EXPLORING THE POTENTIAL OF USING RETINOIC ACID AS AN ORAL ADJUVANT

By

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A thesis submitted to the University of Zambia in fulfilment of the requirements of the degree of Doctor of Philosophy (PhD) in Immunology

The University of Zambia, Department of Internal Medicine

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Dedication

I dedicate this thesis to my beloved husband **Mr Malimba Xavier Lisulo**. You have journeyed with me through it all. Thank you for all your sacrifices, encouragement and patience that have helped me accomplish this.

To my two beautiful daughters **Bupilo** and **Bunde Masheke Lisulo**, this one is for you.

May the good Lord richly bless you.

Quote

The ultimate ignorance is the rejection of something you know nothing about, yet refuse to investigate……

Dr Wayne Dryer

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Abstract

Background: Oral vaccines have been associated with diminished immunogenicity and efficacy in developing country populations. Rotavirus, cholera and oral polio vaccines have shown much lower protection in Africa and Asia than in developed countries. In view of this, there is an urgent need to identify ways to improve the immune responses of these oral vaccines in the settings where they are most needed. It has emerged over the past decade that retinoic acids can up-regulate expression of $\alpha4\beta7$ and CCR9 gut homing molecules on lymphocytes and this can redirect the lymphocytes and vaccine responses towards the gut.

Objective: To establish whether giving oral vaccination alongside all-*trans* retinoic acid (ATRA) can improve the immune response to the vaccine by enhancing expression of gut homing molecules and immunoglobulin A (IgA).

Methods: In order to evaluate the effects of ATRA on gut homing lymphocytes and gut IgA responses to oral vaccines, adult male volunteers (n=94) were randomised to receive one of four oral vaccines: Vivotif, Dukoral, Rotarix, and Polio given with or without 10mg ATRA an hour before vaccination and then ATRA administered daily for 8 days. We measured IgA directed against lipopolysaccharide (LPS) preparations of the vaccines in serum and whole gut lavage fluid (WGLF) one day prior to vaccination and on day 14. We also measured gut homing receptor phenotype on circulating $CD4^+$ T cells.

Results: Vaccine-specific IgA in WGLF was significantly increased (P=0.01) against LPS in the group that received Vivotif alongside ATRA. $\alpha4\beta7$ (P=0.003) and

CCR9 (P=0.002) expression was also increased in the same group and that this enhanced expression was in an entirely coordinated (ρ=0.83, P<0.0001) fashion in 57% of the participants. The change in α4β7 on $CD4^+$ T cells was strongly (ρ= 0.82; P=0.02) associated with an increase in specific IgA response to Vivotif LPS in WGLF. We also found that these individuals had low baseline concentrations of serum retinol (median1.41μmol/L, IQR 1.06-2.48μmol/L; P=0.03). HIV had no effect on any response measured.

Conclusions: ATRA induced α4β7 and CCR9 expression in a coordinated expression shift on $CD4^+$ T cells only when given together with an oral typhoid vaccine. The coordinated phenotypic changes were strongly correlated with enhanced intestinal IgA expression. These findings suggest that ATRA could potentially be a useful oral adjuvant in a subset of individuals.

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List of Abbreviations and Acronyms

ATRA	All-trans retinoic acid
RA	Retinoic acid
α4β7	Alpha 4 beta 7 integrin
CCR9	C-C chemokine receptor 9
WGLF	Whole gut lavage fluid
MAdCAM-1	Mucosal addressin cell adhesion molecule 1
LPS	Lipopolysaccharide
sIgA	Secretory immunoglobulin A
RALDH	Retinyl aldehyde dehydrogenase
RBP	Retinol binding protein
LRAT	Lecithin retinol acyl transferase
GALT	Gut associated lymphoid tissues
APL	Acute promyelocytic leukemia
pIgR	Polymeric immunoglobulin receptor
IL	interleukin
CT	Cholera toxin
LT	Heat labile toxin
TLR	Toll-like receptor
OPV	Oral polio vaccine
IPV	Inactivated poliovirus vaccine
GPEI	Global polio eradication initiative
WHO	World health organisation
NSAIDs	Nonsteroidal anti-inflammatory drugs

Scope of the thesis

Chapter 1introduces the subject matter. This chapter discusses poor vaccine responses in developing countries and how oral vaccine adjuvants specifically retinoic acid can be a promising step in the endeavour to improve vaccine immune responses.

Chapter 2 begins by announcing the global diarrhoea burden then goes on to give a more detailed look at the work has been done in the area of vaccine responses in both industrialised and developing nations to try and reduce this burden. The chapter further describes the current use of adjuvants in vaccination. Vitamin A and its metabolites and the impact on immune response is then discussed then later how retinoic acid affects gut immunity in response to vaccination.

Chapter 3 gives an overview of the problem, the study justification, aims and objectives

Chapter4 describes the methods used throughout the study from recruitment of participants to the ELISA protocols and flow cytometry protocols in accessing immune responses to vaccination and ATRA treatment. It also describes the statistical tests used in analysing the data.

Chapter 5 is the first results chapter that describes the results on the effects of ATRA on gut homing lymphocytes.

Chapter 6 is the second set of result that discusses the effects of ATRA on gut IgA responses to oral vaccines

Chapter 7 the results described in this chapter show the correlation between gut homing profiles of circulating lymphocytes and gut IgA responses

Chapter 8 describes results for the baseline serum retinol concentration of participants. This chapter also outlines the in vitro work done and results of the effect of different ATRA concentrations gut homing expression.

Chapter 9 is the discussion chapter which discusses the findings and interprets them in relation to what others have found, and also outlining the novel findings in this particular study

Chapter 10 is the conclusion chapter. It is the culmination of the whole thesis. It summarises all the findings possible recommendations

CHAPTER 1

INTRODUCTION

Vaccines have been frequently cited as the most equitable low-cost, high-impact health measures, saving millions of lives annually. They are among the most promising interventions to diminish the burden of specific infections in populations in the world (Levine *et al*., 2010). Small pox for example, was completely eradicated through vaccination in 1980 and poliovirus has also been eliminated from much of the world through the oral polio virus vaccines (OPVs) and inactivated poliovirus vaccines (IPVs) (Serazin et al., 2010).

Other licensed vaccines and newer vaccines including conjugate vaccines for *Haemophilus influenza* type b (Hib), pneumococcus, *Neisseria meningitidis* group and rotavirus, have also contributed significantly to the reduction of the mortality and morbidity burdens of these infectious diseases (Del Giudice *et al*. 2003, Serazin et al., 2010).

Although there has been overwhelming success in protection against infectious disease through vaccination, an estimated 1.5 million children died globally in 2010 from vaccine-preventable diseases such as pneumonia and diarrhoea for which there were World Health Organisation (WHO) pre-qualified vaccines available (Greenwood et al., 2011, Machingaidze et al., 2013).Each year, 130 million babies are born and of these, an estimated 23% do not have access to vaccinations including 7.8 million in sub-Saharan Africa (Ehreth *et al*, 2003). For instance, approximately 19.3 million children did not receive diphtheria-tetanus-pertussis vaccine (DTP3) worldwide in 2010, with more than one-third of these children living in Africa (Machingaidze et al., 2013).

While many infectious diseases are controlled by immunizations, this has proved a challenge for diarrhoeal diseases. Few efficacious vaccines for prevention are available even though diarrhoeal disease due to intestinal infections is one of the most important causes of morbidity and mortality in children in developing countries (Petri et al., 2008). Two reasons for these challenges are that, firstly, the gut environment is quite harsh degrading most antigenic epitopes delivered in soluble form, and secondly mucosal tolerance limits immune responses to digested antigens (Lycke, 2012). Notwithstanding these obstacles, a number of oral vaccines developed have been successful in helping reduce mucosal and gastrointestinal infections. However, these vaccines are neither equally immunogenic nor protective in different populations especially those from developing country populations (Levine et al., 2010, Qadri *et al.*2013). Oral rotavirus vaccine for example, affords excellent (80-90%), protection against severe rotavirus diarrhoea in industrialized and middle income countries but has been less efficacious when tested in infants in the developing world (Vesikari et al., 2007, Madhi et al., 2010). However, although there is such low efficacy of rotavirus vaccine, it has shown to alleviate considerable rotavirus disease burden and mortality (Babji et al.,2012) and such, in 2009 WHO recommended that rotavirus vaccination be included in the national immunisation schedule for all member states. The trivalent oral polio vaccine (OPV) has also been found to be less effective in children in the developing world (Grassly et al., 2010) with estimated efficacies of up to 21% in India compared to 50% in the United States (Grassly et al., 2006). Reasons for these low efficacies being reported are not clear. Explanations could include interference from the high titers of antibody in maternal breast milk (Chilengi et al., 2016), interference from other intestinal infections [non-polio enteroviruses can reduce immune responses to OPV (Parker et al.,2014, Parker

et al., 2015)], nutritional factors such as vitamin A deficiency, recurrent diarrhoea episodes (Petri et al., 2008), and environmental enteropathy (Qadri *et al.* 2013).

In view of all this, there is an urgent need to identify ways to improve the immune responses of these oral vaccines in the settings where they are most needed. An adjuvant could have a dramatic impact on vaccination programmes for controlling diarrhoeal disease, with major benefits for child health in developing countries.

Adjuvants

Immunization with purified protein antigens typically elicits a modest antibody response with little or no T cell response (Reed *et al*., 2013). This has resulted in vaccine developers seeking the inclusion of adjuvants in vaccine candidates to enhance the efficacy of weak antigens. Adjuvants are selected based on a number of parameters including the nature of the vaccine, the immune response desired, target population and the route of vaccine administration (Reed *et al*., 2013).

The term adjuvant was derived from the latin word 'adjuvare' meaning 'to help' and was first introduced by Ramon who used reagents such as tapioca and aluminium hydroxide to improve the responses of horses or guinea pigs to diphtheria and tetanus toxoids (Rhee et al., 2012). Adjuvants are components used together with vaccines to help stimulate protective immunity based on antibodies and effector T cell functions (Coffman *et al*., 2010). These adjuvants provide help needed to enhance the immunogenicity of vaccine antigens. In addition, adjuvants can enable the use of lower vaccine doses in instances where multiple immunizations may be required to elicit sufficient antibody responses. For instance, the recombinant influenza vaccine that has been developed requires high doses because of its low immunogenicity. Other uses for adjuvants include increasing the mean antibody titers against the vaccine antigen thus increasing the response of the vaccine in the

general population and this is one of the focuses of my work; and also to increase seroconversion rates where there is reduced responsiveness. Adjuvants can also be used to improve the quality of vaccine immune response by increasing the generation of T cell memory; increasing the speed of initial response and altering the breadth, specificity and affinity of the vaccine response (Coffman *et al*., 2010). Adjuvants generally work by stimulating an innate immune response, which sets the context of the antigen presentation that generates the immune response.

Oral adjuvants

Vaccination through mucosal routes requires potent adjuvants to enhance immunogenicity (Rhee *et al*., 2012, Fujkuyama *et al*. 2012). Mucosal adjuvants can be classified into a number of categories including: (1) Toll-like receptor (TLR) ligands such as AS04 and CpG motifs (Lycke, 2012); (2) Bacterial toxins and their derivatives such as cholera toxin (CT) and *E. coli* heat labile enterotoxin (LT). These are the most powerful mucosal adjuvants identified to date; and (3) Cytokines and chemokines which include IL-1, IL-12, IL-18 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Lycke, 2012).

Globally, one in ten child deaths result from diarrhoeal disease during the first 5 years of life. This has resulted in about 800 000 fatalities worldwide annually, with most occurring in sub-Saharan Africa and South Asia (Liu et al., 2012, Kotloff *et al*., 2013). In Zambia, a study by Amadi *et al,* (2001) showed that high numbers of admissions seen at the children’s malnutrition ward of the University Teaching Hospital, the largest referral hospital in the country, were due to diarrhoeal related disease. Another study done in Ndola, Zambia, revealed that the diarrhoea prevalence in children under 5 years was 44.6% (Chilambwe et al., 2015). The

ministry of health (MOH) in Zambia also reported that 15,000 of Zambia's 2.4 million children under the age of 3, experience diarrhoea every year (MOH 2012).

The introduction of oral rehydration therapy has seen a dramatic reduction in mortality due to diarrhoeal related disease in children in the tropics (Gracey *et al*, 1997), however this has not been enough as high numbers of diarrhoeal deaths are still being reported.

Studies have revealed that vitamin A supplementation helps to reduce deaths due to diarrhoeal disease (Sommer *et al.*, 2008). Previous meta-analyses suggest that vitamin A supplementation for children in developing countries is associated with 30% reductions in mortality, especially deaths from diarrhoea and measles. The Ghana Vast Study revealed a 34% reduction in diarrhoeal-related mortality after vitamin A supplementation (GVST, 1993). In eight major randomized clinical trials, 2 in Africa and 6 in Asia, rural children received periodic vitamin A supplementation at regular intervals. Six of the trials observed clinically and statistically significant reductions in childhood mortality, ranging from 19% to 54%. However, the mechanism of this benefit is still not very clear (Sommer *et al.*, 2008).

Due to its immunological functions mediated by retinoic acid, Vitamin A could be useful in improving the response of oral vaccines. Vitamin A is oxidized to four isoforms of retinoic acid (RA); these include all-*trans* RA (ATRA), 9-*cis* RA, 13-cis RA, and 11-*cis* RA. The retinoic acids have important immunological functions especially on intestinal defence and mucosal immune responses including inhibition of activation-induced cell death in thymocytes and T cells (Iwata *et al.*, 1992, Yang *et al.*, 1993). Most of these immunological functions depend on ATRA and 9-*cis* RA. RA mediates various processes of the immune system including proliferating and differentiation, regulation of apoptosis and alteration of regulation of genes

relevant to the immune response (Ruhl, 2005).For instance, ATRA and 9-*cis* RA have been implicated in enhancing the imprinting of T cells with gut homing phenotype α4β7 integrin and chemokine receptor CCR9, which is required for the rapid migration of the effector T cells to the site of infection (Iwata *et al*., 2004; Hammerschmidt *et al*., 2011). RA is also known to enhance synthesis of IgA in the gut in the presence of IL-5 or IL-6. ATRA secreted by dendritic cells contributes to programming regulatory T cells and Th17 cells. Retinoic acid is also known to affect expression of the polymeric immunoglobulin receptor (pIgR) which transports polymeric immunoglobulin A (pIgA) into external secretions as secretory IgA (S-IgA) which is critical for the defence of mucosal tissues. A study on the human colonic adenocarcinoma cell line, HT-29, data showed that ATRA-treated HT-29 cells expressing pIgR showed a significantly high expression of pIgR in the presence of IL-4 and/or IFN-ɣ, compared to ATRA-untreated cells, suggesting that vitamin A may be required for the proper regulation of IgA transport in response to mucosal infection (Takenouchi et al., 2004).

These are all highly desirable characteristics of an orally active adjuvant as previously defined (Pulendran *et al*., 2011; Reed *et al*., 2013). In a recent study (Lisulo *et al*., 2013), designed to test the hypothesis that all-trans retinoic acid (ATRA) enhances gut immunoglobulin A (IgA) in response to an oral typhoid vaccine in Zambian adults, the authors found that ATRA could enhance gut IgA responses to Vivotif typhoid vaccine. We measured IgA directed against lipopolysaccharide (LPS) and protein preparations of the typhoid vaccine in whole gut fluid and serum and found that ATRA enhanced specific responses to LPS (P=0.02) and protein (P=0.04) in the gut but not in serum. In order to turn this promising observation into a useful public health tool, it was important to determine

if the ATRA adjuvant effect could be generalized to other vaccines, how it works and how little is required to achieve the adjuvant effect earlier observed.

1.1 Statement of the problem

There is evidence that oral vaccines are not as efficacious in developing countries as they are in developed countries as evidenced by rotavirus vaccine (Vesikari et al., 2006, Madhi et al., 2010, Levine et al., 2010); oral polio vaccine (Grassly et al 2006; 2010) and oral typhoid vaccine (Levine et al., 2010). Rotavirus kills half a million children each year worldwide including approximately 230,000 children in sub-Saharan Africa making it the most significant contributor to diarrhoea-related child deaths worldwide (Parashar et al., 2006; Parashar et al.,2009; WHO 2007)

1.2 Study Justification

In view of the impaired efficacy of oral vaccines in developing countries where they are most needed to work effectively, there is an urgent need to identify ways to improve the immune response of these oral vaccines. I have proposed that the use of an oral adjuvant (ATRA) could have a dramatic impact on vaccination programs.

1.3 Research Question

Can giving oral vaccination alongside ATRA improve the immune response to oral vaccines by enhancing expression of IgA and gut homing molecules?

1.4 Aim

To determine whether the findings of the preliminary study (Lisulo et al., 2013) would have general applicability to other oral vaccines and to also investigate the immunological mechanisms of action of ATRA

1.5 Objectives

- *In vivo* analysis of the effects of ATRA on lymphocyte gut homing marker expression.
- To investigate the effects of ATRA on specific IgA response in WGLF and serum pre and post oral vaccination.
- To investigate whether the effect of ATRA is dependent on vitamin A status

CHAPTER 2

LITERATURE REVIEW

2.1 Global Diarrhoea Burden

Diarrhoea kills more children than malaria, measles and AIDS combined, with just over 2195 children said to die from diarrhoea every day (Liu et al., 2012). This number accounts for 11% (Figure 2-1) of child deaths worldwide annually, making diarrhoea the second leading cause of death among children under the age of 5. A multi-centre study (You et al., 2010) done in seven regions in sub-Saharan Africa and south Asia where over 80% of deaths in children younger than 5 years occurs revealed that of the regions studied, Mozambique reported the highest diarrhoea-related mortality in children. The authors also found that the putative pathogens that contributed to the cause of diarrhoea were rotavirus, *Cryptosporidium* heat stable Enterotoxigenic E. *coli* (ST-ETEC) and Shigella. Rotavirus is responsible for at least 36% of all diarrhoea admissions and for an estimated 37% of diarrhoea deaths translating to 453,000 deaths in children <5 years of age across the world (Tate et al., 2012).

Despite all advances in health technology, improved management, and increased use of oral rehydration therapy (ORT) in the past decades, diarrheal diseases continue to be an important cause of morbidity and mortality worldwide, and they remain among the five major killers of children under five years of age (Boschi-Pinto et al 2006).

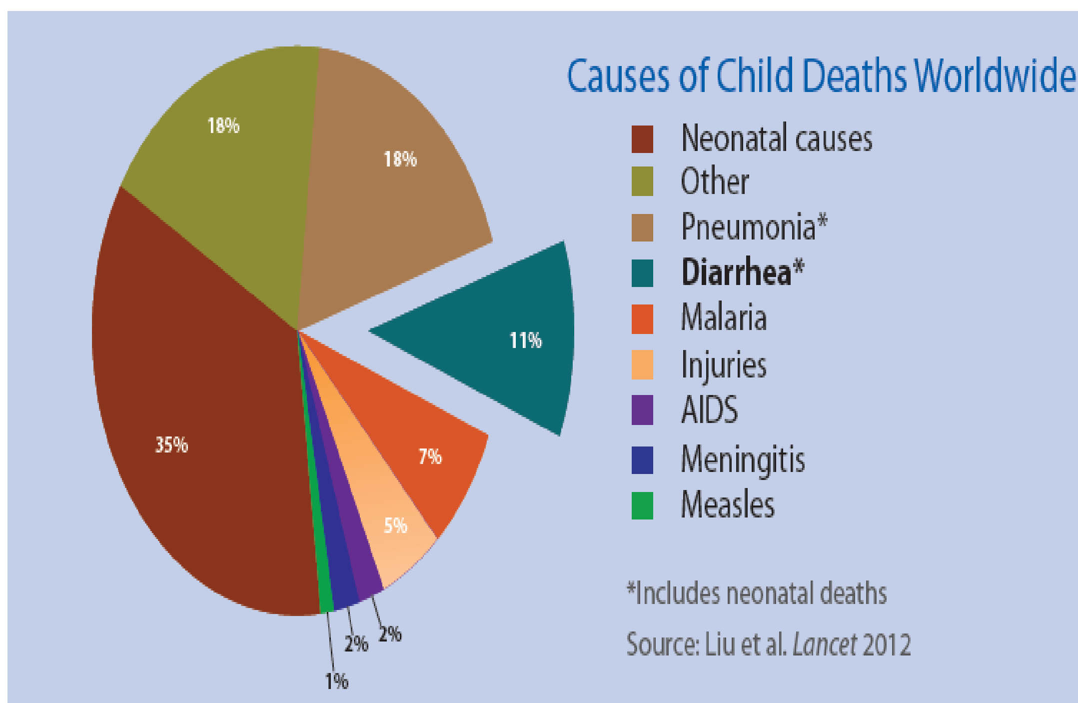


Figure 2-1 Global causes of childhood deaths worldwide

2.1.1 **Diarrhoeal burden in Africa**

Advances in clean water and safe sanitation plus the availability of antibiotics, adequate nutrition (in some parts of the country), the introduction of rotavirus vaccination (Chilengi *et al*, 2015), increase in sensitization on the benefits of exclusive breast-feeding in the first six months of an infant's life and oral rehydration therapy has seen a dramatic reduction in mortality due to diarrhoeal related disease in children in the tropics (Gracey *et al*, 1997, Boschi-Pinto et al 2009). However, this is not enough as many children are still dying from diarrhoeal disease.

In Zambia, diarrhoea is the third largest cause of mortality in children under 5 years after pneumonia and malaria (Ministry of Health Zambia, 2012 report). An estimated 15000 of Zambia's 2.4 million children experience an average of 3-4 episodes of diarrhoea every year (Ministry of Health Zambia, 2012 report). This – despite improvements made in management of acute diarrhoeal diseases i.e. ORT, zinc

therapy given during an acute diarrhoeal episode and addition of vitamin A to the expanded program for immunisation (EPI). Mpabalwani et al., 1995 showed that rotavirus was the leading cause of infantile (children <1year old) diarrhoea (37%) in Zambia. The authors also showed that in the cohort studied, the mortality cases recorded were only children less than 2 years old. This is evidence that there is still more improvement needed in this area.

2.2 **Vaccines**

Vaccines are among the most promising interventions to diminish the burden of specific infections in populations in developing countries (Levine *et al*., 2010). A number of existing vaccines and newer vaccines include those against conjugate pneumococcus, rotavirus, hepatitis A, HPV and meningitis have contributed significantly to the reduction of the mortality and morbidity burdens of many infectious diseases (Del Giudice *et al*. 2003). Each year, 130 million babies are born and of these, an estimated 23% do not have access to vaccinations (Ehreth *et al*, 2003) including 7.8 million in sub-Saharan Africa. This leads to a large number of them dying from vaccine-preventable diseases (Table 2-1).

Table 2-1 Death from Vaccine Preventable Disease - WHO Global Immunization Report 2008

Disease	All age groups	Children <5 yrs
Pneumococal disease	1612000	716000
Measles	242000	200000
Hepatitis B and others	600000	
Rotavirus		449000
Haemophilus influenza b		386000
Pertussis		294000
Tetanus	213000	180000
Meningogoccal disease	26000	10000
Other	36000	19000

2.2.1 **Importance of vaccines**

Global immunizations have reported a number of success stories, with the worldwide eradication of smallpox being at the top. With this triumph and many more diseases to still combat, the World Health Organisation (WHO) launched the Expanded Program on Immunisation (EPI) to upscale vaccination programs that would help eradicate still more vaccine preventable diseases. The EPI vaccines prevent approximately 2.5 million child deaths from measles, pertussis, tetanus and diphtheria, poliomyelitis and tuberculosis.

