlation between duodenum and colon cellular immune responses in terms of the percentage of antigen-specific cytokine-producing CD4+ T cells in each. There are a number of explanations for this result. First, there may genuinely be no relationship between the duodenum and colon. This may be because the duodenum and colon are derived from embryologically distinct compartments, as explained above. Second, the relationship may have been obscured by commensal microorganisms which live in the colon. These commensals may have led to a raised cellular immune response when cells were unstimulated, thereby blunting the response measured when cells were stimulated with Ty21a. Third, the sample size may have been too small to detect a correlation.

In vaccinated individuals, there was also no correlation between blood and duodenal immune responses with regard to the percentage of typhoid antigen-specific cytokine-producing CD4+ T cells in each. This may seem counter-intuitive; it would seem reasonable to think that if there was a high percentage of cytokine-producing T cells in blood, there would also be a high percentage in the duodenum. However, the discrepancy between the responses in the two compartments may be a reflection of the fact that the mucosal immune system is distinct from the systemic immune system. Alternatively, there may have been no significant correlation because the timing of the immune response in blood is different to that in mucosal tissue. It would be expected that the immune response might take longer to develop in the blood compared to the duodenum. Although the number of individuals in this study was small, this result is potentially important because it suggests that the immune response in the blood might not mirror the immune response in mucosal tissue. This will have implications when it comes to using systemic immune responses in the evaluation of future oral typhoid vaccine candidates.

There was a statistically significant increase in the percentage of cytokine-producing CD4+ beta7+ T cells in the duodenum compared to that in peripheral blood. A high percentage of beta7+ T cells would be expected in the duodenum because in order for T cells to home to mucosal surfaces such as the intestine, expression of the integrin α4β7 is required. The ligand for this integrin, MAdCAM-1, is expressed by intestinal endothelial cells and intestinal lamina propria venules (Alford et al., 2008). Homing is important in enabling effector T cells to enter non-lymphoid intestinal tissue (Johansson-Lindbom and Agace, 2007). In the peripheral blood, a certain proportion of antigen-specific T cells would be expected to express beta7 as these cells would have been activated in the gut and would be re-circulating back to the gut. However, T cells activated in non-mucosal sites would not express beta7. The proportion of antigen-specific T cells expressing beta7 in peripheral blood for non-mucosal pathogens would therefore be lower in blood than in the duodenum.

A statistically significant increase was also observed in the percentage of cytokine-producing CD4+ T cells that were beta7+ in blood when stimulated with typhoid as opposed to SEB or flu. Typhoid is presented to the immune system through the gastrointestinal system whereas SEB is not; SEB is not biologically presented. T cells against typhoid are activated in the gut and they then enter the circulation from where they home to the tissue in which they were originally primed due to the homing markers on their surface. One would therefore expect to see a higher proportion of gut-homing T cells in the blood that were specific for typhoid and a lower proportion that were specific for SEB.

The cytokine-producing profiles of T cells from the duodenum of vaccinated and unvaccinated individuals show that single-, double- and triple- cytokine-producing T cells are present. All three cytokines that were measured, IFN-γ, TNF-α and IL-2, contributed to the antigen-specific immune response. The cytokine-producing profiles of T cells from the duodenum and peripheral blood were broadly similar. Surface phenotyping to distinguish between different T cell types was not carried out in this assay so the proportion of each T cell type present in duodenal tissue is not known.

This study has limitations, a major one being the small number of individuals from whom data were collected and analysed. There were not enough individuals in either the vaccine or control group to reach a definitive conclusion about the effect of the vaccine on mucosal immune responses. However, as with the blood study, under the terms of the ethics

approval there was a requirement to conduct an interim analysis partway through the study. More individuals need to be recruited to the study and this is currently taking place.