Ehreth in 2003 reported (Table 2-2) the benefits of vaccination against a number of diseases in terms of annual life years saved (LYS) and disability-adjusted life years (DALY) saved.

Table 2-2 Benefits of disease eradication and control by vaccination, in terms of annual life years saved (LYS) and disability-adjusted life years (DALYs) saved

Disease	**US**		**AFRICA**		**GLOBAL**	
	LYS	**DALY**	**LYS**	**DALY**	**LYS**	**DALY**
Smallpox	1,685,740	NA	933	NA	5,000,000	NA
Polio	212,690	NA	484,230	279,000	35,750,000	1,725,000
Measles	5,811,852	26	2,125,500	17,463,000	71,500,000	29,838,000
Tetanus	42,705	9	2,801,500	3,039,000	56,030,000	12,020,000

NA: not available

Although there have been these successes with vaccines, many infectious diseases still remain important causes of morbidity and mortality especially in developing nations. While many infectious diseases are controlled by immunizations, this has proved a challenge for diarrhoeal diseases. Two reasons for this: firstly the diversity of enteric pathogens and secondly phenomena related to tolerance of luminal antigens. A study in Dhaka, Bangladeshi (Taniuchi et al., 2013) revealed that the

average child in this cohort had between two and four enteropathogens at any one time even as early as the first month of life when breast-feeding was exclusive. These data were contrary to what was found in the infants from the USA who had far less than one enteropathogen detected at any given time.

Oral mucosal vaccines have been developed to help reduce mucosal and gastrointestinal infections. Unfortunately, these vaccines are neither equally immunogenic nor protective in different populations (Qadri *et al*.2013). Data from various clinical trials (Table 2-3) have shown diminished immune response or lower efficacy of oral vaccines in populations from developing countries than from industrialised countries and this low vaccine efficacy is recorded in all age groups from infants to adults (Levine et al., 2010). Levine further points out that oral vaccines implicated include viral and bacterial, both live and non-living.

Table 2-3 Oral vaccines associated with diminished immunogenicity or efficacy in developing country populations

Oral vaccine	**Target ages at which diminished immunogenicity or protection was observed**	**Geographic locations where observed**
Sabin polio vaccine strains	Infants, toddlers, preschool children, school-age children	India, sub-Saharan Africa
RIT 4237 rotavirus	Infants	Gambia
Rotashield rotavirus vaccine (10^4plaque forming unit dosage)	Infants	Brazil and Peru
Rotarix attenuated rotavirus	Infants	Malawi, South Africa, Bangladesh
Rotateq pentavalent attenuated rotavirus	Infants	Ghana, Kenya, Mali
MMU18006 (monovalent Rhesus rotavirus strain)	Infants	Pakistan
CVD 103-HgR live cholera strain	24-59 months; 5-9 years; adults	Indonesia, Thailand, Peru, Ecuador
Dukoral non-living cholera vaccine (killed *V. cholerae* O1 plus B subunit)	1-12 years	Nicaragua
SC602 attenuated *Shigella flexneri* strain	Toddlers and school age children	Bangladesh

From Levine *et.al.,* 2010

2.2.2 **How vaccines work**

Vaccines are designed based on the target microbe, how it infects the cell and what immune response it will trigger (Table 2-4).

Table 2-4 Types of vaccines and their immune responses

Vaccine	**type**	**description**	**Immune response**
Rotarix	Live attenuated	Contain weakened part of microbe	Elicit strong cellular and antibody response
Dukoral	Inactivated	Contain heat or chemical killed microbe + CTB	Stimulate weakened immune response
Hepatitis B vaccine	Subunit	Include only the particulate antigen that best stimulates an immune response	
Diphtheria and tetanus vaccines	Toxoid	Detoxified toxin e.g formalin	Antibodies lock onto and block the toxins

www.niaid.nih.gov/topics/vaccines/2013

When the vaccine is administered, it will not cause sickness but will cause the immune system to respond by producing T and B lymphocytes. The T lymphocytes secrete chemokines that will activate killer T cells that help clear off the infection. T lymphocytes also work closely with the B lymphocytes which secrete antibodies that are also involved in clearing the infection. Once the infection is cleared, the T and B lymphocytes differentiate into memory cells which will recognize and fight off in case of re-exposure to the infectious agent.

Vaccines confer direct protection to the individual and in cases where the immunization coverage in a population is high, people who are not immunized may well be protected indirectly by what is called ‘’herd immunity’’ (Levine, 2010). In ‘’herd immunity’’, the natural reservoir of infected individuals in that population is lowered and so reduces the probability of transmission of infection (Murphy K, 2012).

2.2.3 Oral Vaccines

There are many different types of vaccines available. However, this study focuses on oral vaccines. Oral vaccines can elicit mucosal secretory IgA that is responsible for the prevention of attachment and invasion of antigen and also neutralizes enterotoxins. They can also elicit serum IgG antibodies that are involved in controlling both mucosal and systemic invasive pathogen. They are also able to elicit an array of cell mediated immune responses against cellular bacteria and virus (Pasetti *et al.,* 2011).

There are a number of commercially available vaccines applied parenterally that are efficient in preventing systemic infectious diseases. However this route of vaccination produces weak protection against enteropathogens. Possible reasons could be that the effector T cells and primed B cells induced by the antigen applied either subcutaneously, intramuscularly or intravenously, fail to express gut-homing molecules (Hammerschmidt *et al*, 2011) and thereby the protective shield in the mucosa is not fully induced. Oral vaccination strategy on the other hand generates far more mucosal protection than systemic immunization.

Oral vaccines represent the biggest challenge for vaccine development. Two reasons for this, firstly, the gut environment is quite harsh degrading most antigenic epitopes delivered in soluble form, and secondly mucosal tolerance protects against unwanted immune responses to digested antigens (Lycke, 2012). Notwithstanding these obstacles, a number of oral vaccines developed have been successful.

Commercially available oral vaccines (Table 2-3) include Vivotif a typhoid vaccine, Dukoral for cholera (orochol is also a cholera vaccine although it is no longer commercially available), polio vaccines and the rotavirus vaccine Rotarix. This

thesis discusses four (Vivotif, Rotarix, Dukoral and polio) of the available oral vaccines and the effect ATRA would have on improving their efficacies.

Table 2-5: Commercially available oral vaccines. An indication of percentage efficacy is given

Vaccine Type	Disease	Vaccine constituents	Route	Protection	Commercial name
Live attenuated	Typhoid	*S. typhi* (Ty21a,)	oral	67% over 3yrs	Vivotif
	Cholera	*V. cholera* (CVD103-HgR,	oral	80-90%	Orochol
	poliomyelitis	Attenuated virus	Oral	>95%	Sabin
	Rotavirus	attenuated virus	oral	85-100%	Rotarix
	Rotavirus	human-bovine reassortant viruses	oral	74%	RotaTeq
Inactivated	Cholera	heat-killed V.cholerae + CTB	oral	80-90% over 6months, 60% at 2yrs	Dukoral
Toxoid	Typhoid	Vi capsular polysaccharide	IM	69-77%	Typhim Vi

2.2.3.1 **Vivotif**

Typhoid fever caused by bacterium, *Salmonella enteric* serovar Typhi (S. Typhi) is an important cause of enteric disease in children. It remains a significant health burden especially in low-and middle- income countries. The World Health Organization (WHO) estimated that 2.6 million cases of typhoid occurred worldwide annually, leading to 216,000 deaths with the most deaths being reported in Asia. Aside from this, WHO ranked Southern Africa as a high incidence area (Crump 2004) although data from most African regions was scanty. In 2006, the Ministry of Health (MOH) in Zambia recorded 2141 typhoid fever cases with 292 of these cases being children under 5 years of age (Crump et al., 2010).Recent global estimates of

typhoid fever have remained fairly static at between 11.9 and 13.5 million episodes as of 2010 (Buckle et al., 2012, Mogasale et al., 2014, Wain et al., 2015).Despite the availability of the more recent data on both enteric fevers, additional research is needed in many regions particularly Africa, Latin America and other developing countries(Buckle et al., 2012). Two vaccines are available for the prevention of typhoid fever. Oral Ty21a vaccine (Vivotif Berna) and the parenteral Vi vaccine (Typhim-Vi). In this study, Ty21a was used as it is a live vaccine.

Oral typhoid vaccine, Ty21a was developed by mutagenesis of the wild-type *Salmonella enteric* serovar Typhi (hereafter referred to as *S. typhi*), strain Ty21a.This was the first live oral attenuated whole cell *Salmonella* vaccine containing all other typhoidal structures but lacking both functional galactose-epimerase (*gal*E) gene and the Vi antigen. This vaccine has been licensed in 56 countries of Asia, Africa, USA and Europe (Marathe et al., 2012). Vivotif used in this study was supplied as a dose of 3 capsules taken every other day. Each capsule contained at least $2x10^9$ live S. *typhi.* Other ingredients included lactose, sucrose, amino-acid peptide mixture (Hy-Case SF), ascorbic acid (E300), and magnesium stearate (E470) and inactivated *S. typhi*. The vaccine capsule contained gelatin, titanium dioxide (E171), erythrosine red No.3 (E127) and ferric oxide (E172). The capsule coating contained hydroxypropylmethyl-cellulose-phthalate (HP-MCP)-50, ethylene glycol, dibutyl phthalate and diethyl phthalate.

2.2.3.2 **Rotarix**

Rotavirus is the leading cause of diarrhoeal morbidity and mortality in children under 5 years in developing countries in Africa and Asia. Severe acute diarrhea due to rotavirus is estimated to cause approximately 527,000 deaths globally each year

among children less than 5 years of age (Parashar et al., 2009) with half of them occurring in Africa (Mwenda et al., 2010). Due to the high numbers of cases of rotavirus disease, the World Health Organisation (WHO) has recommended the inclusion of rotavirus vaccines into the EPI, national immunization programmes. Two oral rotavirus vaccines have been made available worldwide, a monovalent human rotavirus vaccine (Rotarix, GlaxoSmithkline Biologicals) and a pentavalent bovine-human reasortment rotavirus vaccine (RotaTeq, Merck Vaccines). The live attenuated oral Rotavirus vaccine Rotarix was approved in the USA in 2008 and has only recently been introduced in Zambia in 2012 (Chilengi et al., 2015). Rotarix contains the RIX4414 rotavirus strain developed from attenuation of a human strain (G1P). In this study each participant received 1ml dose that contained 10^6 $CCID_{50}$ (10 million infectious rotavirus particles are in the 50% cell culture infective dose) of live attenuated rotavirus.

2.2.3.3 **Dukoral**

Cholera is a diarrhoeal disease caused by *Vibrio cholerae 01 and 0139*. This bacterium causes high volume of watery diarrhoea which can be fatal in both adults and children. The global burden of cholera remains high with sub-Saharan Africa accounting for the majority of this burden. There are an estimated 2.9 million cases of cholera reported annually in endemic countries (Figure 2-2) with about 95 000 deaths annually (Ali et al., 2015).

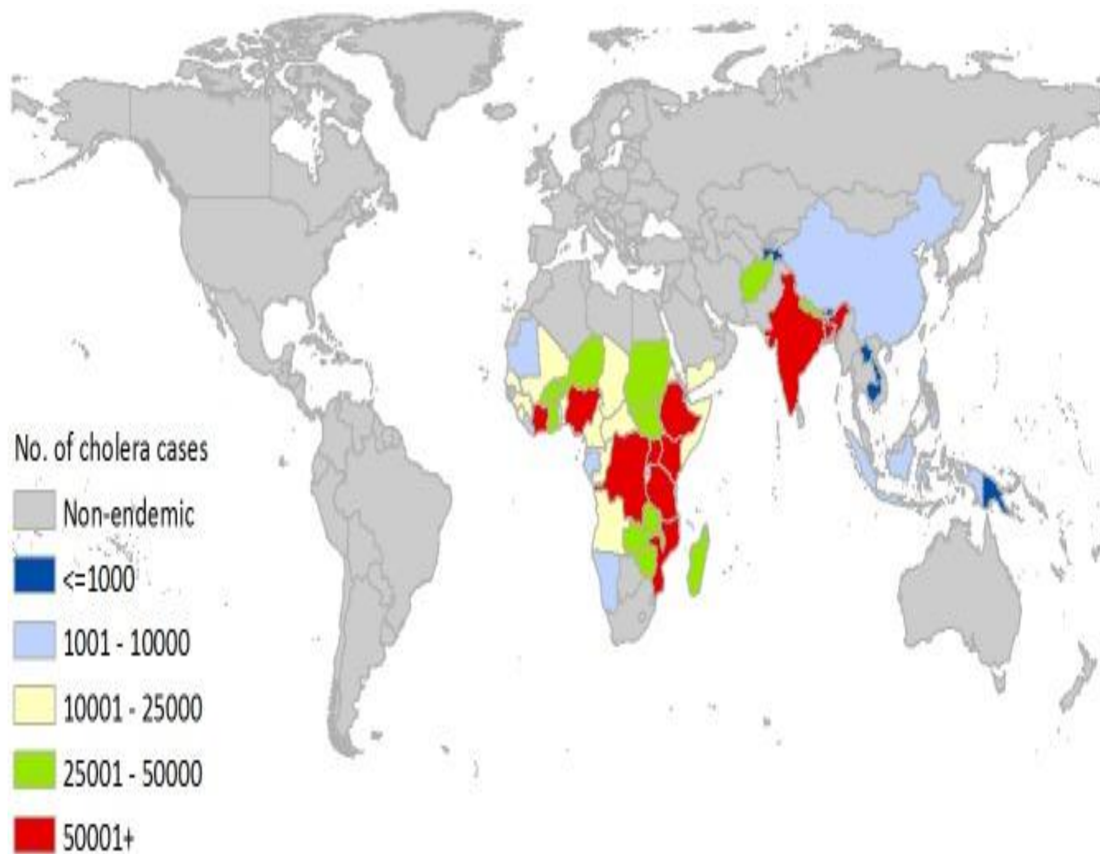


Figure 2-2 Annual number of cholera cases in endemic countries (Ali et al., 2015)

Vibrio cholerae causes diarrhea by producing several toxins and most importantly cholera toxin (CT) subunits A and B. The toxic action of CT is initiated by its binding of the B-subunit to high affinity monosialoganglioside GM1 receptors this leads to cellular signaling disruption and chloride ion secretion (Bharati et al., 2011). The main strategies to prevent and control cholera have been to promote hygiene and to ensure safe water and sanitation is made available to populations at risk. This however has not always been achieved and as a result additional strategies have been implemented through the introduction of cholera vaccines (Martin et al., 2014). Of the internationally licensed oral cholera vaccines, the most widely used vaccine Dukoral (SBL, Vaccines, Sweden), consists of a mixture rCTB and heat or formalin-inactivated V. cholerae 01 whole cells ofdifferent serotypes and biotypes (Bharati et al., 2011).

Primary Dukoral vaccination is 2 doses taken with an interval of 1 to 6 weeks. Participants in this study received 1 dose of the vaccine. Active substances in the vaccine include 31.25×10^{9} bacteria (bacterial content prior to inactivation) of the following Vibrio *cholerae* O1 strains: Inaba classical biotype (heat inactivated), Inaba E1 Tor biotype (formalin inactivated), Ogawa classical biotype (heat inactivated) and Ogawa classical biotype (formalin inactivated). Also present is 1 mg of the recombinant cholera toxin B subunit (rCTB). Other ingredients include sodium di-hydrogen phosphate, disodium hydrogen phosphate and sodium chloride.

2.2.3.4 **Oral Polio Vaccine (OPV)**

Poliomyelitis is an infectious disease caused by one of 3 related enteroviruses (P1, P2 and P3). The virus enters through the oral fecal route, multiplies in the intestine and invades local lymphoid tissues and passes into the blood stream and may infect cells of the central nervous system. Several milestones in polio eradication effort have now been reached. In 1988 when the WHO began the campaign for a polio free world, over 350,000 cases had been reported and 125 countries were considered endemic. In 2014 the number of cases reported had dropped tremendously to only 39 cases detected in two countries (Garon, 2015) and only Afghanistan, Nigeria and Pakistan considered still endemic (Lycke 2014). The Global Polio Eradication Initiative (GPEI) in November 2015 reported 16 and 41 wild poliovirus cases from Afganistan and Pakistan respectively and all were wild poliovirus type 1(WPV1). No polio cases caused by WPV2 have been identified since 1999. In September 2015, WPV2 was declared eradicated and Nigeria removed from the list of polio endemic countries. All this has been achieved through the widespread use of trivalent oral polio vaccine (tOPV). This vaccine was first developed in 1961 by Albert Sabin and

in 2006, WHO issued a statement to affirm its quality and safety. It contains type 1, 2 and 3 live attenuated polioviruses. The only challenge is that there is competition among the 3 viruses to cause immunity resulting in protection but unequal efficacy for each type. OPV has been the vaccine of choice for GPEI because of its ease of administration in mass campaigns, low cost, and ability to induce strong intestinal mucosal immunity against poliovirus shedding and transmission (John et al., 2014) although this response wanes substantially within a year of vaccination (Grassly et al 2012). Therefore, although vaccinees may mount a neutralizing antibody response to the 3 poliovirus subtypes, they may still be susceptible to infection and transmit wild poliovirus (Grassly et al., 2010, Mach et al., 2009).

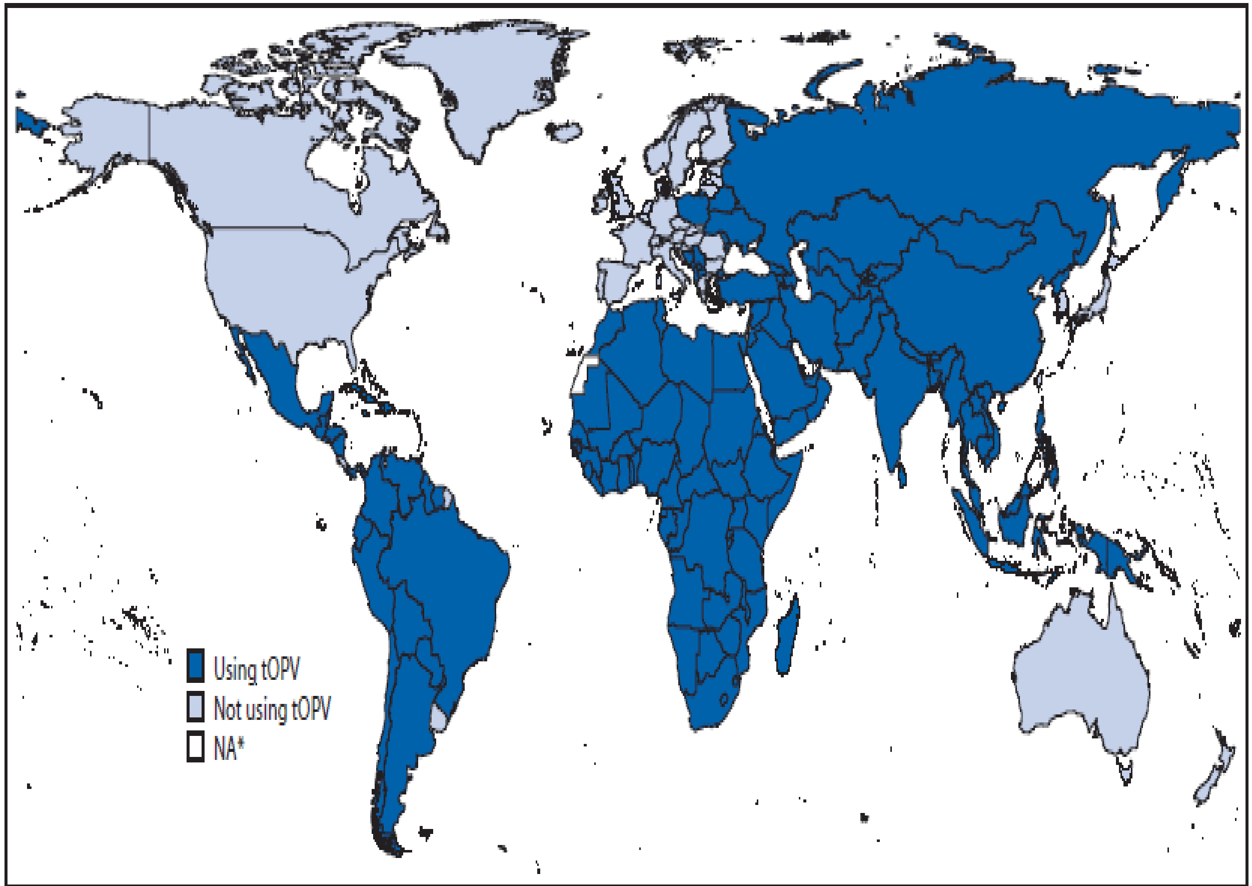


Figure 2-3 Status of trivalent oral poliovirus vaccine use by country

Continued use of tOPV has been found to cause circulating vaccine-derived polioviruses (cVDPV) which are genetically divergent and neurovirulent and capable of causing paralytic poliomyelitis (Diop et al., 2015). The majority of cVDPV case isolates are type 2 accounting for over 97% of all VDPV cases reported in 2006 (Diop et al., 2006). In order to reduce or eliminate the risks of VDVP, WHO has recommended the withdrawal of tOPV by April 2016 and replacement with bivalent OPV (b OPV) which only has type 1 and 3 polioviruses. Removing the type 2 component of tOPV reduces the risk of type 2 VDPVs.