A second limitation of this study was that staining of duodenal and colonic tissue with the fluorochrome CD4-APC-Cy7 was unsuccessful. There is no obvious biological explanation for why this would be the case. Consequently, CD4+ T cells could not be positively gated on. CD8- T cells had to be gated on instead, on the assumption that the CD8- T cell population was the same as the CD4+ T cell population. Work carried out in this lab, in which gut tissue was successfully stained with CD4-Pacific Blue supports this assumption. The CD4+ T cell population was shown to be equivalent to the CD8- T cell population.

A further limitation of this study, as with the blood study, involves the low potency of the vaccine batch that was used for vaccination and cell stimulation. Although the vaccine was still clinically potent, this may have influenced the results that were acquired.

In the future, as with the blood study (chapter 3), more individuals need to be recruited to increase the sample size and the reduced potency batch of the vaccine should be compared with another batch of the vaccine. Additionally, a fluorochrome other than CD4-APC-Cy7 should be used in the flow panel so that positive gating can be used to identify CD4+ T cells.

4.6 CONCLUSION

In conclusion, this study shows an increase in the typhoid antigen-specific CD4+ T cell immune response in duodenal tissue after vaccination with Ty21a. This result is consistent with the idea that local immune responses against pathogens that enter the body via a mucosal route are important. However, it is unclear if this result was truly antigen-specific as there was also an increase in the antigen-specific CD4+ T cell response when cells were stimulated with flu. The difference between the systemic and mucosal immune response to the vaccine suggests that only investigating peripheral immune responses to mucosal pathogens or vaccine candidates may not give a representative picture.

CHAPTER 5: FINAL DISCUSSION

Mucosal surfaces are protected by a highly specialised mucosal immune system which is distinct from the systemic immune system. Pathogens such as *S.* Typhi, which invade the body through the gut mucosa but can cause bacteraemia, should elicit both mucosal and systemic immune responses. This study used two flow cytometric assays to measure mucosal and peripheral blood antigen-specific T cell responses in cells stimulated with heat-killed Ty21a in vaccinated and unvaccinated individuals.

Firstly, antigen-specific cellular immune responses against Ty21a were measured in peripheral blood. There was no statistically significant increase in the mean percentage of cytokine-producing or proliferating antigen-specific T cells in vaccinated individuals between day 0 and day 18. Secondly, antigen-specific immune responses against Ty21a were measured in T lymphocytes from two mucosal tissues, duodenum and colon. There was a statistically significant increase in the mean percentage of cytokine-producing T cells in vaccinated individuals compared to unvaccinated individuals in the duodenum but not in the colon. However, a statistically significant difference between the two groups was also observed in the duodenum in response to stimulation with flu, making it unclear whether the response measured after stimulation with Ty21a was actually antigen-specific. Thirdly, the correlation between the percentage of typhoid antigen-specific cytokine-producing T cells in duodenum and that in colon was investigated, as was the correlation between duodenum and blood. Neither correlation was strong. Lastly, the percentage of antigen-specific cytokine-producing CD4+ T cells that were beta7+ was measured in the duodenum and in peripheral blood. There was a statistically significant difference between the two samples regardless of the antigen with which cells were stimulated.

Although statistical significance was not reached, there was a trend towards increased antigen-specific cytokine production and lymphoproliferation in vaccinated individuals compared to controls in blood, which is consistent with other studies. With regard to mucosal tissue, this was the first time that duodenal and colonic biopsies have been used to assess cellular immune responses against Ty21a. Previously, duodenal cellular responses have been estimated by using PBMCs expressing the mucosal homing marker beta7. Furthermore, such studies did not use Ty21a, but new generation unlicensed live oral

vaccines. In this study, more T cells from the duodenum expressed beta 7 than T cells from peripheral blood, a finding that is also consistent with previous work.

The results from this study support the theory that local cellular immune responses against pathogens such as *S.* Typhi, which are presented across mucosal membranes, are important. This finding, coupled with the finding that the correlation between peripheral blood and mucosal cellular immune responses is not strong, highlights the need to identify correlates of protection in peripheral blood that reflect what is happening at the mucosal surface. This is necessary for the accurate evaluation of future vaccine candidates against mucosal pathogens.