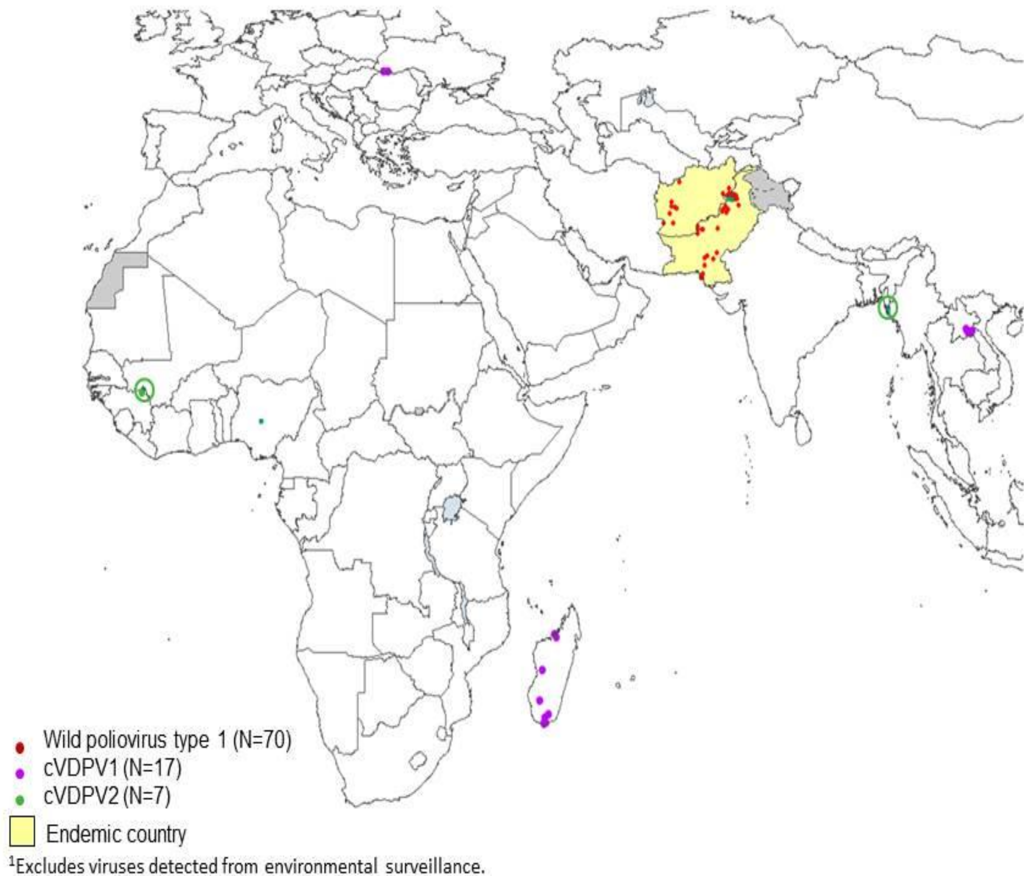


Figure 2-4 Wild poliovirus and cVDPV cases 2015

The GPEI has also recommended that at least one dose of injectable inactivated poliovirus vaccine (IPV) followed by OPV be introduced in routine immunizations worldwide. A number of countries have since introduced the vaccine into their schedules. Zambia intends to introduce the vaccine in the 1st quarter of 2016. IPV is effective in preventing VDPV and has been shown to be efficacious in both industralized and developing countries (Okayasu et al., 2011). Although IPV alone does not induce an effective mucosal immune response, some studies demonstrate that adults who received OPV as children show increases in poliovirus-specific IgA and memory CD4+ T cells expressing the gut homing integrin α4β7 after

administration of IPV (Herremans et al., 1999, Krieg et al., 2004). This finding indicates that IPV could be used as a complement to OPV by boosting intestinal immunity which may have waned, hence the recommendation by GPEI.

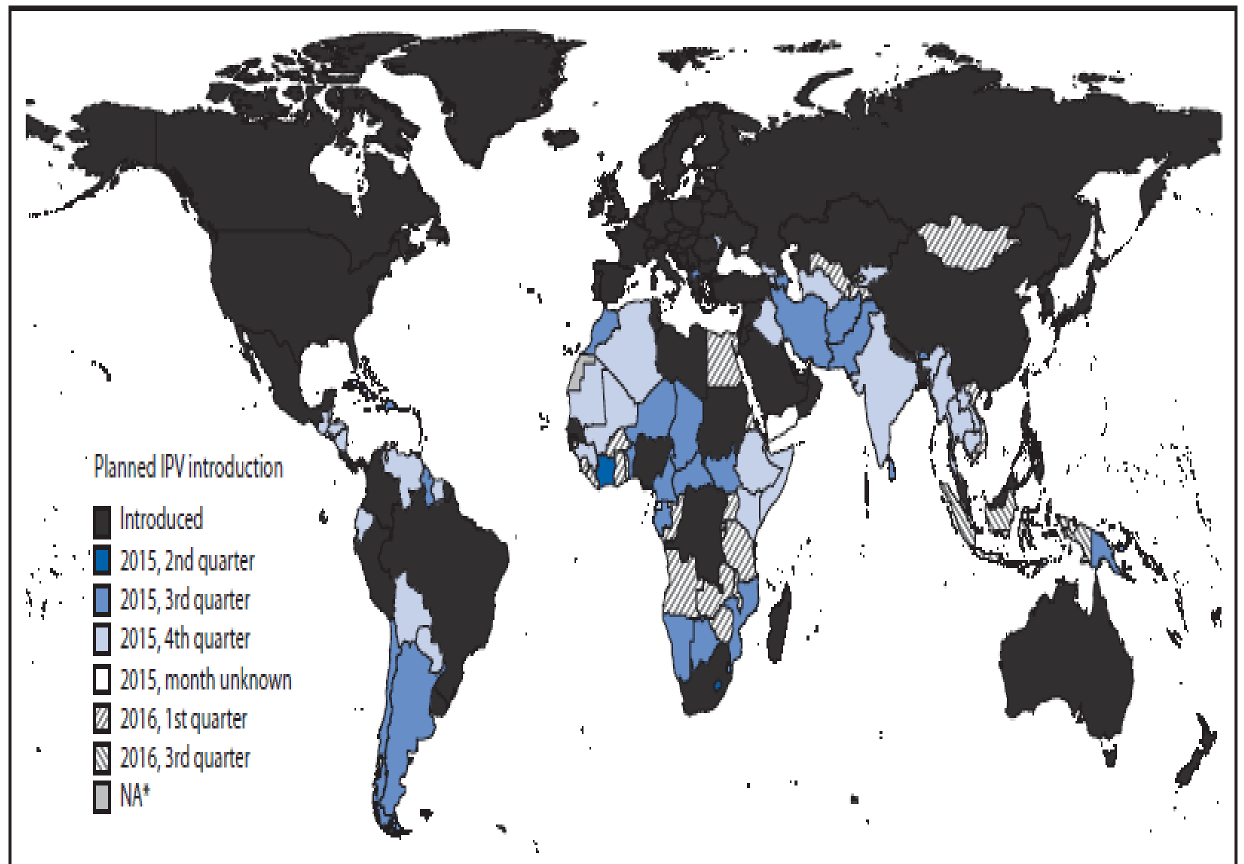


Figure 2-5 Status of introduction of inactivated poliovirus vaccine, by country-worldwide

This study used the tOPV to investigate the effect of ATRA on immune responses to this particular vaccine. Each participant received 2 drops (0.1ml) of the vaccination directly into the mouth alongside ATRA or without ATRA. The active substances in 0.1ml of OPV are: 106 infectious particles of LS-c2ab strain of Type 1 poliovirus of 50% cell culture infective dose; 105 infectious particles of P712, Ch, 2ab strain Type 2 poliovirus of 50% cell culture infective dose and 105.8 infectious particles of Leon 12a1b strain Type 3 poliovirus of 50% cell culture infective dose. at least 105.8.

2.3 **Problems with vaccine efficacy globally and in Africa**

Although oral vaccines are available, they have shown high efficacy in industrialised countries but much lower efficacy in developing countries (Levine *et al*, 2010). This is confirmed for rotavirus, cholera and may also be true of poliovirus. Oral rotavirus (RV) vaccine is 78% effective against severe RV diarrhoea in Finland (Vesikari *et al*, 2006, Ruiz-Palacios *et al*, 2006), but was only 35% effective in Malawi (Madhi *et al*, 2010). In another study, the live cholera vaccine CVD 103-HgR elicited significant (four-fold or greater) rise in serum vibriocidal antibody in America adults however when the same vaccine was given to Indonesian children, it demonstrated diminished immunogenicity (Levine *et al*., 2010, Sack *et al*., 2008). As mentioned earlier, reasons for these low efficacies being reported are not clear.

2.4 **Adjuvants**

Immunisation with purified protein antigens typically elicits a modest antibody response with little or no T cell response (Reed *et al*., 2013). This has resulted in vaccine developers seeking the inclusion of adjuvants in vaccine candidates to enhance the efficacy of weak antigens. Adjuvants are components used together with vaccines to help stimulate protective immunity based on antibodies and effector T cell functions (Coffman *et al*., 2010). These adjuvants provide help needed to enhance the immunogenicity of vaccine antigens. In addition, adjuvants can enable the use of lower vaccine doses in instances where multiple immunisations may be required to elicit sufficient antibody responses. For instance, the recombinant influenza vaccine that has been developed requires high doses because of its low immunogenicity. Adjuvants can also be employed to optimise a desired immune response, for example with respect to immunoglobulin classes and induction of cytotoxic or helper T lymphocyte responses. Some antigens may also be used to

promote antibody responses at mucosal surfaces. The essential roles of adjuvants are summarised below:

- Dose sparing
- Helps to confer a more rapid immune response
- Helps to broaden antibody immune response profile
- Induce a greater magnitude and functionality of antibody responses
- Promotes effective T cell responses by either optimising the quality and durability of antibody responses or inducing effector $CD4^+$ or $CD8^+$ T cells to kill intracellular pathogens

Adjuvants can be grouped into several classes depending on their application. There are 3 classes:

1. Immunomodulatory molecules (IM): this class of adjuvants includes ligands that directly activate innate immune receptors. They target receptors on antigen presenting cells which stimulate signaling that leads to the activation of key transcription factors.
2. Delivery systems: Adjuvants that fall in this category are responsible for the effective delivery of vaccine antigens and or immunomodulatory molecules.
3. Combination systems: These are formulation systems that include both delivery and immunomodulatory components.

Table 2-6: Classes of clinically used and tested adjuvants

Adjuvant Name	Class	Mechanism or receptor	Type of immune response
dsRNA analogues eg poly(I:C)	IM	TLR3	Ab, T_H1, $CD8^+$ T cells
Lipid A analogues (MPL)	IM	TLR4	Ab, T_H1
Flagellin	IM	TLR5	Ab, T_H1, T_H2
Imidazoquinolines (Imiquimod)	IM	TLR7, TLR8	Ab, T_H1
CpG ODN	IM	TLR9	Ab, T_H1, $CD8^+$ T cells
Saponins (QS21)	IM	unknown	Ab, T_H1, T_H2, $CD8^+$ T cells
C-type lectin (TDB)	IM	Mincle, Nalp3	Ab, T_H1, T_H17
CD1d ligands (α-galactosylceramide)	IM	CD1d	Ab, T_H1, T_H2, $CD8^+$ NK T cells
Aluminium salts	PF	Nalp3, ITAM, Ag delivery	Ab, T_H2
Emulsions (MF59, AS03, AF03)	PF	Immune cell recruitment, ASC, Ag uptake	Ab, T_H1, T_H2
Virosomes	PF	Ag delivery	Ab, T_H1, T_H2
ISCOMs (saponin, phospholipid)	C	unknown	Ab, T_H1, T_H2, $CD8^+$ T cells
AS01 (MPL, QS21,liposomes)	C	TLR4	Ab, T_H1, $CD8^+$ T cells
AS04 (MPL, aluminium salt)	C	TLR4	Ab, T_H1

Ab, antibody; IM, immunomodulatory molecule; PF, particulate formulation; C, combination of immunomodulatory molecule and particulate formulation.

2.4.1 **Adjuvant Selection**

Adjuvant selection is based on a number of parameters including the nature of the vaccine, the immune response desired, target population and the route of vaccine administration (Reed *et al*., 2013). Recent evidence suggests that adjuvants engage components of the innate immune system and do not directly affect the T and B cells (Coffman *et al*., 2010; McKee *et al*., 2010; O'Hagan and De Gregorio, 2009). There are two uses for adjuvants firstly to increase the mean antibody titers thus increasing the response of the vaccine in the general population and this is the main focus of my work; secondly adjuvants are used to increase seroconversion rates where there is reduced responsiveness.

Adjuvants can also alter the immune response in a qualitative manner. Adjuvants help to provide functionally appropriate types of immune responses; increase the generation of memory; increase the spread of initial response and alter the breadth specificity and affinity of the response (Coffman *et al*., 2010).

2.4.2 Oral Adjuvants

Vaccination through mucosal routes requires potent adjuvants to enhance immunogenicity. There are several mucosal adjuvants available with the major category being that of Toll-like Receptor agonists. This category of adjuvants is based on pathogen-associated molecular patterns (PAMPs) often formulated in oil-in-water emulsions (Lycke, 2012). Other mucosal adjuvants fall under immunomodulators like the bacterial enterotoxins- cholera toxin and *E. coli* toxin. Another group is the Immunostimulating complexes (ISCOMs) that form from a mixture of cholesterol phospholipids and Quillajasaponins these are known to target dendritic cells (Lycke, 2012). The focus of my work will be all-*trans* retinoic acid which in preliminary experiments shows promise as an oral adjuvant.

2.5 Vitamin A

Vitamin A is a fat-soluble vitamin that is essential for humans and other vertebrates. Vitamin A comprises a family of molecules containing a 20 carbon structure with a methyl substituted cyclohexenyl ring (beta-ionone ring) and a tetraene side chain with a hydroxyl group (retinol), aldehyde group (retinal), carboxylic acid group (retinoic acid), or ester group (retinyl ester) at carbon-15.

The term vitamin A includes any molecule which can cure vitamin A deficiency and pro-vitamin A carotenoids that are dietary precursors of retinol. The term retinoid refers to retinol, its metabolites, and synthetic analogues that have a similar structure. Carotenoids are poly-isoprenoids, of which more than 600 forms exist. The all-*trans* isomer is the most common and stable form of each carotenoid; however, many *cis* isomers also exist. Carotenoids usually contain 40 carbon atoms, have an extensive system of conjugated double bonds, and contain one or two cyclic structures at the end of their conjugated chain. An exception is lycopene, which has no ring structure and does not have vitamin A activity. Preformed vitamin A is found only in animal-derived food products, whereas dietary carotenoids are present primarily in oils, fruits, and vegetables.

Vitamin A is a valuable component of many biological functions. It is obtained from the diet in form of pro-retinoid carotenoids such as β-carotene. The most abundant form of carotenoids is found in plants. Other forms of vitamin A which are taken up from diet include retinol and retinyl esters plus small amounts of RA.

2.5.1 **Vitamin A supplementation in children**

There is no question that vitamin A is beneficial for child health as shown by data from a number of clinical trials. Vitamin A supplementation appears to have the greatest effects on prevention of severe diarrhoea and treatment of measles. Introduction of vitamin A supplementation into EPI programs in developing countries including Zambia has seen a 27% reduction in deaths from diarrhoea in children under 5 years of age. A study in Gambia (Thurnham et al., 2000) showed that gut integrity measurements were best at the time of year when dietary vitamin A was most abundant and that intervention with vitamin A supplementation accelerated the improvement in gut integrity in sick infants both in the community and those admitted to hospital.

Children with conditions of malnutrition and measles are at high risk of vitamin A deficiency and as such high dose supplements of vitamin A are used as part of their treatment (Sommer et al., 1996). Benn et al., 2011 argued that vitamin A when given with measles vaccine at age 9 months may improve the antibody response to measles vaccines and prolong the period during which children are protected against measles. This however was not the case when vitamin A and vaccine were given at age 6 months when there seemed to be impaired seroconversion. It is assumed that vitamin A exerts its effect on overall mortality by preventing and treating vitamin A deficiency, which would otherwise lead to increased risk of infections and mortality., and that the effect of vitamin A depends on the time of vaccination.

2.5.2 **Metabolism and absorption of vitamin A**

Retinol undergoes enzymatic catabolism to convert it to its metabolically active form retinoic acid. Other forms of the vitamin resulting from this catabolism include the aldehyde form retinal and alcohol form retinol. Several enzymes have been implicated in the process of converting vitamin A to its active form. These include a family of alcohol dehydrogenases (ADHs) and aldehyde dehydrogenase/reductase (RALDH).

Wolf (1984) showed that enzymatic conversion of retinyl esters to retinol occurred in the intestinal lumen before absorption by the enterocytes. He showed that the carotenoids are absorbed and then partially converted to retinol in the enterocytes. He further argued that because the retinoids are highly insoluble, they have to be hydrolysed to ease their uptake into enterocytes where the metabolism continues. In the enterocytes, the retinol is bound to retinol binding proteins. There are three main types binding proteins including retinol-binding protein (RBP), cellular retinol/retinal binding protein (cRBP-I and II) and cellular retinoic acid-binding protein (cRABP). The cRBP bound retinol undergoes re-esterification and the resulting retinyl esters are complexed to the intestinal lipoproteins, chylomicrons (Blomhoff *et al*, 1990). Lecithin: retinol acyl transferase (LRAT) is the enzyme responsible for the conversion of retinol to retinyl ester which is then packed into the chylomicrons and absorbed in the liver and retinyl esters are stored in the liver stellate cells in large cytoplasmic lipid droplets. About 50 to 80% of vitamin A in the body is stored in these cells (Blomhoff *et al*, 1990) (Figure 2-6).

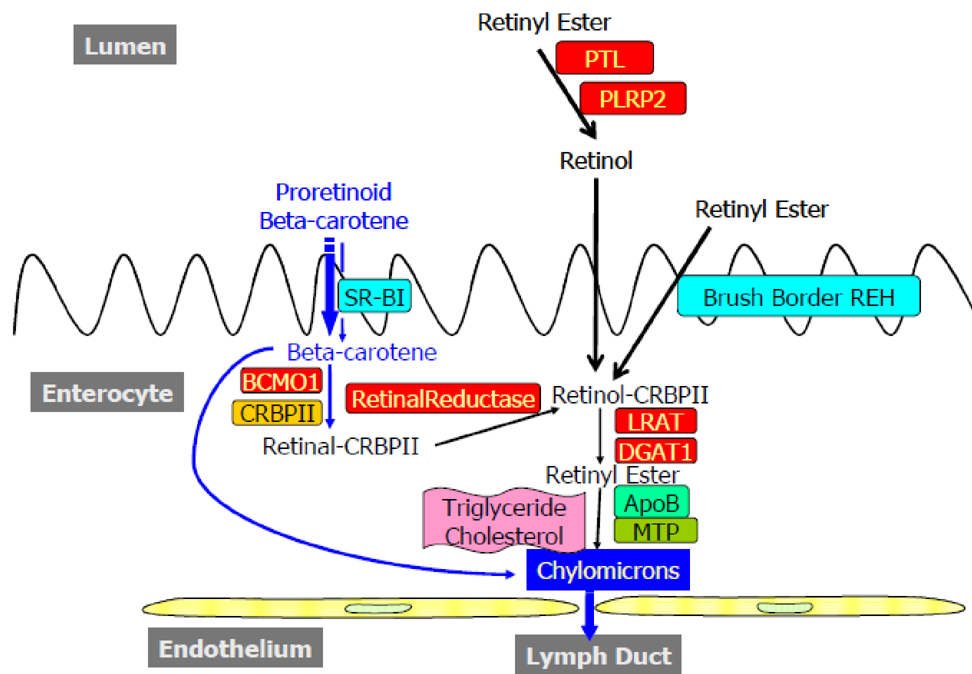


Figure 2-6 Metabolism and absorption of retinyl esters in the intestinal lumen and enterocyte (D'Ambrosio, 2011)

2.5.3 **Retinoids**

Retinoids are a group of nutritionally-relevant compounds present in many foods of plant and animal origin (Ruhl, 2007). They are signaling molecules that are similar in chemical composition to vitamin A (Tang *et al*, 2011). Retinoids contain a β-ionone ring, a polyene side chain, and a polar end group (Tang *et al*, 2011). Of the retinoids, retinoic acid (RA) is known to be the most relevant in mediating various processes in the immune system. It is known to inhibit activation-induced cell death in thymocytes and T cells according to studies done by Iwata *et al*., (1992) and Yang *et al*., (1993).

2.5.4 **Retinoic Acid**

The biological effect of vitamin A at molecular level appears to be largely through retinoic acids (RA). As earlier discussed, there are four major isoforms of retinoic acid. These include 13-*cis*, 11-*cis*, 9-*cis* and all-*trans* retinoic acid (ATRA). Most immunological functions depend on ATRA and 9-*cis* RA. RA mediates various processes of the immune system including proliferating and differentiation, regulation of apoptosis and alteration of regulation of genes relevant to the immune response (Ruhl, 2007).

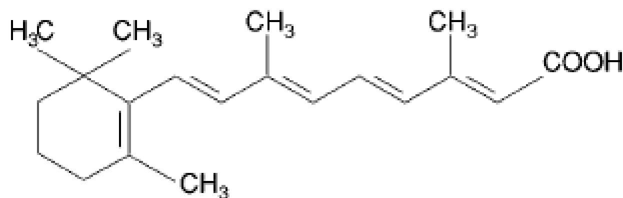


Figure 2-7 Chemical structure of ATRA

2.5.4.1 Therapeutic use of ATRA

ATRA has been used in the therapy of acute promyelocytic leukemia (APL) and significantly improves the outcome of the disease by differentiating leukemic promyelocytes into mature cells. It has been shown to successfully induce differentiation in APL patients (Castaigne et al., 1990, Warrell et al., 1991)

2.6 **Gut Immunity**

The gastrointestinal tract is lined with a thin layer of mucus secreting epithelium. This epithelium is the only physical barrier protecting the gut from pathogens and the many commensal microorganisms present in the gut. The epithelium is therefore part of the mucosal immune system. The main adaptive effector immune mechanisms are the secretory IgA (SIgA) antibodies that prevent the colonisation replication of intestinal pathogens and neutralise viruses and enterotoxins, and T-cell

mediated and antibody-dependent cytotoxic mechanisms which eliminate target host cells infected by intracellular bacteria and viruses (Czerkinsky et al., 2015).

Cells of the mucosal immune system include lymphocytes, dendritic cells (DCs) and macrophages. These cells are found in organised gut-associated lymphoid tissues (GALT) comprising the Payer's patches and the isolated lymphoid follicles. They are also found in connective tissue called lamina propria and are scattered throughout the surface epithelium (Figure 2-8).

The role that mucosal lymphocytes play in gut immunity depends on their location. For instance, those in the organised lymphoid tissues are involved in induction of immune responses while those scattered throughout the intestinal mucosa and in the lamina propria are involved in effector functions.

Two types of cells make up the follicle-associated epithelium that forms the barrier between the gut lumen and the lymphoid tissues, the enterocytes and microfold cells. The microfold cell is a specialised form of cell that has a folded luminal surface through which antigens can enter. It lacks microvilli and mucus layer found on enterocytes. For an antigen to cause stimulation of the mucosal immune system it must first cross the epithelial barrier and it does so by being taken up by the microfold cells through phagocytosis or endocytosis, transported to the basal membrane and through transytosis released to the extracellular space.

The basal membranes of microfold cells contain pockets in which lymphocytes and dendritic cells reside. So, as the antigen is transported from the lumen across the epithelium to the basal membrane, it is taken up by the dendritic cells, processed and presented to the T lymphocytes. The cells then move from the sub epithelial dome to the T cell area of the Payer's patches where they interact with naïve and antigen-specific T cells and cause activation of B cells which involves class switching to

IgA. The following sections describe the immunological components involved in gut immune responses that were investigated in this study.