The main limitation of this study is the small sample size. Under the terms of the ethics approval, there was a requirement to conduct an interim analysis after a small number of individuals had been recruited. This study reports the results of this interim analysis. More individuals are currently being recruited to increase the sample size.

Work currently being carried out includes recruiting more individuals to increase sample size and using frozen serum samples collected from individuals who were recruited to the study to look for humoral responses to the vaccine. Correlations between humoral and cellular responses are also being investigated.

Future work should involve measuring cellular immune responses at mucosal sites such as the lung because mouse models of *S.* Typhimurium vector vaccines containing respiratory antigens have provided protection against challenge by respiratory pathogens such as *Streptococcus pneumoniae*. Such work would inform the development of *S.* Typhi vector vaccines against respiratory pathogens for use in humans. Mucosal cellular immune responses should be measured against unlicensed new generation oral vaccines such as M01ZH09 and CVD 909 because these vaccines are more immunogenic than Ty21a.

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APPENDICES

Appendix 1

SUMMARY OF PRODUCT CHARACTERISTICS

1 NAME OF THE MEDICINAL PRODUCT

Vivotif®

2 QUALITATIVE AND QUANTITATIVE COMPOSITION

The composition in terms of active ingredients is as follows:

- *Salmonella enterica* serovar Typhi (abbr. *S. typhi*) Ty21a not less than 2 x 10^9 viable cells

Quantities expressed per capsule.

3 PHARMACEUTICAL FORM

Enteric-coated capsule, for oral administration to humans.

4 CLINICAL PARTICULARS

4.1 Therapeutic indications

For active oral immunisation against typhoid fever in children aged 6 years and over, adults and elderly.

4.2 Posology and method of administration

Posology

Children aged 6 years and above, adults and elderly: One capsule is to be taken on day 1. The second capsule should be taken on day 3 and the third capsule on day 5.

Unless the immunisation schedule of 3 vaccine capsules is completed, an optimal immune response may not be achieved.

Even after three doses, not all recipients of Vivotif will be fully protected against typhoid fever. Therefore, travellers should take all necessary precautions to avoid contact with or ingestion of potentially contaminated food or water.

Protection against typhoid fever commences approximately 7-10 days after ingesting the third dose of vaccine.

Under conditions of repeated or continuous exposure to *S. typhi* protection persists for at least 3 years.

In the case of travel from a non-endemic area to an area where typhoid fever is endemic, an annual booster consisting of three doses is recommended.

Children under 6 years: Safety and efficacy have not been established in children under 6 years of age.

Method of administration

The blister containing the vaccine capsules should be inspected to ensure that the foil seal and capsules are intact.

The capsule should be taken approximately one hour before a meal with a cold or lukewarm (temperature not to exceed body temperature, e.g. 37°C [98.6°F]) drink on alternate days, e.g. days 1, 3 and 5. The vaccine capsule should not be chewed and should be swallowed as soon as possible after placing in the mouth.

4.3 Contraindications

Vivotif must not be administered:

- To persons known to be hypersensitive to any component of the vaccine or the enteric-coated capsule (see section 6.1).

- To persons with congenital or acquired immune deficiency (including patients receiving immunosuppressive or antimitotic drugs).

- During an acute febrile illness or during an acute gastrointestinal illness. Vaccination should be postponed until after recovery.

4.4 Special warnings and precautions for use

None known

4.5 Interaction with other medicinal products and other forms of interaction

As the growth of vaccine organisms may be inhibited by sulphonamides or antibiotics, vaccination should not commence within 3 days after completing treatment with any antibacterial agents. Also, it is preferable that antibacterial therapy should not commence within 3 days after the last dose of Vivotif.

If malaria prophylaxis is also required, the fixed combination of atovaquone and proguanil can be given concomitantly with Vivotif. Doses of mefloquine and Vivotif should be separated by at least 12 hours. For other antimalarials, there should be an interval of at least 3 days between the last dose of Vivotif and the first dose of malaria prophylaxis.