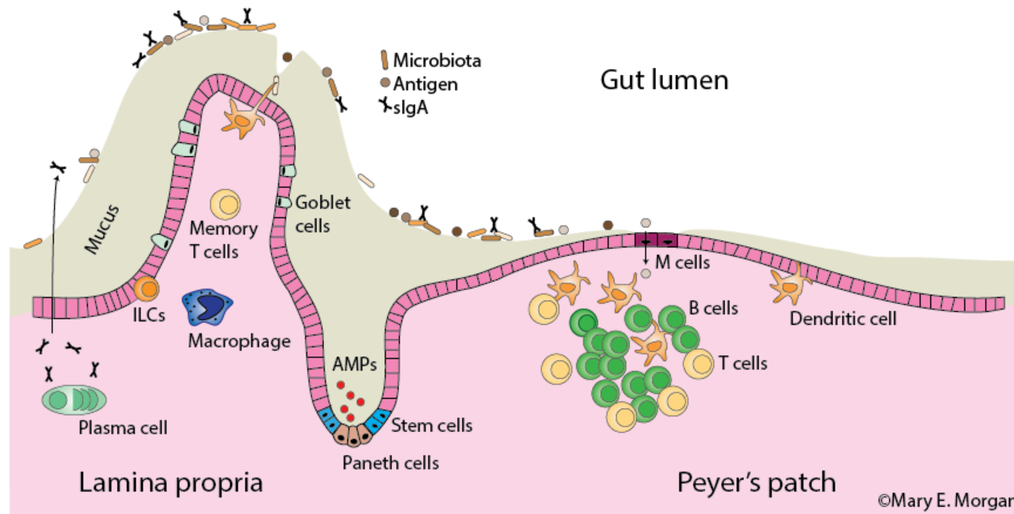


Figure 2-8 Cells involved in maintaining health at the intestinal-lumen interface. ILC: innate lymphoid cells; AMP: antimicrobial peptides; sIgA: secretory IgA

2.7 **Whole gut lavage fluid (WGLF)**

The mucosal immune system is largely independent of the systemic immune system and as a result direct investigation of intestinal humoral immunity requires collection of either intestinal secretions, mucosal biopsies or both (Mahony et al., 1990). It is possible to infer important information about mucosal responses from blood, such as the protective correlation between serum anti-LPS antibodies predicting protection from Ty21a vaccination (Levine et al., 2010), but intestinal samples are closer to the cells generating the immune response. Whole gut lavage fluid is ideal for studies of luminal cells, immunoregulatory and pro-inflammatory cytokines, mucosal secreted antibodies to food and microbial antigens (Gaspari 1988, Poxton 1995, Mahony 1990, Mahony 1991). Gaspari et al., 1988 described the method of obtaining and analysing immune responses in gut secretions. Oral gut lavage with a polyethylene

glycol based isotonic solution is a non-invasive clinical method that is used to obtain the whole gut secretions (Poxton et al., 1995). The clear fluid passed per rectum will contain total protein, protease activity, IgA, IgM, IgG, albumin, alpha-1-antitrypsin and polyethylene glycol. This thesis describes the use of gut fluid to investigate the effects on ATRA on oral vaccine responses.

2.8 **Immunoglobulin A (IgA)**

Secretory IgA accounts for two-thirds of all immunoglobulins produced locally in the plasma cells in the intestinal lamina propria (Crabbe 1965, Brandtzaeg 2009). The biological function of IgA is the neutralisation of harmful antigens in the luminal side of the gut, preventing adherence to, or penetration of, the epithelium, so-called "immune exclusion". The IgA produced is translocated to the gut lumen by the transmembrane glycoprotein polymeric Ig receptor (pIgR) across mucosal epithelial cells (Murphy, 2012). A molecule of pIgR is consumed with every round of SIgA transported and as a result, the regulation of it is dependent on the expression and epithelial trafficking of pIgR (Johansen et al., 2011). PIgR is solely produced by intestinal epithelial cells and regulated by proinflammatory cytokines including IFN-ɣ, TNF and IL-1 (Asano et al., 2011). Studies by Takenuchi, 2004 showed that ATRA up-regulates pIgR in the gut mucosa.

SIgA ensures immune exclusion at mucosal surfaces by specific binding to the pathogens within the mucus and prevents the mucosal barrier from being colonised and invasion of the epithelium (Phalipon 2003, Brandtzaeg 2009). The functions for SIgA above are illustrated in the excerpt from Johansen et al., 2004 (Figure 2-9).

2.9 **Retinoic acid and gut immunity**

Like their counter part lymphocytes, naïve B cells are primed in the Peyer's patches and mesenteric lymph nodes. They are first stimulated via B cell receptors (BcRs) to IgM-producing B cells and then undergo class switching to IgA production which is controlled by the cytokine TGF-β (Murphy, 2012). Effector B cells just like effector T cells need gut-homing molecules in order to be redirected to the gut.

It has been well documented that class switching is T cell dependent. However, Mora *et al.,* 2006 showed that gut-associated dendritic cells were able to induce T cell-independent expression of IgA and gut homing receptors on B cells.

With the hypothesis that RA induced expression of α4β7-integrin and CCR9 molecules on effector T cells, Mora and colleagues set out to investigate if this too were true for antibody-secreting B cells. They found the addition of RA to activated murine spleen B cells induced high levels of α4β7 and maintained a robust CCR9 expression on B cells consistent with what Iwata had revealed on $CD4^{+}$ T cells.

Following this result, Mora further investigated whether RA also had an effect on IgA secretion by mucosal B cells. The team found that the B cells that were cultured with Peyer's patches dendritic cells secreted much more IgA than the B cells cultured without dendritic cells from Peyer's patches.

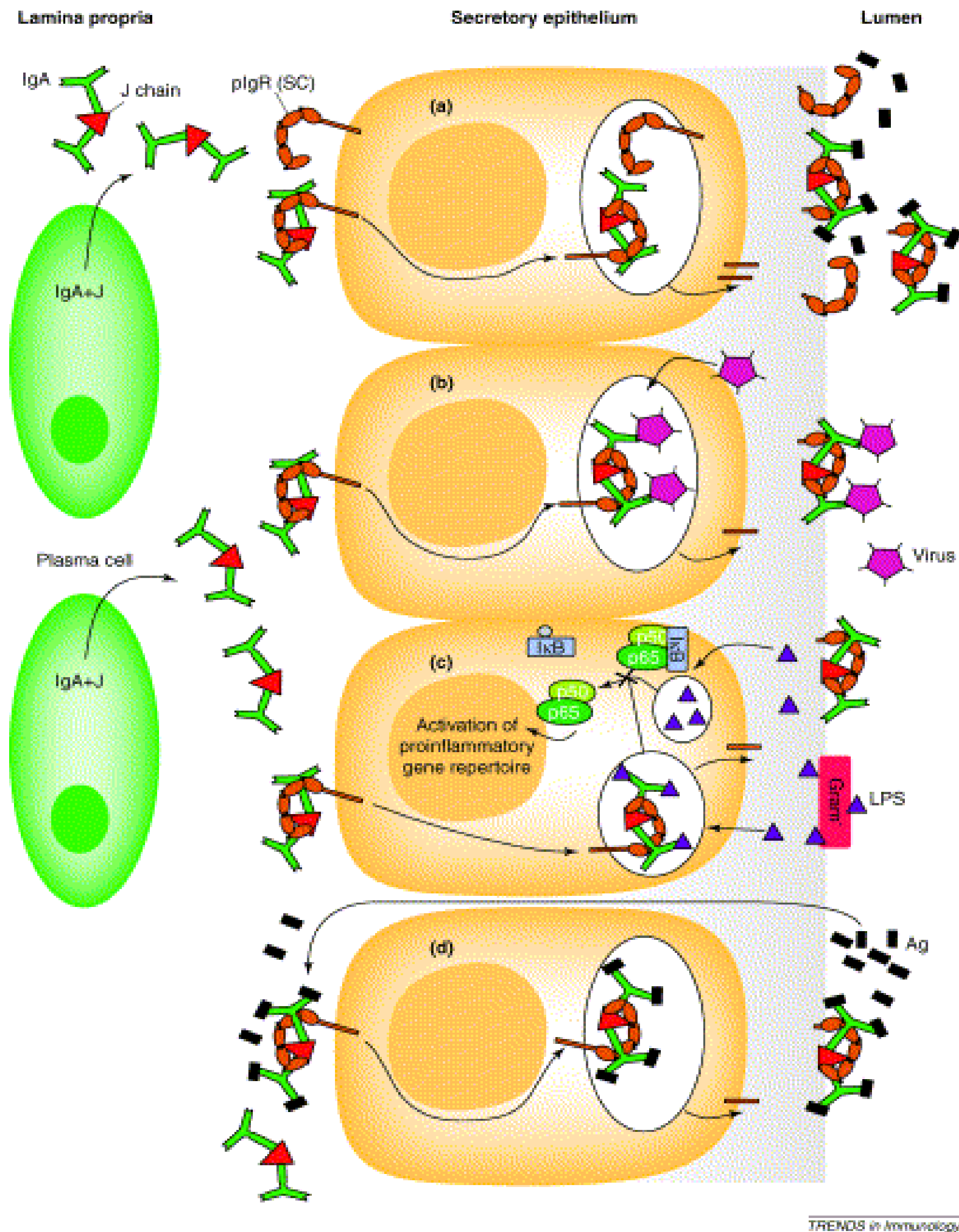


Figure 2-9 Four suggested non-inflammatory modes of defence mediated by dimeric IgA binding to pIgR. **(a)**SIgA generated from pIgR-driven export of locally produced dimeric IgA function as blocking antibodies in immune exclusion at mucosal surfaces. **(b)** Intracellular neutralisation of an infecting virus by specific dimeric IgA, perhaps followed by transport of viral products to the lumen (note, this kind of neutralisation requires occupation of virus by the pIgR transport vesicles). **(c)** Intracellular neutralisation of endotoxin [lipopolysaccharide (LPS)] from Gram-negative bacteria, which inhibits potentially harmful activation of the proinflammatory NF-κB pathway in the epithelial cell. **(d)** Stromal clearance of antigen (Ag) that has breached the mucosal barrier. The Fab arms of dimeric IgA are more perpendicular to the Fc arm than in IgG, a feature that makes dimeric IgA particularly suitable for agglutinating particulate antigens. (Johansen et al., 2004)

2.10 Retinoic acid and T cell activation

2.10 **Lymphocytes express different cell-surface protein**

T lymphocytes are derived from multipotent hematopoietic stem cells in the bone marrow. These progenitor cells then migrate to the thymus where they mature (Janeway 2012). In the thymus, the cells receive Notch 1 signals instructing them to commit to the T-cell lineage and not the B cell lineage. This signalling is also important in the regulation of the T-cell lineage choices including α: β verses γ: δ choice and CD4 verses CD8 decision. Lymphocytes undergo functional maturation at different stages and these are reflected by the expression of different cell-surface proteins described in the following sections.

2.10.1 **Cluster of differentiation 3 (CD3)**

Lymphocytes express the T cell receptor complex, CD3 on their surfaces. This receptor protein complex has an extracellular immunoglobulin-like domain.

2.10.2 **Cluster of differentiation 4(CD4)**

The lamina propria of the intestine is home to between 70-80% of the body's immune cells with the majority of T cells being those that express the CD4 glycoprotein on their surface. These cells referred to as helper $CD4^+$ T cells (Th), are involved in interacting with antigens presented by the major histocompatibility complex class II proteins and augmenting the ability of B cells to produce antibodies. $CD4^+$ T cells are involved in a number of functions including activation of cells of the innate immune system, B-lymphocytes, cytotoxic T cells, as well as non-immune cells. $CD4^+$ T cells can be differentiated into effector subtypes, T-helper 1 (Th1) andT-helper 2 (Th2) cells depending on the cytokine. The Th1 cells are involved in elimination of intracellular pathogens while the Th2 cells mount an immune response to extra-cellular parasites including helminths (Luckheeram et al., 2011,

Smith et al., 2011). CD4+ T cells also differentiate into Th17 cells. These cells are known to contribute to the resistance to infections such as Listeria, Salmonella, Toxoplasma, Cryptococcus and Leishmania (Wan et al., 2009). The major signalling cytokines involved in Th17 differentiation include IL6, IL21, IL23 and TGF-β with the main regulator retinoic acid receptor-related orphan receptor gamma-T (RORɣt). T reg cells are a distinct lineage of CD4 cells that are essential for maintaining immune system homeostasis by promoting self-tolerance and restraining excessive immune responses and ATRA has been shown to induce both the Foxp3 T regs and IL-10-producing Tregs (Liu et al., 2015).

2.10.3 **Cluster of differentiation 8 (CD8)**

T cells that carry the co-receptor CD8 and recognise antigens, for example viral antigens, that are synthesised in the cytoplasm of a cell and become bound to MHC Class I molecules. CD8 T cells differentiate into cytotoxic CD8 T cells (Janeway, 2012). Based on T cell subset depletion studies and the analysis of gene knockout mice, it is evident that CD8 (+) T cells contribute to resistance against intracellular infections with certain viral, protozoan, and bacterial pathogens. Although they are known primarily for their capacity to kill infected cells, CD8 (+) T cells elaborate a variety of effector mechanisms with the potential to defend against infection (Harty et al., 2000).

2.10.4 **Alpha4 beta7 integrin (α4β7)**

Effective clearance of pathogen requires rapid migration of effector T and B cells to the site of infection. Vaccination against intestinal infections requires immunisation by a mucosal route because of the tissue-specific consequences of lymphocyte

priming. During oral vaccination, a strong immune response is required to be mounted in the gut mucosa. Gut-specific migration of the antigen stimulated T and B cells is determined by the expression of trafficking/homing integrins. The integrins are heterodimeric cell surface adhesion molecules composed of α and β subunits. On T cells, the α4 integrin subunit associates with β7 subunit to form α4β7 integrin (DeNucci et al., 2010). Naïve T cells circulating the bloodstream express receptor CCR7 and L-selectin. These markers help the T cells migrate to the Peyer's patches where they are presented with antigen complexed to dendritic cells causing them to become activated. This activation leads to the loss of the CCR7 and L-selectin molecules and the gain of α4β7-integrin (Murphy, 2012). The adhesion molecule α4β7-integrin expressed by antigen-stimulated T cells helps them to bind to the endothelial cells lining the blood vessels in mucosal tissues via the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (Iwata *et al*., 2004). This binding triggers the signal for migration of effector T cells into the lamina propria.

2.10.5 **CC-chemokine receptor 9 (CCR9)**

The α4β7-integrin works together with another gut homing marker chemokine receptor CCR9. The ligand for this receptor is the chemokine, CCL25 which is expressed by the epithelial cells of the small intestine. This receptor is also important in the selective recruitment of the effector T cells. CCR9 is the small intestine (SI) - specific homing receptor, whereas α4β7 is the homing receptor to both the small and large intestine (Kim et al., 2015).

2.10.6 **Human lymphocyte antigen -DR (HLA-DR)**

During T cell activation, several surface molecules are expressed at different stages in the activation process of the T cells. HLA-DR is one of the activation markers or antigens expressed (Salgado et al., 2002). HLA-DR is a glycosylated cell surface transmembrane protein expressed on antigen presenting cells and monocytes. Expression of HLA-DR by monocytes is essential for the presentation of peptides derived from ingested microbes to $CD4^{+}$ T cells to initiate a specific immune response (Perry et al., 2004).

2.10.7 **Cluster of differentiation 69 (CD69)**

CD69 antigen is expressed on activated T and B cells, activated macrophages and NK cells. This antigen is normally associated with acute activation of T cells, and is reviewed as an early activation marker (Rea et al.1999).

2.10.8 **Cluster of differentiation 25 (CD25)**

CD25is an activation marker found on activated cells including T, NK and B cells and monocytes. It is the α subunit receptor for interleukin-2 (IL-2) cytokine which is produced by the activated T cell itself. The IL-2 cytokine drives proliferation and differentiation (Janeway, 2012). CD25 does not participate in signal transduction resulting from IL-2 interaction. CD25 is also found on regulatory T cells.

2.10.9 **Cluster of differentiation 45RA (CD45RA)**

CD45 is a protein tyrosine phosphatase expressed on all hematopoietic cells. CD45 can be expressed as one of several isoforms by alternative splicing of exons that comprise the extracellular domain. CD45RA is expressed on naïve T cells, as well as

the effector cells in both CD4 and CD8. After antigen experience, central and effector memory T cells gain expression of CD45RO and lose expression of CD45RA. Thus either CD45RA or CD45RO is used to generally differentiate the naïve from memory populations. Naive cells continuously recycle between peripheral blood and secondary lymphoid organs and this trafficking is highly dependent on the expression of L-selectin, CD62L (Picker et al., 1993).

2.11 **T cell activation**

Effective clearance of pathogen requires rapid migration of effector T cells to the site of infection (Hammerschmidt et al., 2011). Selective migration of the effector T cells to the gut requires expression of α4β7-integrin and chemokine receptor CCR9. Naïve T cells circulating the bloodstream express receptor CCR7 and L-selectin (CD62L). The markers help the T cells migrate to the Payer's patches. Here they are presented with antigen complexed to dendritic cells causing them to become activated. This leads to the loss of CCR7 and L-selectin molecules and the gain of α4β7-integrin and CCR9 chemokine receptor (Murphy, 2012). The adhesion molecule α4β7-integrin expressed by antigen-stimulated T cells helps them to bind to the endothelial cells lining the blood vessels in mucosal tissues via the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (Iwata *et al.*, 2004). This binding triggers the signal for migration of effector T cells into the lamina propria.

2.11.1 **ATRA and T cell homing**

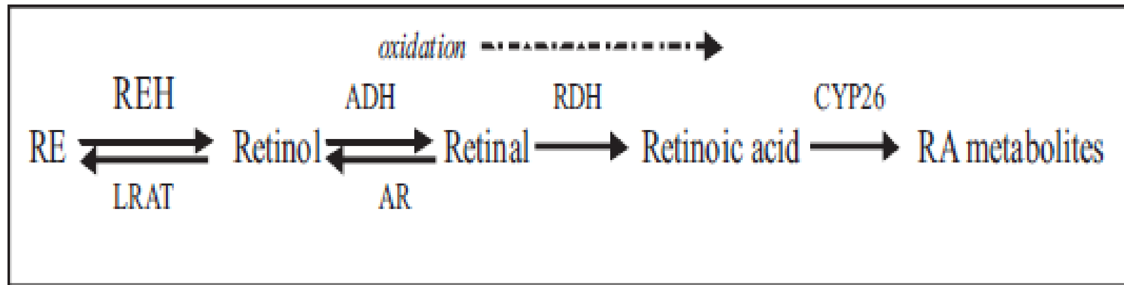
Several studies (Iwata, 2004; Hammerschmidt, 2011) have shown that RA is a key mediator in T cell homing to the gut. Iwata and colleagues demonstrated that stimulated $CD4^{+}$ T cells cultured in vitro with all-*trans* RA enhanced the expression of gut-homing receptors α4β7-integrin. They further demonstrated that RA treatment

induced a strong chemotactic activity in $CD4^+$ T cells toward the CCR9 ligand TECK (CCL25). Recent studies (Kim et al., 2015) have also revealed that RA regulates gut homing expression on the innate lymphoid cells (ILCs), a new cell population that plays an important role in host defence (Kumar et al., 2014). Kim and colleagues showed that RA induced homing receptor switch for the migration of ILC3 and ILC1 (ILCs subsets) to the gut. They showed that α4β7 and CCR9 is highly expressed by these cell subsets in the presence of RA.

2.12 **Dendritic Cells**

Dendritic cells (DCs) have been shown to induce imprinting of tissue tropism of effector T cells. During vitamin A metabolism, the irreversible pathway from retinal to RA is catalysed by RALDH (Iwata, 2004). Iwata showed that the mRNA of 3 different isoenzymes of RALDH was expressed by Peyer's patches (PP) and mesenteric lymph node (MLN) DCs. These were *RALDH1, RALDH2* and *RALDH3.* The RALDH allows the intestinal DCs to convert retinal to RA which in turn induces T cell expression of the gut homing receptors α4β7 and CCR9 (Zeng et al.,2012). Also, Lamina propria-derived $CD103^+$DC are responsible for the imprinting of gut homing receptors on T cells in PP and MLN (Edele et al., 2008).

In the enterocyte, retinol is oxidised to retinoic acid under enzymatic control as illustrated below:



RE: Retinyl ester; REH: Retinyl ester hydrolase; LRAT: Lecithin retinol acyl transferase; ADH: aldehyde dehydrogenase; AR: Aldehyde reductase; RDH: Retinol dehydrogenase; CYP26: Cytochrome 26 (Kelly et al., 2009)

REH converts retinyl esters to retinol which is then oxidised to retinal by alcohol while LRAT catalyses the esterification of retinol for storage. CYP26 a member of the cytochrome P450 system converts ATRA into hydroxyl-, keto- and glucuronyl-metabolites having a reduced bioavailability and may present an excretion pathway (Kelly, 2009).

2.13 **Transcriptional effect of Retinoic acid**

RA is known to influence and regulate gene transcription. RA directs transcriptional responses by binding nuclear receptors. There are two classes of these receptors including retinoic acid receptors (RARs) and retinoid X receptors (RXRs). The receptors are part of the steroid/thyroid retinoid nuclear receptor family (Tang *et al.*, 2010). The receptors exist in three different isotypes, α, β and ɣ. Chambon et al in 2005 revealed that the gene sequence for each of the RAR isotypes was different from the other two, and that the sequences for each isotype were highly conserved between humans and mice, leading to the speculation that each RAR isotype has a specific function.

These receptors form either homodimers (RXR-RXR) or heterodimers (RAR-RXR) (Blomhoff et al; 2005). RAR-RXR regulates the transcriptional activitation of all-trans retinoic acid target genes by binding to DNA-response elements called RA

response elements (RAREs) (Tang *et al*., 2010). RAREs consist of a direct repeat of a core hexameric sequence 5'-(A/G)G(G/T)TCA-3' or a more relaxed 5'-(A/G)G(G/T)(G/T)G/C)A-3' motif separated by 1,2 or 5 base pairs (Rhinn, 2012). Binding of RA to RAR-RXR heterodimer, alters the interaction with co-regulatory proteins enhancing the recruitment of co-activator complexes (Tang et al. 2010; Rhinn, 2012). These complexes then cause chromatin remodelling facilitating the assembly of transcription pre-initiation complex which contains RNA polymerase II (Pol II), TATA-binding protein (TBP) and TBP-associated factors (TAFs).

2.14 Retinoic acid effects on vaccine responses

As previously discussed, RA signals through its nuclear retinoic acid receptors, has effects on development and differentiation of different cell types including those of the innate and adaptive immune system and enhances T cell proliferation and migration to gut mucosa. With all these attributes of RA, Tan et al 2011 reasoned that the provision of ATRA during vaccination might augment the ability of T cell-based viral vaccines to promote the gut mucosal homing of CD8 T cells in order to provide increased protection from mucosal viral challenge. Their mouse data revealed that antigen-specific T cells primed in *vivo* by vaccination in the presence of ATRA exhibited increased accumulation at mucosa-associated sites. They showed that ATRA can influence T cell responses during activation. Other animal data (Hammerschmidt et al 2011) showed that RA-assisted subcutaneous immunisation generated intestinal immunity and conferred the benefits of mucosal protection otherwise generated only when antigen was applied orally. The authors concluded that RA could be used as gut-homing navigators for vaccinations given subcutaneously or intramuscularly in order to generate protective immunity against enteric pathogens. A number of studies (Table 2-7) have shown the immunological

importance of vitamin A and its derivative ATRA. In our recent study in adult male volunteers (Lisulo et al 2013), oral ATRA administration 1 h prior to dosing with oral typhoid vaccine increased secretion of specific IgA against vaccine derived LPS into the gut.