Vivotif may be administered concomitantly with the live attenuated vaccines yellow fever vaccine and oral polio vaccine.

4.6 Pregnancy and lactation

Animal reproduction studies have not been conducted with Vivotif. It is not known whether Vivotif can cause foetal harm when administered to pregnant women or can affect reproduction capacity. Vivotif should be given to a pregnant woman only if clearly needed.

There are no data regarding administration of Vivotif to nursing mothers. It is not known if Vivotif is excreted in human milk.

4.7 Effects on ability to drive and use machines

None known.

4.8 Undesirable effects

The following adverse reactions were reported commonly (<1/10 but>1/100) in clinical studies:

Gastrointestinal disorders

Abdominal pain, nausea, diarrhoea, vomiting

General disorders and administration site conditions

Fever, influenza-like illness

Nervous system disorders

Headache

Skin and subcutaneous tissue disorders

Rash

The following additional adverse reactions have been reported very rarely (approximately <1/10,000) during post-marketing surveillance:

Skin reactions such as dermatitis, exanthema, pruritus, urticaria.

Anaphylaxis.

Asthenia, malaise, tiredness, shivering.

Paraesthesiae, dizziness.

Arthralgia, myalgia.

4.9 Overdose

Doses five-fold higher than the recommended dose do not produce vomiting, abdominal distress or fever. However overdosing can increase the possibility of shedding the *S. typhi* Ty21a organisms in the faeces.

5 PHARMACOLOGICAL PROPERTIES

5.1 Pharmacodynamic properties

As a result of irreversible changes in cell wall biosynthesis, the Ty21a strain is devoid of pathogenicity but is able to elicit an immune response against *S. typhi*.

Excretion of the vaccine strain after administering doses approximately 50 times greater than those in the present vaccine was assessed by taking stool or rectal swabs daily for 7 days following the last dose of vaccine. The rate of excretion of the vaccine strain in the stools was low, and the vaccine strain could not be recovered from small bowel aspirates one or more days after vaccination. Sera for determination of antibodies to O, H and Vi antigens were obtained prior to vaccination and biweekly for 8 weeks. Fourfold or greater responses in titre of O antibody only were observed. There was no correlation between faecal excretion of the strain Ty21a organisms and seroconversion with respect to titre of any of the antibodies tested.

5.2 Pharmacokinetic properties

Not applicable.

5.3 Preclinical safety data

There is no other relevant information other than presented in the sections above.

6 PHARMACEUTICAL PARTICULARS

6.1 List of excipients

The excipients contained in the preparation are as follows:

Sucrose (Saccharose) Ph. Eur
Ascorbic acid (E300) Ph. Eur
Casein hydrolysate HSE
(Hy-Case SF Sheffield)
Lactose anhydrous NF/USP, Ph. Eur
Magnesium stearate (E470) Ph. Eur.
Inactivated *S. typhi* Ty21a bacteria HSE

Capsule:

Gelatin
Titanium dioxide (white) (E171)
Titanium dioxide (red) (E171)
Erythrosine red No.3 (E127)
Ferric oxide (yellow) (E172)
Ferric oxide (red) (E172)

Capsule coating:

HydroxypropyImethyl-cellulose- phthalate
(HP-MCP) - 50
Ethylene glycol
Dibutyl phthalate
Diethyl phthalate

6.2 Incompatibilities

None known.

6.3 Shelf life

In blister packs: 18 months from date of packing, unopened, at 2-8°C.

After opening blister: not applicable.

6.4 Special precautions for storage

Store at 2-8°C. Protect from light

6.5 Nature and contents of container

Blister packs (PVC/PE/PVDC 250/30/90). Each blister pack contains 3 capsules.

6.6 Special precautions for disposal

No special instructions.

7 MARKETING AUTHORISATION HOLDER

Berna Biotech Italia s.r.l.

Via Bellinzona 39

I-22100 Como

Italy

8 MARKETING AUTHORISATION NUMBER(S)

PL 15747/0001

9 DATE OF FIRST AUTHORISATION/RENEWAL OF THE AUTHORISATION

23/06/2009

10 DATE OF REVISION OF THE TEXT

25/06/2009

Appendix 2

Volunteers required

The development of non-injected vaccines requires a good understanding of the immune responses produced as a result of oral vaccination.

Healthy, non smoking volunteers are invited to participate in research investigating immune responses to a licensed, oral (by mouth) typhoid vaccine. It does not matter if you have previously received a typhoid vaccine. Participation will be as an outpatient and will include blood tests and one or two endoscopic (camera) procedures (gastroscopy and sigmoidoscopy or bronchoscopy) over the course of four weeks.

Remuneration will be paid for your time and inconvenience

Please contact Dr. Sherouk El Batrawy, Sr. Lorna Roche or Dr. Stephen Gordon for further information on:

0151 706 4863 or 0151 705 3172

(or email lorna.roche@rlbuht.nhs.uk)

Appendix 3

NHS

National Research Ethics Service

North West 2 Research Ethics Committee вЂ“ Liverpool Central
3rd Floor
Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0161 625 7818
Facsimile: 0161 237 9427

23 March 2010

Dr Stephen B Gordon
Reader in Respiratory Medicine
Liverpool School of Tropical Medicine
Respiratory Infection Group
LSTM
Pembroke Place
L3 5QA

Dear Dr Gordon

Study Title:	**Mucosal and peripheral blood responses to vaccination with the licensed live oral typhoid vaccine Ty21a**
REC reference number:	**10/H1005/20**
Protocol number:	**1.0**

Thank you for your letter of 09 March 2010, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. I will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to

the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk. *Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.*

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter		01 February 2010
REC application	2.5	28 January 2010
Protocol	1.0	25 January 2010
Investigator CV	S.Gordon	22 December 2009
Participant Information Sheet: Remuneration	1.0	28 January 2010
GP/Consultant Information Sheets	1.0	28 January 2010
Letter from Sponsor		28 January 2010
Letter from Sponsor		03 February 2010
Letter from Statistician		25 January 2010
Referees or other scientific critique report	1	26 January 2010
Evidence of insurance or indemnity		28 January 2010
Summary/Synopsis	1.0	25 January 2010
Referees or other scientific critique report	R.Read	27 January 2010
Investigator CV	A.Thompson	27 January 2010
Referees or other scientific critique report	D.Lalloo	15 January 2010
Participant Information Sheet	2	04 March 2010
Participant Consent Form	2	04 March 2010
Recruitment Poster	2	04 March 2010
Response to Request for Further Information		09 March 2010

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

10/H1005/20	**Please quote this number on all correspondence**

Yours sincerely

Professor Sobhan Vinjamuri
Chair

Email: carol.ebenezer@northwest.nhs.uk

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Miss Ameeka Thompson*
Heather Rogers
Sian Roberts

Appendix 4

LSTM
LIVERPOOL SCHOOL
OF TROPICAL MEDICINE

Pembroke Place,
Liverpool, L3 5QA, UK
Tel: +44 (0)151 705 3100
Fax: +44 (0)151 705 3370

www.liv.ac.uk/lstm

Doctor Stephen Gordon
Liverpool School of Tropical Medicine
Pembroke Place
Liverpool
L3 5QA

Thursday, 28 January 2010

Dear **Doctor Stephen Gordon**

Sponsorship and Indemnity Request: **(SG10.01) Mucosal responses to oral vaccination.**

PhD Student: **Ameeka Thompson**

I am pleased to confirm that LSTM has agreed, in principle, to act as co-Sponsor for the above mentioned clinical research study in collaboration with **The Royal Liverpool and Broadgreen University Hospital NHS Trust.**

Please note that this letter **does not** constitute final LSTM Approval to allow your study to proceed. LSTM approval will be given when the final research ethics approval, financial and other regulatory requirements for the study have been met.

Yours sincerely

Mrs Sian Roberts
Head of Research Management