Table 2-7 Summary of studies looking at the immunological mechanism of ATRA on vaccine response

Author	**study design population**	**Intervention**	**Main findings**
Iwata (2004)	Naïve $CD4^+$ T cells from mouse model	various doses of all-trans RA,retinaldehyde, retinol (nM)	RA gave significant upregulation of α4β7,induced mRNA expression of CCR9
Hammerschmidt (2011)	transgenic mice	sub-cutaneous application of RA	$CD4^+$T cells upregulated α4β7-integrin and CCR9 after sub-cutaneous antigen plus RA application
Tan (2011)	6-8 week old female mice, HLA-A human T cells from healthy blood donors	induction of exogenous ATRA during systemic vaccination	All trans RA doses $\geq$10nM increased levels of CCR9, α4β7and CD103 in mouse T cells. Expression of α4β7 on $CD8^+$ T cells was enhanced by exposure to ATRA
Bernardo (2013)	colonic biopsies from ulcerative colitis patients, monocytes from healthy volunteers	culturing of cells in complete medium with different doses (10^{-6}M,10^{-7}M,10^{-8}M) of RA and LPS (0.1ug/ml)	RA induced an immature, gut –homing phenotype on monocyte-derived DC (MoDC) although expression was in a dose-dependent manner. RA-conditioned MoDC had decreased T cell stimulatory capacity and increased gut homing imprinting capacity on stimulated T cells.
Lisulo (2013)	randomized control trial, Zambian adult men	ATRA given alongside oral typhoid vaccine	IgA in wglf against vaccine LPS and protein extract was increased in vaccine recipients who were given ATRA compared to those who were not given ATRA.
Evans (2013)	rhesus macaques, chimpanzee cells, PBMCs from human donors	exogenous ATRA treatment of cells in a dose and time dependent-manner	Up-regulation of α4β7and CCR9 time-dependent. expression of α4β7 was conserved among 3 primate species

CHAPTER 3

METHODOLOGY I

3.0 Statement of the problem

Recent work has revealed that oral vaccine efficacy and immunogenicity is much less effective in developing countries than in developed countries (Levine *et al*., 2010). For example, oral rotavirus vaccine affords excellent (80-90%) protection against severe rotavirus diarrhoea in industrialised and middle income countries (Vesikari et al, 2006) but has been less efficacious when tested in infants in the developing world (Madhi et al, 2010). The trivalent oral polio vaccine (OPV) has also been found to be less effective in children in the developing world (Grassly et al. 2010) with estimated efficacies of up to 21% in India compared to 50% in the United States (Grassly et al. 2006). The live oral typhoid vaccine has also been shown to elicit a diminished serum antibody response although it stimulates intestinal IgA antibodies (Levine et al 1989, Levine et al, 2010). Several reasons have been identified as possible causes for the diminished immunogenicity of oral vaccines in these developing populations. These could include poor sanitation, intestinal flora overgrowth, persistent infections with helminths or other parasites, for example for polio virus type1; it is likely that interference by concurrent infections such as non-polio enteroviruses contribute substantially to impaired vaccine efficacy (Parker et al., 2014). Differences in nutritional status, such as vitamin A deficiency and environmental enteropathy which leads to malabsorption of nutrients (Lycke 2012) could also contribute to reduced vaccine efficacy. In view of this, there is an urgent need to identify ways to improve the immune responses of these oral vaccines in the settings where they are most needed. An oral adjuvant

could have a dramatic impact on vaccination programmes for controlling diarrhoeal disease, with major benefits for child health in developing countries.

3.1 Study Justification

Impaired efficacy of oral vaccines is a major problem in Africa as demonstrated by rotavirus studies. In preliminary studies in Zambian adults (Lisulo et al., 2013), ATRA enhanced specific IgA responses directed against oral typhoid vaccine antigens. In order to turn this promising observation into a useful public health tool it was necessary to determine if the ATRA adjuvant effect can be generalised to other vaccines. Furthermore, few studies have thoroughly explored the adjuvanticity and immunological mechanisms of ATRA in human participants and in vaccination settings.

3.2 Research Question

Can giving oral vaccination alongside ATRA improve the immune response to the vaccine by enhancing expression of IgA & gut homing molecules?

3.3 Conceptual Framework

The relationship between high burden of infection and increased morbidity and mortality can be conceptualised at a fairly general level (Figure 3.3) as a four stage relationship where a set of causal factors (bacterial overload, non-polio enteroviruses, environmental enteropathy, intestinal malabsorption, vitamin A deficiency) impact on a series of intermediate indicators (reduced vaccine efficacy) which in turn determine the final outcome. An attempt to improve one of the causal factors (increase vaccine efficacy) may result in quite a different outcome.

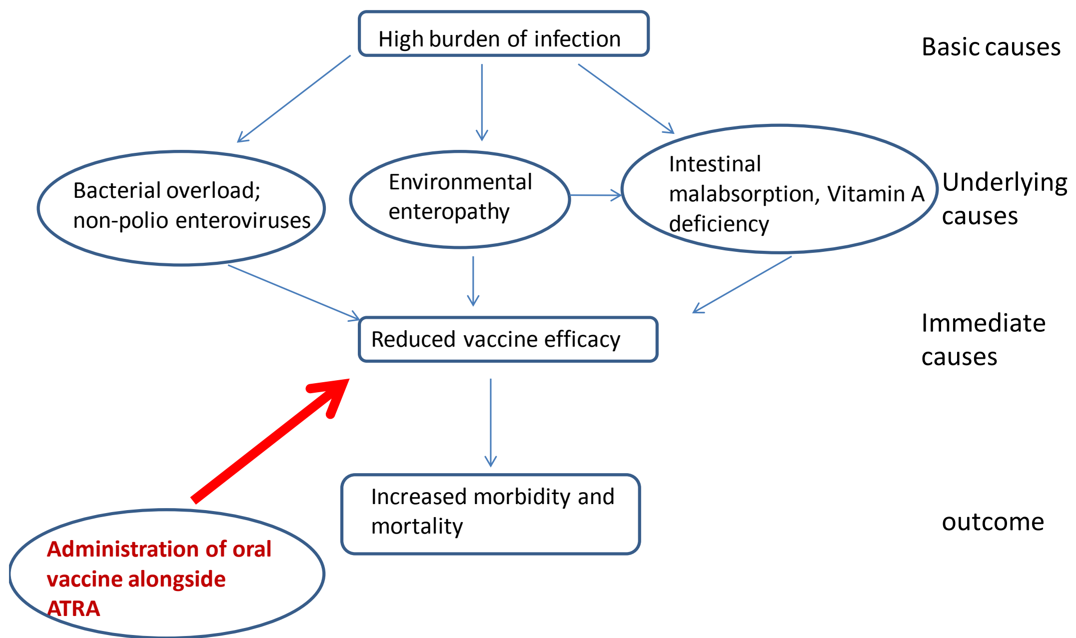


Figure 3.3 Conceptual framework

3.4 Aim

The overall aim of the study was to determine if the findings of the preliminary study (Lisulo et al., 2013) had general applicability to other oral vaccines and to investigate the immunological mechanisms of action of ATRA.

3.5 General objective

To investigate the immunological effects of ATRA on responses to oral vaccination

3.6 Specific objectives

- *In vivo* analysis of effects of ATRA on $CD4^+$ T cell expression of α4β7, CCR9, HLADR, CD69 and CD25 at different time points: baseline, 6hrs, 24hrs, 72hrs, day 8 and day14.
- *In vitro* study of the effects of different concentrations of ATRA on expression of gut homing markers on $CD4^+$ T cells.
- To investigate the specific IgA response to vaccine preparations of different oral vaccines (Vivotif, Dukoral, Rotarix, Polio) in whole gut lavage fluid and serum after vaccination alongside ATRA
- To investigate whether the dose of ATRA has an effect on its mechanism of action
- To investigate whether the effect of ATRA is dependent on vitamin A status

CHAPTER 4

METHODOLOGY II

This chapter describes the investigations carried out in order to answer the five questions (Chapter 3, 3.6) that would ultimately give a general answer as to whether giving oral vaccination alongside ATRA would improve the immune response to the vaccine by enhancing expression of IgA & gut homing molecules.

4.0 Study design

The study was an open-label clinical trial

4.1 Study site

The study recruited participants from Misisi compound. Misisi is an unplanned residential area situated just south of Lusaka the capital of Zambia. It is home to an estimated 80,000 people most of who live on less than a dollar per day. The poor sanitation and inadequate water supply leaves much to be desired. Mortality rates in adults and children is high probably attributable to the severity of the HIV epidemic and the many infectious diseases attributed to poor sanitation and poverty. The community was chosen based on a large survey conducted in 1995 where Misisi was categorised as a high risk area (Kelly et al., 1997 and Nchito et al., 1998). It is in such environments that vaccines are not working well and where they are most needed.

4.2 Subject recruitment and selection

The study recruited adult male volunteers from Misisi compound aged between 18 and 60 years were recruited. The consent process was as follows:

1) House-to-house sensitisation & focus group discussions: This involved focus group discussions (FGDs) with volunteers who responded to the study advertisement. During the discussions, the research aims were explained and

discussed in depth and the volunteers were allowed to ask questions. An information sheet (see appendix) explaining the study was also provided. The volunteers were then asked to return in a week if they decided to take part in the study.

2) Screening and Recruitment: During this phase volunteers were asked a number of questions from a questionnaire (see appendix) to establish their eligibility for the study. Then signing of the consent form (see appendix) as record of agreement to be part of the study. A full history was taken and examination performed for each subject who fulfilled the inclusion criteria.

Inclusion criteria

i. Males aged 18-60
ii. Naïve to the vaccine in question
iii. Willing and able to participate in the study after giving written informed consent
iv. No history of diarrhoea within the previous month prior to the study
v. No use of broad-spectrum antibiotic within two weeks prior to study
vi. No administration of any vaccine 6 months prior to study
vii. No helminth infection at screening (Kapulu et al., 2011)
viii. No ethanol dependency
ix. No NSAIDs
x. No participation in another study

Nutritional assessment of each volunteer was carried out by measuring the body mass index (BMI) and mid upper arm circumference (MUAC). HIV testing was also offered to the participants. Participants were invited to the laboratory facilities to

explain and show them how their samples would be processed. No financial incentives or other gifts were offered to participants except for transport refunds.

4.3 Sample size

In order to draw valid conclusions from a minimum sample size, I based my assumptions on results from preliminary investigations (Lisulo et al., 2013). Specific IgA expression in whole gut lavage fluid against vivotif lipopolysaccharide (LPS) and protein extract was increased by a mean of 0.024 and standard deviation (SD) of 0.027.

In this study the aim was to see a doubling (effect size) in the mean increase in IgA expression. Based on this, the sample size calculation was:

$$n = 2\,(Z\alpha + Z1\text{-}\beta)^2(\delta)^2 / \Delta^2$$ **(Kadam and Bhalerao, 2010)**

n = minimum required sample size

$Z\alpha$ = accepted error

δ = standard deviation

$Z1\text{-}\beta$ = power of study

Δ = effect size (clinical importance)

Substituting the values into the equation:

n=?

δ= 0.027

Δ = 0.024

$Z\alpha$ = 1.96 (5% error)

$Z\,1\text{-}\beta$ = 0.8416 (80% power)

$n = [2(1.96 + 0.8416)^2 (0.027)^2] / (0.024)^2$

n= 19.86

Thus a total of 20 participants were to be sampled per group.

Four vaccines were being evaluated and so there were to be 8 groups each consisting of 20 people and amounting to 160 participants. At 10% loss to follow up the total sample size would then need to be 176.

4.4 Ethical considerations

Approval for the study was obtained from the Biomedical Research Ethics Committee of the University of Zambia (013-01-14). See appendix

4.5 ATRA and Vaccine administration.

Recruited volunteers were randomised to receive one of the four oral vaccines: Vivotif, Dukoral, Rotarix, and Polio (Table 4-1), care was taken that all study participants were naive to the vaccine they were given. All vaccines were taken under supervision and participants that received vaccine with ATRA were given ATRA 1 hour before vaccination. Subsequently, ATRA was administered as a 10mg capsule taken daily for 8 consecutive days.

Table 4-1 Summary of vaccine schedule

	Day				
	1	2	3	4	5
Vaccine/ dose					
Vivotif (3 doses)	X		X		X
Rotarix (1dose)	X				
Dukoral (1 dose)	X				
Polio (1 dose)	X				

4.6 Sample collection

Blood and WGLF samples were collected for analysis at times specified in Table 4-2.

Table 4-2 Schedule of sample collection

	Day 1		Day 2	Day3	Day 8	Day 14
	baseline	6hrs				
Blood	X	X	X	X	X	X
WGLF	X					X

4.6.1 Blood collection

Blood (5ml) was collected and divided into three specimen bottles [a plain tube and two anti-coagulant EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA)] and kept in the dark awaiting processing. Blood in the plain tubes was allowed to clot at room temperature then centrifuged using the Eppendorf Centrifuge 5430R (Hamburg, Germany) at 3500 rpm for 5 minutes and the serum stored at -80° C until analysis. One lot of EDTA treated blood was stained for specific monoclonal antibodies and analysed by flow cytometry and the other EDTA blood sample was taken to UTH-virology lab for HIV testing using two rapid test kits: *DetermineHIV-1/2* (Abbott Laboratories, Tokyo, Japan) and *Uni-Gold IV* (Trinity Biotech, Ireland).

4.6.2 Whole gut lavage fluid (WGLF) collection

WGLF was used to assess mucosal immune responses as previously described (Lisulo et al 2013, Daley et al., 2007). The gut fluid was obtained on days outlined in Table 4-2. Following an overnight fast, gut lavage solution (Klean-prep; Norgine, Uxbridge, UK) was prepared as per manufacturer's instructions. The active ingredient in Klean prep, macrogol 3350, a polymer of ethylene glycol, is an inert substance that passes through the gut without being absorbed. This increases the

volume and water content of stool making them watery and easier to pass (laxative effect). The electrolytes in the Klean prep help to ensure the laxative works without causing the body to gain or lose significant amounts of sodium, potassium or water. Other ingredients include sodium sulphate, sodium bicarbonate, sodium chloride and potassium chloride. Each participant drank on average 3 litres of gut lavage solution before passing watery stool that eventually cleared into a gut lavage effluent having minimal particulates. Approximately 50ml of effluent was collected into a 50ml centrifuge tube (Figure 4-1) and 1 tablet of complete EDTA-free Protease inhibitor cocktail (Sigma) was added to each 50ml sample of gut fluid within 5-15 minutes of collection in order to inhibit serine, cysteine and aspartic proteases.1ml of 0.5M EDTA (Sigma) was also added immediately to a final concentration of 10mmol/l, inhibiting metalloproteases. Samples were spun in a refrigerated (4° C) centrifuge at 6000rpm for 20 minutes and supernatant was aliquoted and stored at -80^oC.

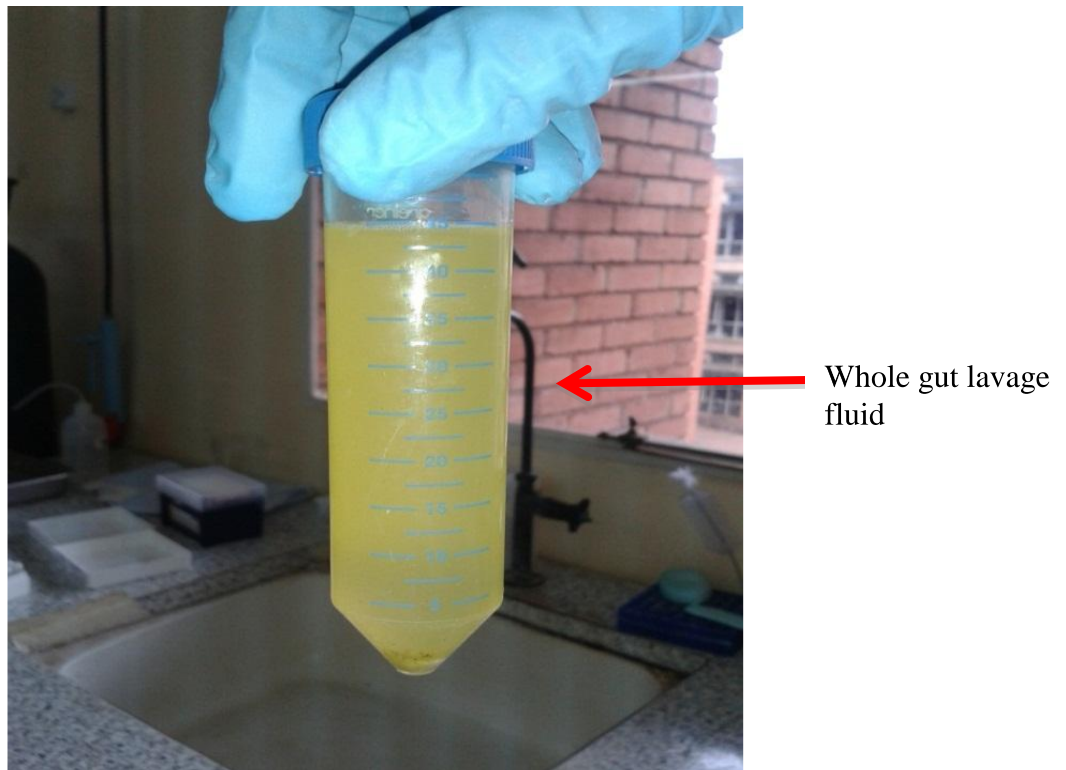


Figure 4-1 Whole gut lavage fluid with minimal fecal particulates collected in 50ml falcon tube.

4.7 Flow cytometric (immunophenotyping) analysis of α4β7 and CCR9 gut homing surface markers at different time points

Immunophenotyping is the analysis of heterogeneous populations of cells in order to identify the proportions of various subpopulations of interest. Flow cytometry is a technique that allows for measurement of single cells (immunophenotyping) using a laser beam. Lasers simultaneously assess each cell according to size (Forward scatter), internal complexity and granularity (Side scatter) and cell surface antigens. This information can be used to sort or separate sub-populations of cells. In this process, antibodies artificially conjugated to fluorochromes will recognise cells expressing antigens for which the antibody is specific and will fluoresce. This part of the chapter describes how flow cytometry was used to identify cell surface markers being affected by ATRA.

4.7.1 Panel design

Four panels were designed: Naïve, Homing activated I, II and III. The homing panels were differentiated by 3 activation markers (HLA-DR, CD69 or CD25). Our cell population of interest was the lymphocytes ($CD3^+$) as these are the ones responsible for cell-mediated immune responses of adaptive immunity. The lymphocytes would then be subdivided into $CD4^+$ and $CD8^+$ T cell subsets expressing gut homing antigens, α4β7 and CCR9. To design these panels, three principles were followed:

1. Fluorochromes were matched by brightness to level of antigen expression on the cell (the brightest fluorochrome was conjugated to the least expressed antigen);
2. Potential spectral overlap was kept at a minimal;
3. when using tandem dyes, consideration of their technical limitations was taken into account

4.7.1.1 Picking suitable antigens

The antigens of interest were CD3, CD4, α4β7, CCR9, CD25, CD69, HLA-DR and CD45RA. The number of cells expressing these antigens varies (Table 4-3). This was important to know as it would help what fluorochromes would be used to conjugate to the antibodies against these antigens.

Table 4-3 Expression of antigen on cells

Cell	Antigen	Molecules per Cell
T cell	TCR	100,000
	CD2	55,000
	CD3	124,000
	CD5	90,000
	CD7	20,000
	CD45	>200,000
CD4+ T cell	CD4	100,000
	CD28	20,000
	CCR5	4,000-24,000
CD8+ T cell	CD8	90,000
	CD28	15,000
B cell	CD19	18,000
	CD20	109,000
	CD21	210,000
	CD22	14,000
	HLA-DR	85,000
	CD11a	10,000
	CD40	2,000
	CD86	16,000
	CD80	2,000
Dendritic cell	CD11a	27,000
	CD40	17,000
	CD80	132,000
	CD86	208,000
Monocyte	CD14	110,000
	CD32	21,000
	CD64	13,000
Neutrophil	CD14	3,500
	CD16	225,000
NK cell	CD56	10,000
Red Blood Cell	Glycophorin A	340,000
Basophil	CD23	15,000

From *www.bdbiosciences.com*

4.7.1.2 Conjugation of antibodies to fluorochromes

In order for the antibody-antigen complex in flow cytometry to be detected, the antibodies needed to be conjugated to a fluorochrome that would fluoresce upon excitation. Assigning of fluorochrome to antibodies was dependent on the level of expression of the antigen by the cell (antigen density) and the brightness of the fluorochrome. The BD FACSVerse used in the study has 2 lasers; blue and red and 6 colours [4-2-0] (Table 4-4).

Table 4-4 Fluorochrome Reference Chart

Laser	Fluorochrome	Relative Brightness
Blue Laser (488nm)	fluorescein isothiocyanate (FITC)	Moderate
	Phycoerythrin (PE)	Brightest
	Peridinin (PerCP)	Dim
	Phycoerythrin cyanine (Pe-Cy7)	Bright
Red Laser (640nm)	Allophycocyanin (APC)	Brightest
	Allophycocyanin-H7 (APC-H7)	Dim

Fluorochromes have a wide emission spectrum and as such when multiple fluorescences are measured, there is the likelihood of spectral overlap. This was taken into consideration when designing the panels using the BD fluorescence spectrum viewer tool. Least abundant antigens were conjugated to brightest fluorophore (Table 4-5).

Table 4-5 FACS Panels

Fluorochrome	APC-H7	PerCP	FITC	APC	PE-CY7	PE
Homing Activated (i)	CD3	CD4	α4 (CD49d)	β7	HLA-DR	CCR9
Homing Activated (ii)	CD3	CD4	α4 (CD49d)	β7	CD69	CCR9
Homing Activated (iii)	CD3	CD4	α4 (CD49d)	β7	CD25	CCR9
Naïve	CD3	CD4	α4 (CD49d)	β7	CD45RA	CCR9

5 Antibody titrations

Titrating antibodies is important in achieving the optimal separation between positive and negative populations while using the least amount of antibody. Two-fold serial dilutions of the antibodies were done starting with the recommended manufacturer's concentration or volume. Briefly, 100µl whole blood was added to the tubes each with different volumes of antibody and vortexed to mix well. The mixture was incubated at room temperature in the dark for 15 minutes. 2ml of lysing solution (Table 4-7) that had been diluted to a working concentration of x1 (supplied as x10) was added to each tube vortexed and incubated for 10 minutes then centrifuged at 1500 rpm for 5 minutes. The supernatants were discarded and the tubes vortexed to resuspend the cells. 2ml PBS was then added and centrifuged at 1500rpm for 5 minutes. Again supernatant was discarded. The cells were vortexed and 250ul of 1X cell fix was added. The cells were acquired and analysed on the flow cytometer to ascertain the optimal dilution. (Table 4-6).

Table 4-6 Antibody titrations

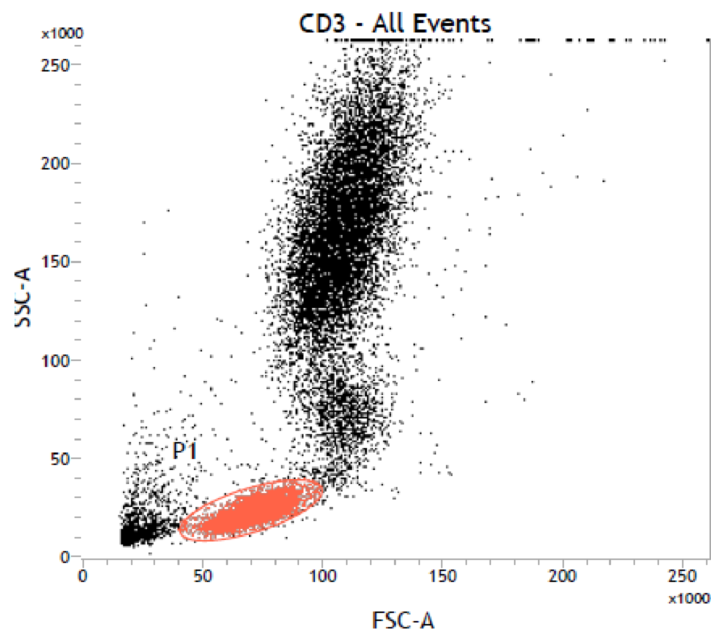
Antibody	Antibody volume (μl)	
	recommended volume	Optimal Working volume
CD3	20	1
CD4	20	1
α4 (CD49d)	5	2.5
β7	20	1
HLA-DR	5	5
CD69	5	5
CD25	5	5
CCR9	20	1

Table 4-7 Materials needed for immunophenotyping

Product	Supplier	Catalogue Number
CD3 APC-H7	Becton Dickinson	641397
CD4 PerCP	Becton Dickinson	550631
α4 (CD49d) FITC	Becton Dickinson	560840
β7 APC	Becton Dickinson	551082
HLA-DR PE-CY7	Becton Dickinson	560651
CD69 PE-CY7	Becton Dickinson	557745
CD25 PE-CY7	Becton Dickinson	557741
CCR9 PE	Becton Dickinson	561607
FACS lysing solution (10X concentration)	Becton Dickinson	349202
Cell fix (10X concentration)	Becton Dickinson	340181
5ml polystyrene FACS tubes	Becton Dickinson	352054
Cell wash	Becton Dickinson	349524

6 Gating strategies for negative cells population using Fluorescence-Minus-One (FMO) controls

Defining the boundary between positive and negative cells has always been a challenge when low-staining subsets need to be resolved. FMO controls reveal the maximum fluorescence expected for a given subset in a given channel when the antibody used in that channel is omitted from the stain set. Thus, these controls allow a simple decision as to where to place the upper boundary for negative cells in the channel (Tung et al., 2007). This was used to help in setting the gates and quadrants for each cell population of interest (Figure 4-2A, B).



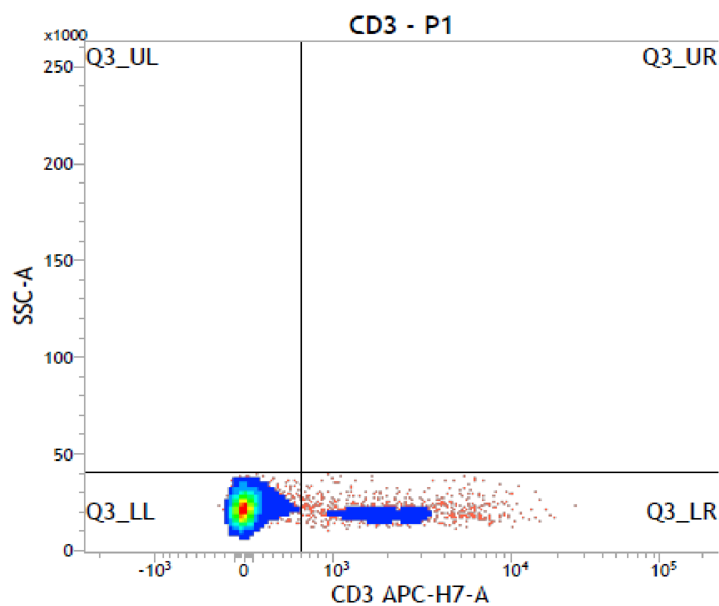


Figure 4-2A Example of FMO gating strategy for T cell sub-population expressing CD3. Panel 1(top) shows the dot plot 'gated on' or restricted to the lymphocyte population (in red). Panel 2 (bottom); X-axis labeled with name of fluorescently labeled cell marker (CD3) omitted from the stain set. Quadrants on the X and Y axes are set where the $CD3^-$ negative population ends. X –axis set at 10^2 and Y-axis set at 40. Populations of interest will start at this mark.

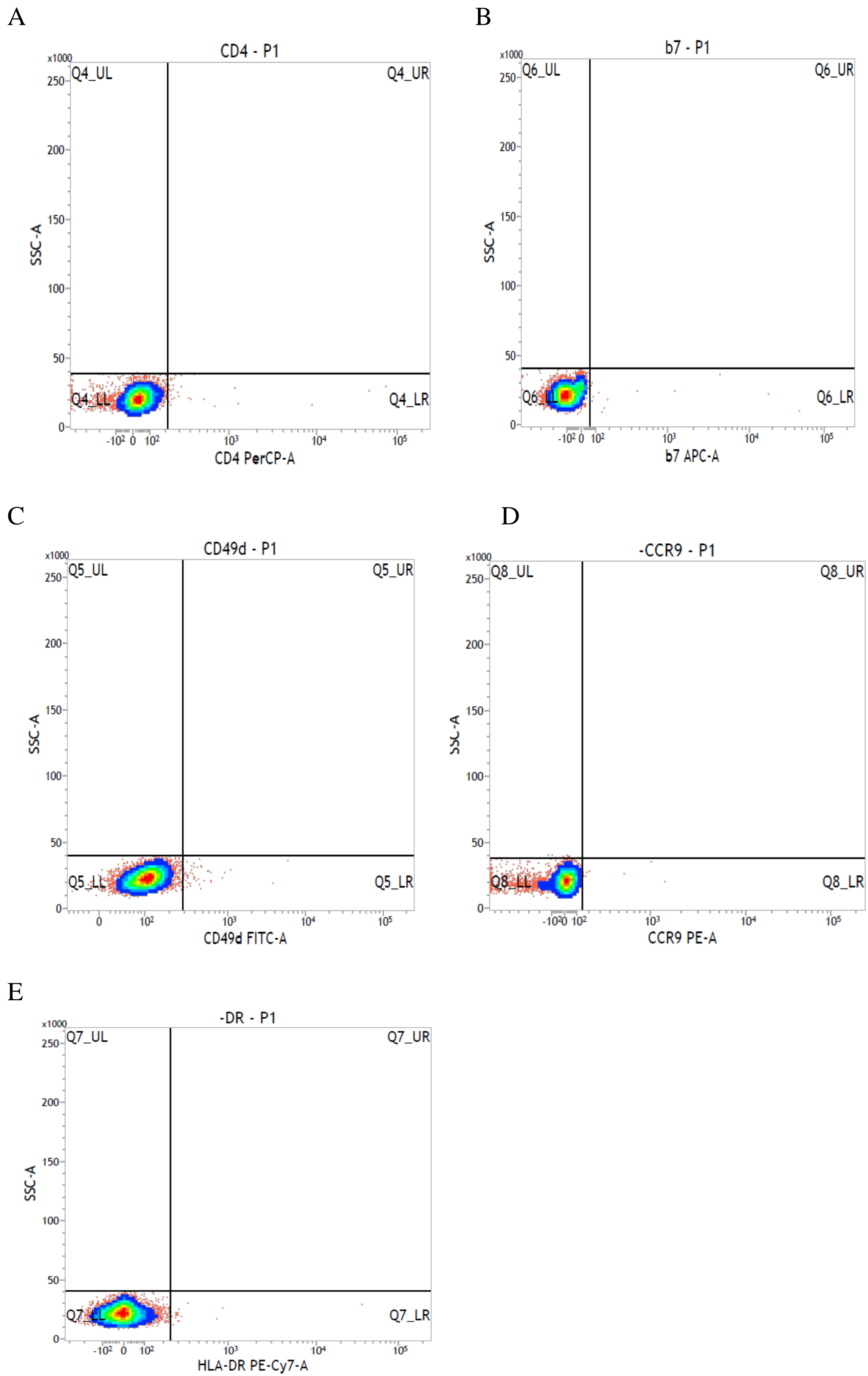


Figure 4-2B Examples of FMO gating strategies for T cell sub-populations. X-axis labeled with the Pseudocolor dot plots for (A) CD4$^-$ population (B) β7$^-$ population (C) α4$^-$ population (D) CCR9$^-$ population (E) HLA-DR$^-$ population. The lower right quadrant for each population determines where the positive population of interest will begin.

7 Gating strategies to define lymphocyte subsets according to marker expression

“Gating” is the term used to describe the selection of a sub population of cells for analysis. For example, if further characterisation of only lymphocytes within the white blood cells is desired, the region of the lymphocytes on the FSC–SSC plot would be outlined and a gate placed on the lymphocyte cell population (Figure 4-3.1 A). The lymphocytes are then further characterised by lymphocyte subsets defined by marker expression.

A

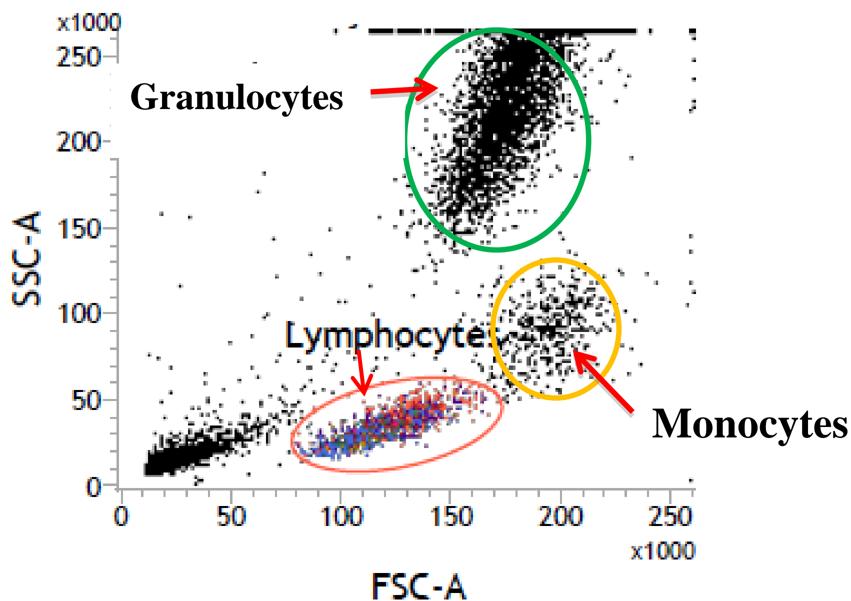


Figure 4-3.1 Scatter plot displaying the FSC and SSC of lysed whole peripheral blood. The major leukocyte populations as defined by level of cell granularity and cell size are shown. Population of interest is the lymphocytes

Once the lymphocyte population had been defined, the next population of interest was the CD3+ cells within the sorted lymphocytes (Figure 4-3.2A). CD3+ population consists of CD4+ and CD8+ cells. In order to sort the CD3+CD4+cells, a pseudocolor plot of CD3 verses CD4 was used. Our population of interest was positive for both markers and falls in the upper right quadrant (Figure 4-3.2B).

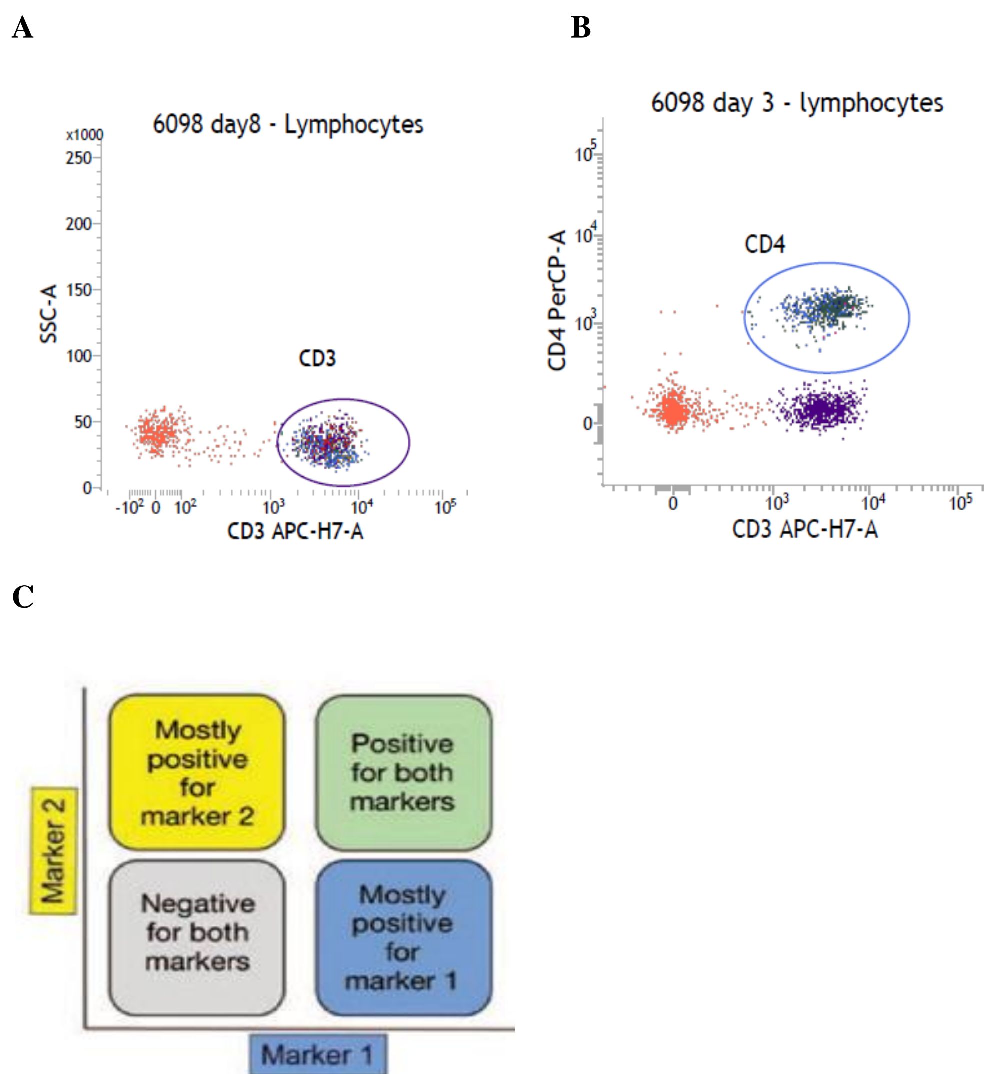


Figure 4-3.2 Dot plots of lymphocyte subset population. (A) Gate placed on CD3+ cell population of interest. (B) Population positive for both CD3 and CD4 markers. (C) Cartoon detailing interpretation of quadrant gates

The next population to be sorted was the activated CD4+ cells. Dot plot graph of HLA-DR (marker of activation) verses CD4 was analysed (Figure 4-3.3A). Then from this population (HLA-DR+CD4+), the activated gut homing cells (α4β7+HLA-DR+CD4+) were sorted (Figure 4-3.3B)

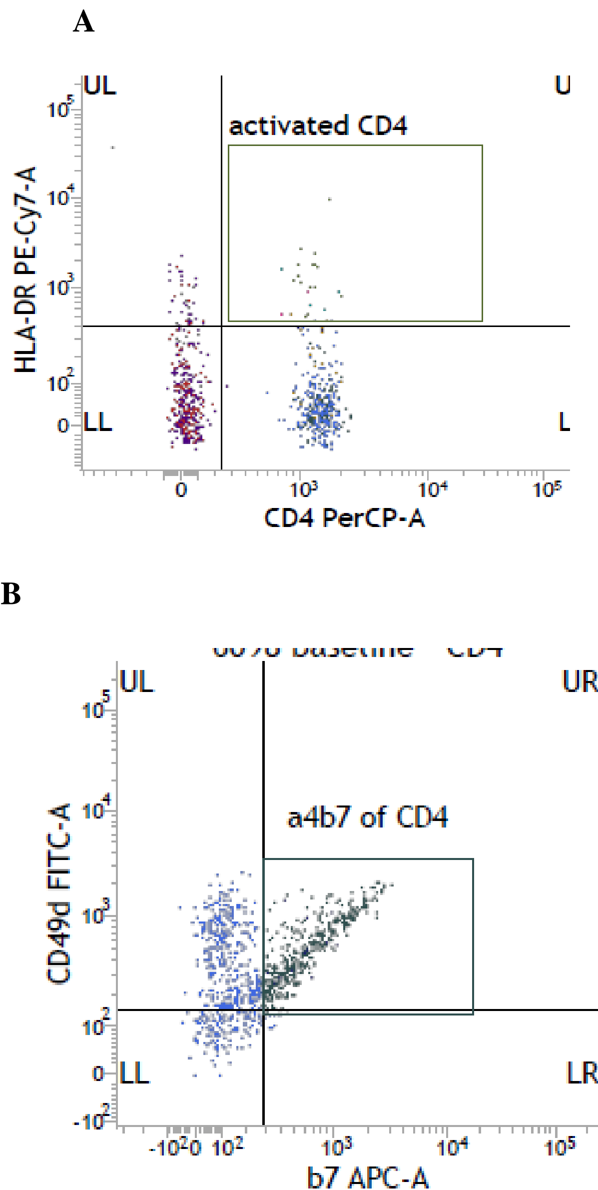


Figure 4-3.3 Fluorescence plots gated on activated CD4+ cells that are gut homing. (A) Activated CD4+ cells (CD4 verses HLA-DR) (B) of the activated CD4+ cells, plot is gated on the cells expressing both α4 and β7 markers – gut homing cells. α4 and β7 are commercially available as single antibodies

The same principle for sorting α4β7+ cells was applied for sorting CCR9+ cells (Figure 4-3.4)

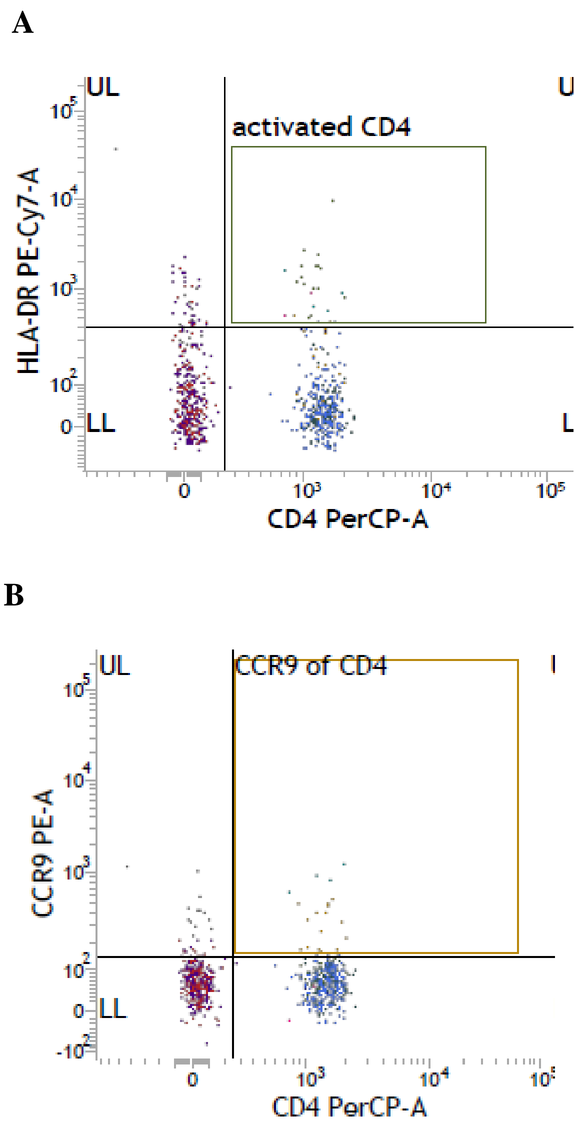


Figure 4-3.4 Fluorescence plots gated on activated CD4+ cells that are gut homing. (A) Activated CD4+ cells (CD4 verses HLA-DR) (B) of the activated CD4+ cells, plot is gated on the cells expressing CCR9

Once the lymphocyte subset populations were determined, the number of cells “events” in each population of interest was obtained using the FACSuite software (Figure 4-3.5):

Statistics

Name	Events	% Parent	% Grandparent	% Total	FSC-A Mean	SSC-A Mean
6098 day8:Lymphocytes	1,184	11.84	***	11.84	123,384	33,618
6098 day8:CD3	749	63.26	7.49	7.49	120,361	30,777
6098 day8:CD4	360	48.06	30.41	3.60	114,737	27,254
6098 day8:a4b7 of CD3	205	27.37	17.31	2.05	120,458	32,253
6098 day8:a4b7 of CD4	88	24.44	11.75	0.88	112,747	27,888
6098 day8:activated a4b7 of CD4	4	4.55	1.11	0.04	120,728	35,712
6098 day8:a4b7 of activated CD4	2	8.33	0.27	0.02	116,492	31,159
6098 day8:CCR9 of CD3	28	3.74	2.36	0.28	120,952	33,097
6098 day8:CCR9 of CD4	20	2.67	1.69	0.20	112,910	28,103
6098 day8:activated CCR9	4	1.11	0.53	0.04	111,058	29,968

Figure 4-3.5 Example of statistics table for the events represented for each lymphocyte subset.

8 Instrument Setting

The BD FACSVerse was the flow cytometer used for all immunophenotyping. Several procedures were followed to set the instrument prior to running samples:

Daily clean protocol (See Appendix)

Performance QC protocol (See Appendix)

Characterization QCprotocol (See Appendix)

Compensation: Manual compensation using Fluorescence Characterisation (FC) research beads were prepared according to manufacturer’s instructions. An experiment was set up and a tube added for each of the fluorochromes (FITC, PE, Per-CP, APC, PerCP-Cy5.5, PE-Cy7, APC-Cy7 and APC-H7) to be used. A dot plot of forward scatter area (FSC-A) verses side-scatter area (SSC-A) was drawn and

duplicated for each fluorochrome in the tube against all others being compensated. The FACSVerse voltage settings and threshold were optimized to acquire 10 000 events (cells). After acquiring the samples, the populations of interest were gated. The median value for fluorochromes on the x and y axis were edited accordingly. The populations were then compensated and values adjusted. The medians of the negative and positive populations of the fluorochrome not in the tube were compared. The positive in this case was a false positive and as such was reduced until value reached close to that of the negative value.

4.7.2 Whole blood staining protocol

After designing the panels, titrating the antibodies and setting the gates, the samples were then prepared using the lyse wash protocol which was similar to the one used when titrating the antibodies. The antibody master mix (Table 4-8) was prepared first by combining all antibodies in the panel.

Table 4-8 Master mix preparation template

Antibody	volume per sample (µl)	total volume(µl)
CD3 APC-H7	1	1x(no. of samples + 1)
CD4 PerCP	1	1x(no. of samples + 1)
α4 (CD49d) FITC	2.5	2.5x(no. of samples + 1)
β7 APC	1	1x(no. of samples + 1)
HLA-DR PE-CY7/ CD69/CD25/ CD45RA	5	5x(no. of samples + 1)
CCR9	1	1x(no. of samples + 1)

Three 5ml polystyrene round bottom FACS tubes were labeled with participant ID, date and panel name i.e. Naïve (CD45RA), Homing Activated I (HLA-DR), Homing Activated II (CD69) and Homing Activated III (CD25). 100µl of EDTA anti-coagulated whole blood and 11.5ul of antibody master mix was added to each tube.

Tubes were vortexed and incubated at room temperature (25^oC) in the dark (so the fluorochromes do not dim) for 15 minutes. This was optimal time to stain the cells. FACS lysing solution was then diluted to x1 concentration with distilled water. 2ml of the diluted lysing solution (lyse the red cells) was added to the tubes after incubation, vortexed and incubated further for 10minutes. The tubes were then centrifuged at 1500 rpm for 5minutes and supernatant discarded leaving behind about 50ul. Cells were resuspended by vortexing and washed in 2ml cell wash at 1500rpm for 5minutes. Supernatant was discarded and tubes vortexed. 250ul cell fix was added and sample was ready for acquisition on the flow cytometer using the FACSuite software. The total number of cells for each subpopulation and the percentage were recorded (Appendix).

4.8 Analysis of specific IgA response in WGLF and serum to antigen preparations of different oral vaccines (Vivotif, Dukoral, Rotarix, Polio)

In order to measure the amount of antibodies that were present in WGLF and serum in response to vaccination, we used an Endpoint Enzyme-linked Immunosorbent assay (ELISA). ELISA is a biochemical technique that is used to measure the amount of antigen or antibody in a sample. It involves at least one antibody with specificity for a particular antigen.

4.8.1 Vaccine Antigen Preparations

Lipopolysaccharides (LPS) and protein extract from Vivotif and Dukoral vaccines were prepared and used in the ELISA immunogenicity assays as follows:

4.8.1.1 Vivotif LPS extraction

LPS was extracted from the typhoid vaccine using a modified phenol water extraction technique (Westphal et al, 1965). Briefly, Vaccine capsule content was weighed and re-suspended at 5% in distilled water and heated to 67°C. An equal volume of hot (67°C) phenol (Sigma) was added and the mixture stirred vigorously every 5 minutes for 15 minutes. The suspension was then chilled to allow the phases to separate and centrifuged at 10000 x g for 15 minutes. The supernatant (with LPS) was transferred to 1.5ml eppendorf tubes. Extraction was repeated with an equal volume of distilled water and the aqueous phases were pooled giving a final volume of 12ml. 6.25ml sodium acetate (final concentration 0.5M) was added to the solution. 10X volume of 95% ethanol was added and kept at -20°C overnight to precipitate the LPS. The precipitate was centrifuged at 2000 x g at 4°C for 10min. Pellet was suspended in 100ul distilled water then precipitate dried and re-suspended in 50ul distilled water and stored at -20°C.

4.8.1.2 Vivotif protein extraction

Protein was extracted from Vivotif vaccine using B-Per bacterial protein extraction reagent kit (Thermo Scientific, Loughborough, UK). Contents from 3 Vivotif capsules were pooled and weighed. B-PER reagent was added following manufacturer's instructions and suspension pipetted up and down until homogeneous. Solution was then incubated at room temperature for 15minutes and centrifuged at 15000g for 5minutes in order to separate the soluble protein from the insoluble proteins. The protein extract was quantified using the BCA protein assay kit microplate procedure (Thermo Scientific, Loughborough, UK).

4.8.1.3 Dukoral LPS extraction

LPS was extracted from Dukoral vaccine also using the phenol-water extraction technique (Westphal et al, 1965). Dukoral was made up to 5% using distilled water and heated to 67^oC. An equal volume of 90% phenol, was also heated to 67^oC and added to the heated Dukoral solution. The mixture was then heated for 15minutes at 67^oC with vigorous stirring every 5minutes.The mixture was then chilled on ice to allow for separation of the phases and centrifuged for 25minutes at 7000rpm for complete phase separation. The upper aqueous layer containing LPS was carefully removed and transferred to a 50ml tube. The process was repeated several times and the aqueous phases (LPS) pooled. Sodium acetate was added to a final concentration of 0.5M and 10 volumes of 95% ethanol and solution placed at -20ºC overnight in order to precipitate the LPS. The precipitate was then centrifuged at (4128rpm) 2000 x g at 4ºC for 10 min and supernatant discarded. Pellet was suspended in 100ul of distilled water and stored at -20^oC until use.

4.8.1.4 Cholera toxin B subunit (CTB)

CTB was purchased from Sigma as 1mg lyophilized powder containing 5% protein. This was then reconstituted in distilled water to a final concentration of 1mg of CTB per ml. This solution contained 0.05 M Tris buffer, pH 7.5, 0.2M NaCl, 3mM NaN_3 and 1mM sodium EDTA. and this was reconstituted in 1ml of distilled water. The stock solution was stored at 4^oC until use.

4.8.2 Vaccine antigen titrations

Vivotif and Dukoral LPS titration

To determine appropriate concentration of LPS antigen to coat plates with, 4 different dilutions (1:10, 1:100, 1:1000, and 1:10000) of antigen was made up in PBS and coated at 100ul /well on microplate (according to the plate layout). The

plate was covered in aluminium foil and incubated at 4°C overnight. The rest of the assay was done according to the ELISA protocol. The dilution picked for the rest of the assays was 1:100.

Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank					blank	blank					blank
B		1:10	1:10	1:10	1:10			1:100	1:100	1:100	1:100	
C		1:10	1:10	1:10	1:10			1:100	1:100	1:100	1:100	
D												
E												
F		1:10000	1:10000	1:10000	1:10000			1:1000	1:1000	1:1000	1:1000	
G		1:10000	1:10000	1:10000	1:10000			1:1000	1:1000	1:1000	1:1000	
H	blank					blank	blank					blank

Vivotif Protein titration

Plates were coated with different protein antigen concentrations: 0.5ug/ml, 1ug/ml, 2ug/ml, 2.5ug/ml, 5ug/ml, 10ug/ml, 15ug/ml, and 20ug/ml. The plates were covered in aluminium foil and incubated at 4°C overnight. The rest of the assay was done according to the ELISA protocol. The working concentration used for ELISA assays was 2.5ug/ml.

Once all the antigens were extracted and titrated, the rest of the ELISA buffers were then prepared:

Table 4-9 ELISA Reagents

Product	**Code**	**Supplier**
Blocker casein in PBS	37528	Thermo scientific
PBS	P4417-100TAB	Sigma
Tween 20	P-1379	Sigma
goat anti-human IgA alkaline phosphatase conjugate	2050-04	Southern biotech
carbonate bicarbonate	C3041-100CAP	Sigma
4-Nitrophenyl phosphate disodium salt hexahydrate (pNPP)	N2765-50TAB	Sigma
ELISA plates (NUNC)	442404	Thermo scientific

1. *Coating buffer*: 10 capsules of carbonate bicarbonate were diluted in 1L distilled water and stored at room temperature
2. *Dilution buffer*: 100ul casein (0.05%) was added to 100ul tween-20 (0.05%) and made up to 200ml with PBS. Buffer was used within a week of preparing.
3. *Washing buffer*: 0.05% Tween-20 in PBS
4. *Blocking buffer*: 1% casein in PBS ready made

4.8.3 Endpoint ELISA assay protocol

Following optimisation of antigen concentrations, 96 well plates were coated with specific antigen diluted in PBS at appropriate concentrations at 100ul per well and incubated at 4°C overnight. Antigen solution was discarded and plates washed 6 times. Plates were then blocked with casein at 200ul/well and incubated for 1 hour at room temperature. During the 1hour incubation, samples diluted at 1:3 in dilution buffer were prepared in 1.5ml eppendorfs. After blocking, the plates were washed 6 times and the prepared samples were added as 3-fold serial dilution down the plate at 100ul per well starting with undiluted WGLF and 1:3 dilutions for serum. All samples were added in replicate and incubated for 2 hours. Just before the 2 hours was up, the detecting secondary antibody, goat anti-human IgA alkaline phosphatase conjugate was prepared in dilution according to manufacturer's specifications (1:4000). At the end of 2 hours, the plate was washed 6 times and secondary antibody added 100µl per well. Plates were incubated for 1 hour. The developing agent *p*-nitrophenyl phosphate disodium salt (*p*NPP) was prepared by dissolving a 20mg tablet into 20ml coating buffer. After incubation, plates were washed 6 times and developing agent added at 100µl per well. Plates were developed in the dark for

30mins then read at 405nm in the EL800 plate reader (BioTek, Potton, UK). Data were analysed using Gen5 software.

4.8.4 Detection of Rotarix-specific IgA and IgG in serum and WGLF.

Analysis of IgA and IgG antibody responses was also done using ELISA. Briefly, 96 well microtiter plates (Thermo Scientific, Denmark) were coated with rabbit hyperimmune serum to rhesus rotavirus (RRV) at a 1: 10,000 dilution and incubated at 4°C overnight. The next day, plates were blocked with blocking buffer (phosphate buffer saline [PBS] supplemented with 5% skim milk), incubated at 37°C, and were subsequently treated with a 1:10 dilution of supernatant from clarified Rotarix vaccine. WGLF and serum samples were diluted serially in diluent buffer (PBS supplemented with 1% skim milk and 0.5% [vol/vol] of 10% polyoxyethyleneether (W1) starting with 1:20 dilution and added to plates at 37°C. Following a 2hour incubation, antibodies were detected with biotin-labeled affinity purified antibody to human IgA (dilution of 1:2000) or IgG (dilution of 1:3000) produced in goat (Kirkegaard and Perry Laboratories, USA), followed by ExtraAvidin (dilution of 1:3000), then visualized by incubation with tetramethylbenzidine (TMB, Sigma). Reaction was stopped with 1 N hydrochloric acid. Plates were read using the MRXe plate reader at wavelength 450 nm.

4.8.5 Detection of polio-specific IgA and IgG in serum.

Analysis of oral polio virus (OPV) specific antibodies in serum was performed by neutralization (kindly performed by Dr David Allen, Public Health England, Colindale, UK).

4.9 Analysis of the effects of different ATRA doses on gut IgA responses and gut homing marker expression

In order to analyse the effect of reducing the frequency of ATRA administration from 8 doses to a single dose, participants were vaccinated with Vivotif alongside either 1dose of ATRA or the full 8 doses. Blood and WGLF were taken at baseline (before vaccination) and 14 days after vaccination as previously described in section 4.6. FACS analysis was done on the whole blood samples and specific IgA responses were analysed using ELISA as described in sections 4.7 and 4.8 respectively.

4.10 *In vitro* analysis of the effects of different concentrations of ATRA on expression of gut homing markers on CD4$^+$ T cells

Participants gave 5ml blood sample at baseline before vaccination (section 4.6.1). A total of 500µl of EDTA treated blood from each participant was used in the *in vitro* studies. 10mg (1capsule) of ATRA was dissolved into 3.32ml of dimethyl sulfoxide (DMSO) to give a 10mM stock solution. 100µl of blood was placed into 5 FACS tubes each. ATRA was added to the blood at final concentrations of 0nM, 1nM, 10nM, 100nM and 1000nM respectively and incubated at room temperature in the dark for 24 hours. The bloods were then stained with appropriate antibodies and sorted as in section 4.7.2.

Figure 4-4: Incubation of whole blood sample at different ATRA concentrations

4.11 To determine the baseline vitamin A status of participants

The quantification of retinol was done using high performance liquid chromatography (HPLC) modified version (Youn-Kyung et al., 2010). This was done at the Tropical disease research centre (TDRC), Ndola Central hospital (Kindly performed by Ng'andwe Kalungwana and Sydney Mwanza). Solvents used were HPLC grade methanol, absolute ethanol, HPLC grade water, all *trans* retinol and retinyl acetate standards all from Sigma-Aldrich. 100µl of serum sample was mixed with 50µl of retinyl acetate and 50µl of ethanol in a clean capped glass tube to release the retinoic acids from its complex. Mixture was vortexed for 30 seconds. The released retinoid was then recovered from the ethanol solution by two extractions with 400µl of n-hexane mixed into a separate clean capped glass tube containing 500µl HPLC grade water. Retinyl acetate served as the internal standard for the analysis. The mixture was then vortexed and the resulting upper hexane extracts evaporated to dryness under a gentle stream of nitrogen gas. The resulting residue in the evaporating tube was dissolved in 50µl of mobile phase and a 20µl of solution was applied on the reverse phase HPLC column and eluted at 1.5ml/min, using 85% HPLC grade methanol and 15% 0.01 M sodium acetate buffer, adjusted to pH 5.2, with detection at 325nm, 343nm and 400nm. The quantification was achieved using the SHIMADZU Prominence LC-2010C HT Liquid Chromatograph with PDA detection. Results were calculated based on the standard equations. Acceptance of results was based on the recovery of $\geq$ 80% and on the results of the pool serum analysed with each batch of samples. The coefficient of variation was calculated and $\leq$5% was acceptable.

4.12 Data Analysis

All data were entered and analysed using STATA 12 (Stata Corp, College Station, TX, USA) and GraphPad Prism 6.0 (La Jolla, CA). Continuous variables were presented as median and interquartiles range. Statistical comparisons were done using nonparametric tests; Mann-Whitney for paired variables and Kruskal-Wallis for multiple comparisons. For parametric analysis, paired or unpaired *t*-test was performed to compare two groups. Spearman's rank correlation coefficient was used for correlations. P values <0.05 were assumed statistically significant.

Chapter 5

5.0 RESULTS I: Baseline characteristics of study participants

Adult male volunteers from a high density community in Lusaka, Zambia were recruited as previously described (Lisulo et al, 2013). Participants were screened for helminth infection and HIV infection and a nutritional assessment was carried out. Altogether, 50 volunteers received Vivotif (an oral typhoid vaccine, see chapter 2), 13 received Rotarix (a rotavirus vaccine), 13 received Dukoral (an oral cholera vaccine) and 9 received an oral polio vaccine. Thirteen participants did not receive any vaccine. Volunteers were randomised to receive one or none of the vaccines with or without 10mg ATRA given daily for 8 days. The 37 participants given Vivotif alongside ATRA participated in either WGLF studies (13 volunteers), dose reduction studies (12 volunteers) or the time course studies (12 volunteers), so these are shown as pooled Vivotif vaccine groups (Table 5-1).

Table 5-1. Baseline characteristics of study participants

										P
n	37	13	7	6	4	5	7	6	13	
Vaccine	Vivotif	Vivotif	Rotarix	Rotarix	polio	polio	Dukoral	Dukoral	none	
ATRA	+	-	+	-	+	-	+	-	+	
Age (years)	34(22-42)	38(29-40)	21(19-25)	39(27-53)	29 (25-33)	20(19-42)	31(28-40)	39(37-40)	28(22-43)	0.43
HIV positive (n)	5	2	0	1	1	1	3	4	3	
BMI (kg/m^2)	20 (19-23)	22(19-23)	20(19-21)	20(19-20)	21 (19-24)	19 (17-25)	20(18-24)	19(18-19)	19 (18-24)	0.43

Volunteers were all healthy adult men randomized to receive one or none of the vaccines with or without 10mg ATRA given daily for 8 days. The 37 participants given Vivotif + ATRA participated in either WGLF studies (13 volunteers), dose reduction studies (12 volunteers) or the time course studies (12 volunteers), so these are shown as pooled Vivotif vaccine groups
Continuous variables are shown as median and interquartile range (IQR).
ATRA: all-*trans* retinoic acid.
BMI: body mass index.

5.1 ATRA enhances gut homing marker expression on circulating $CD4^{+}$T cells when given alongside Vivotif

Homing capacity is important for effector function of T cells *in vivo*. Gut homing α4β7 and CCR9 receptor phenotype on circulating $CD4^{+}$ T cells during oral vaccination in the presence or absence of ATRA was measured at 6 different time points over 14 days. In the time course study, 12 subjects received the first dose of Vivotif an hour after receiving 10mg ATRA then the second and third dose of vaccine on day 3 and day 5 respectively. Six received only Vivotif and another 6 received only ATRA. ATRA was taken as a 10mg capsule for 8 consecutive days. 120 blood samples were obtained at the different time-points from participants and analysed for T cell marker expression.

5.1.1 FACS analysis of participants given Vivotif plus ATRA

There was an overall increase in α4β7 (P=0.003) and CCR9 (P=0.0002) expression on $CD4^{+}$ T cells and total CD4 count (P<0.001) in participants that received Vivotif alongside ATRA (Figure 5-1A-C). Post-hoc analysis day by day revealed a slight fall in $\alpha4\beta7^{+}$ $CD4^{+}$ T cells at day 3 (P=0.02) and day 8 (P=0.01) Figure 5-1A.

A

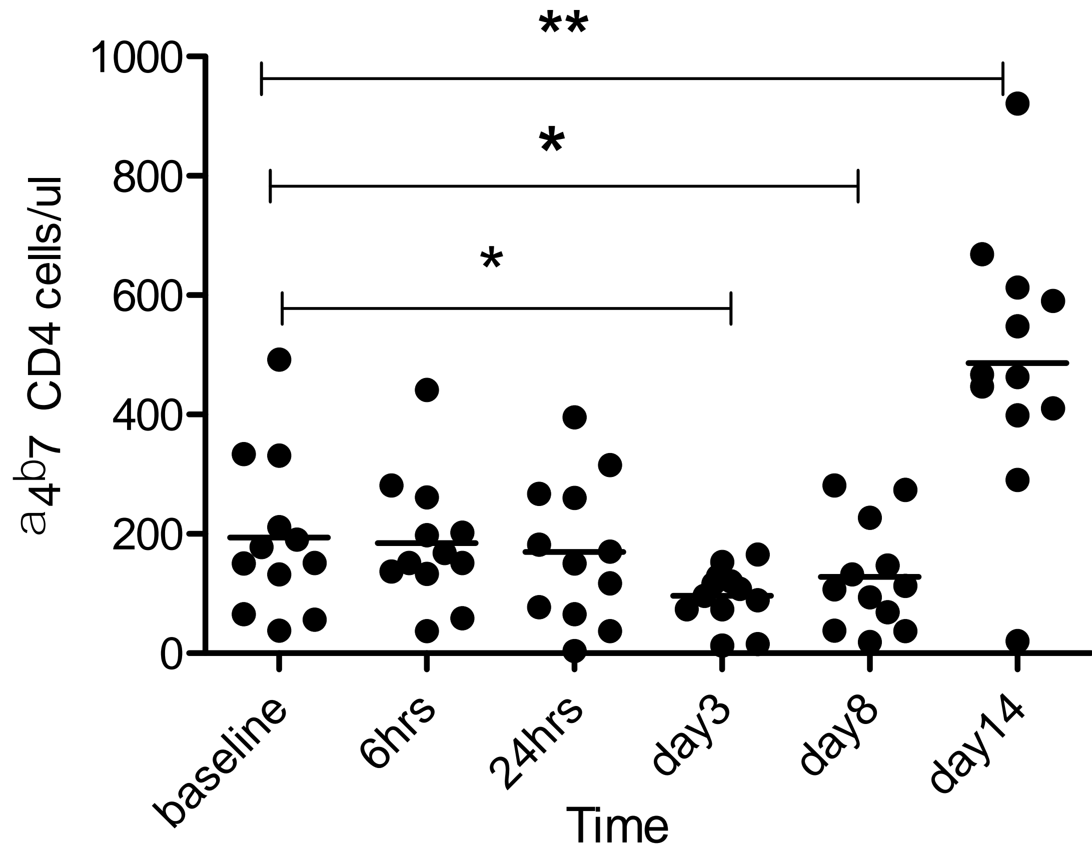


Figure 5-1A: 10mg ATRA following oral Vivotif vaccination enhanced α4β7-integrin expression on circulating $CD4^+$ T cells (P=0.003). integrin expression on T cells were measured at different time points over 14 days. There was gradual drop in expression of α4β7 on day 3 (**P*=0.021) and day 8 (**P*=0.01)

B

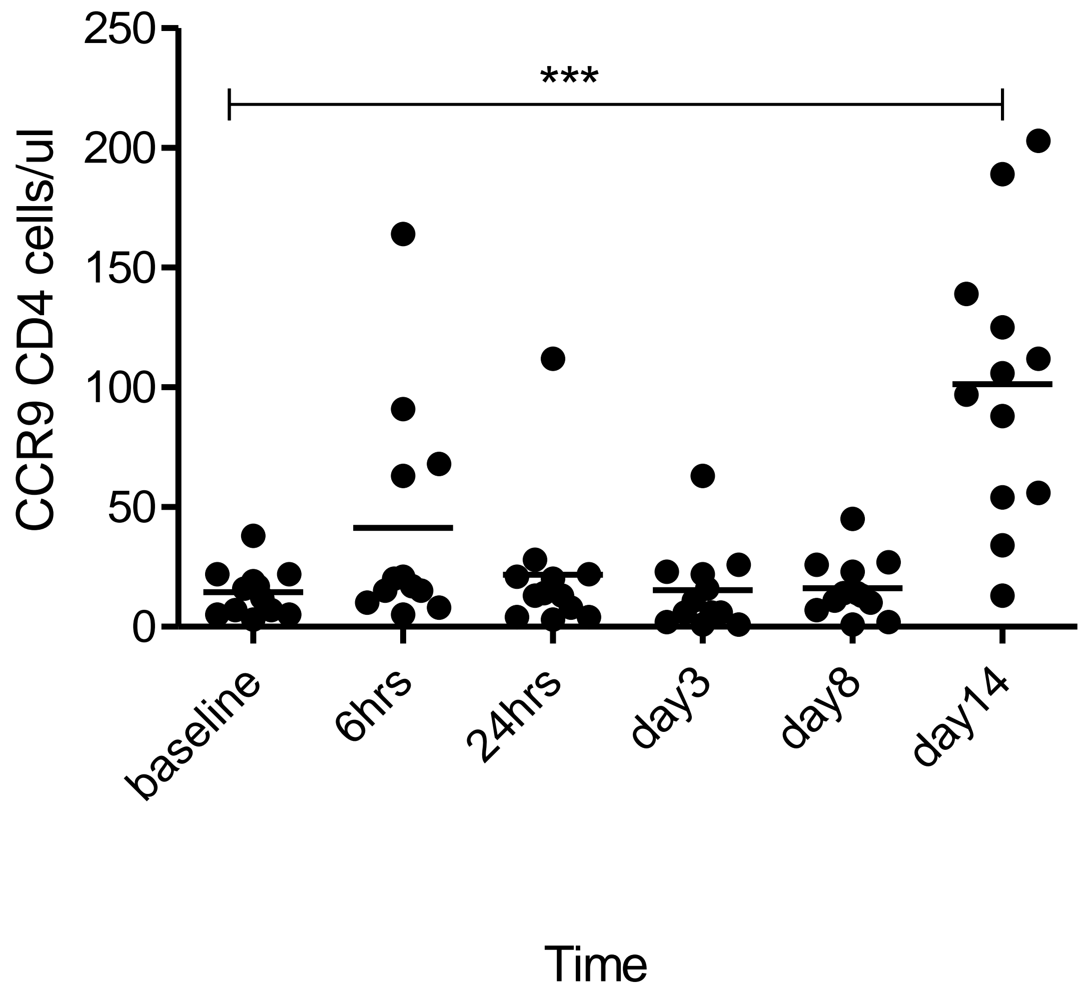


Figure 5-1B: CCR9 expression on circulating $CD4^+$ T cells was measured at different time points. Expression of the gut homing marker was increased significantly (****P*=0.0002) in the group that received Vivotif with ATRA

C

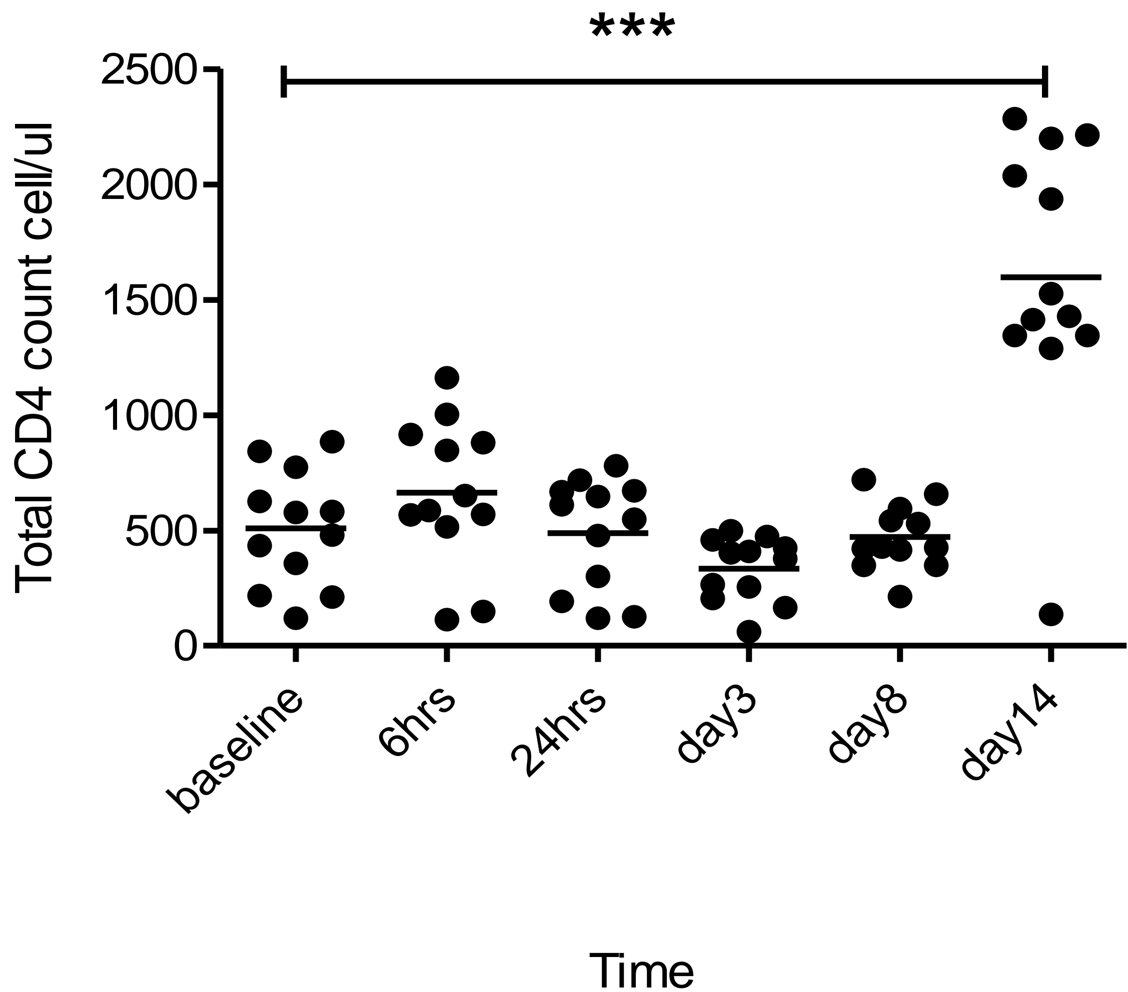


Figure 5-1C: Total CD4 count was measured at different time-points over 14days. There was significant increase (***P*<0.0001) in the CD4 count in the participants that had received Vivotif alongside ATRA.

5.1.2 FACS analysis of participants given Vivotif without ATRA

Participants who received Vivotif vaccine alone (Figure 5-2A-C) without ATRA treatment showed no overall increase in gut homing marker expression, α4β7 and CCR9, or the total CD4 count when measured over 14 days. However, post-hoc analysis day by day revealed a fall (P=0.025) in $\alpha4\beta7^{+}$ $CD4^{+}$ T cells at day 14

(Figure 5-2A) and in total CD4 count (P=0.001) (Figure 5-2C). No difference was seen on CCR9 expression (Figure 5-2B).

A

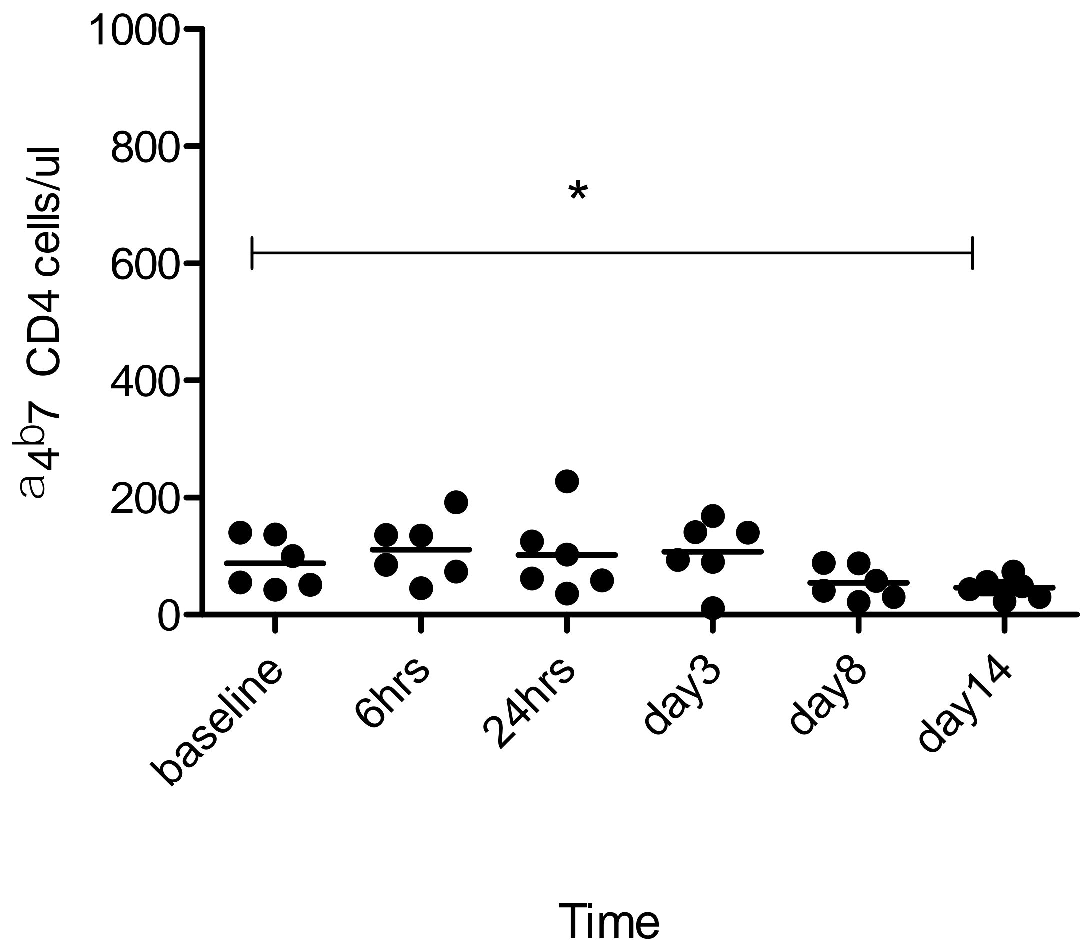


Figure 5-2A: Vivotif vaccination alone did not show any significant increase in α4β7-integrin expression on circulating $CD4^+$ T cells.

B

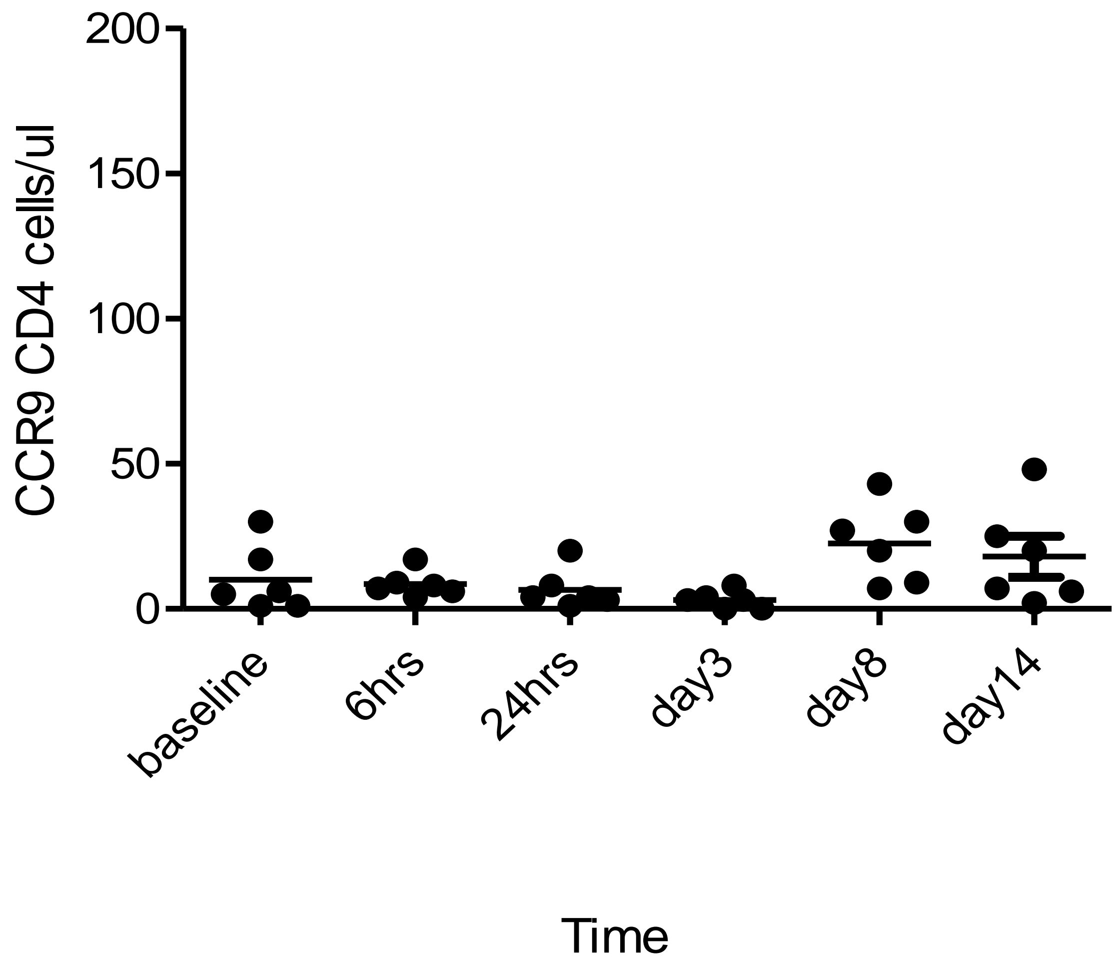


Figure 5-2B: CCR9 expression on circulating $CD4^+$ T cells was measured at different time points. There was no significant difference in expression of the gut homing marker on CD4 cells in participants given Vivotif without ATRA.

C

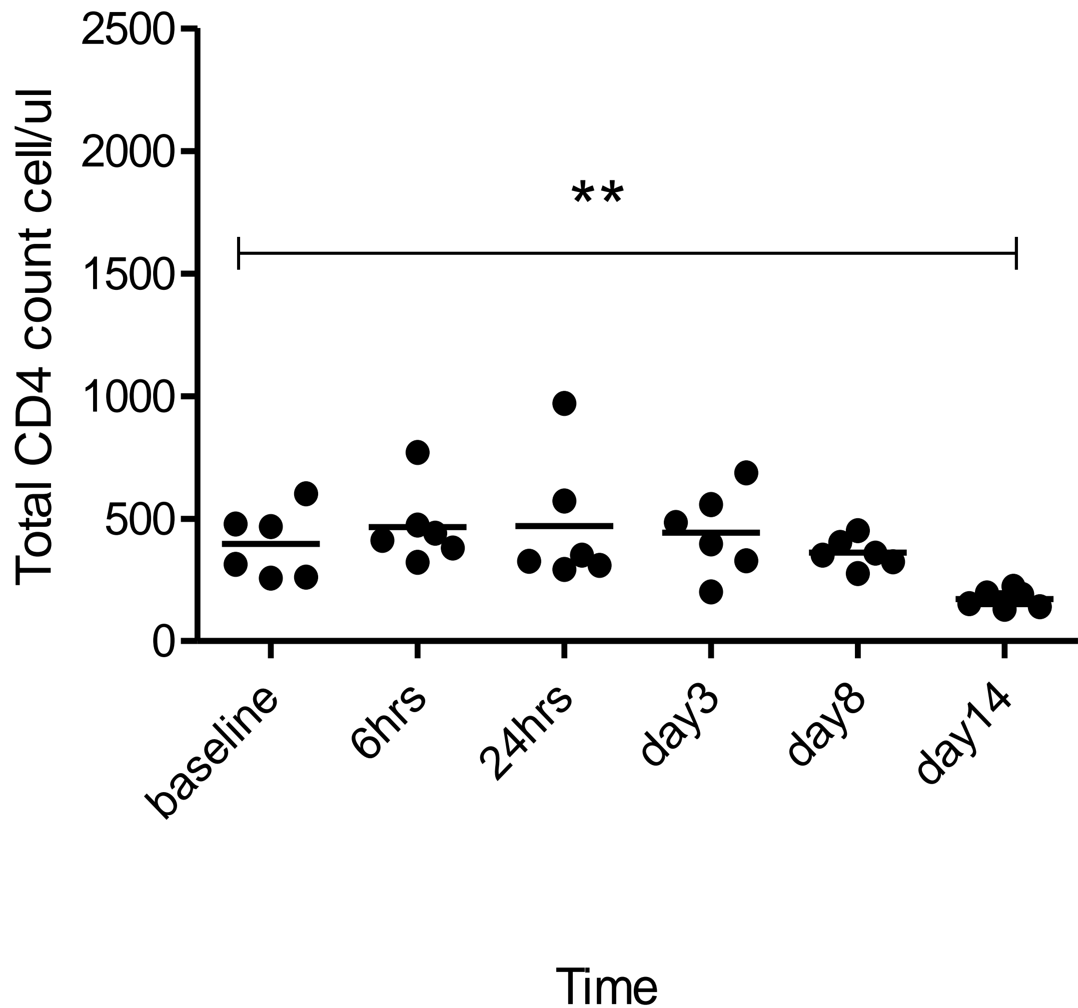


Figure 5-2C: Total CD4 count was measured at different time-points over 14days. There was a significant decrease (P=0.001) in the CD4 count in the participants that had received Vivotif without ATRA.

5.1.3 FACS analysis of participants given ATRA alone

Participants who received ATRA treatment without vaccination showed no overall increase in gut homing marker expression, α4β7 and CCR9, or the total CD4 count when measured over 14 days (Figure 5-3A-C). Post-hoc analysis day by day

revealed a fall (P=0.01) in $\alpha4\beta7^{+}$ CD4 $^{+}$ T cells at day 14 (Figure 5-3A). No difference was seen on CCR9 expression (Figure 5-3B) or CD4 count (Figure 5-3C)

A

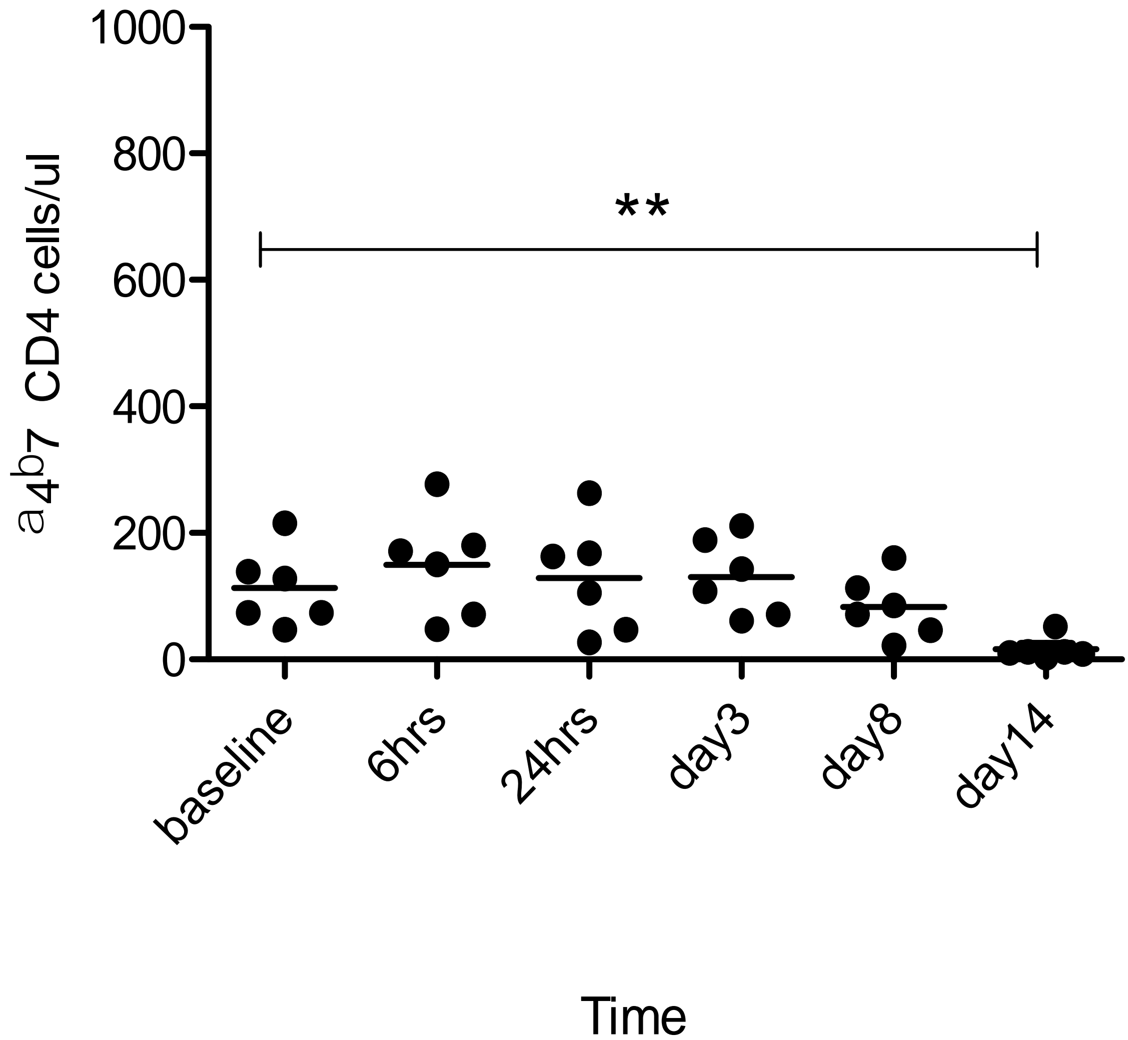


Figure 5-3A: Data revealed a drop in α4β7 expression (***P*=0.01) in participants who receive ATRA alone.

B

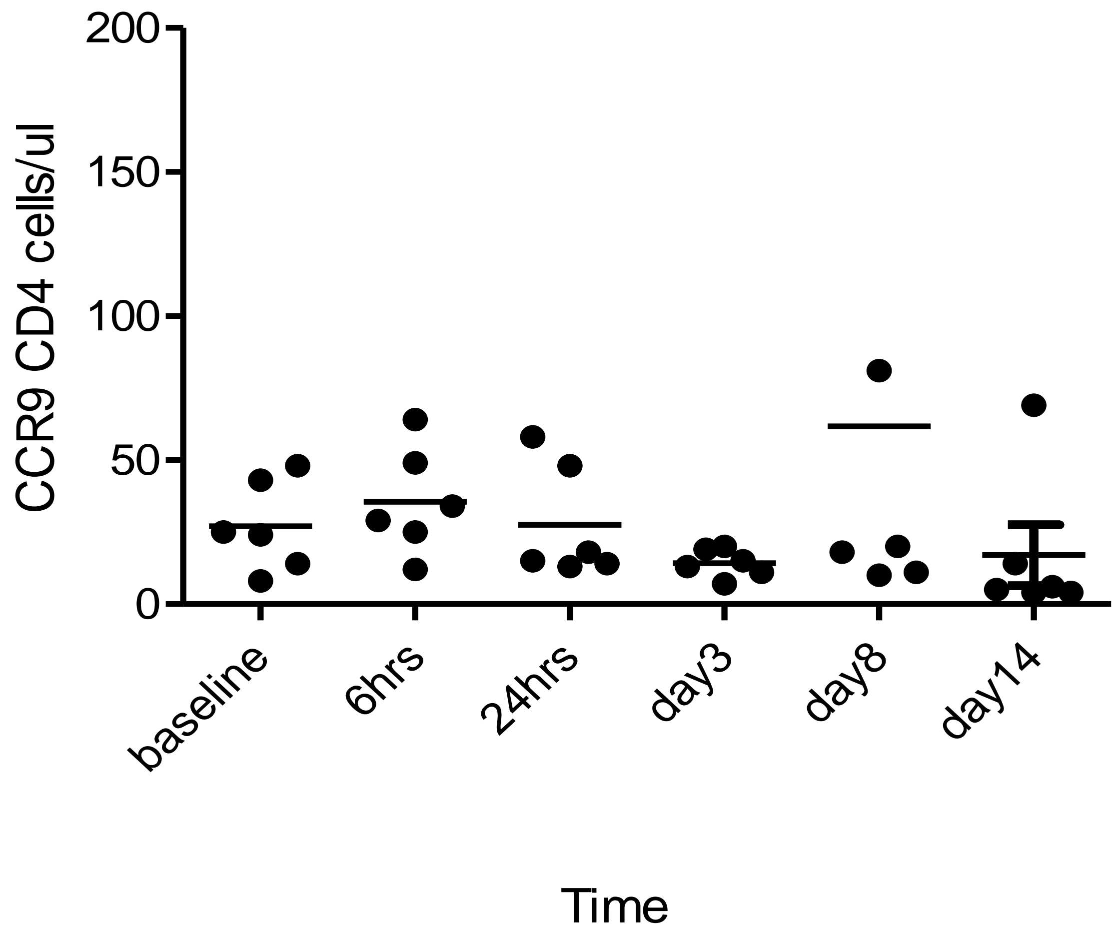


Figure 5-3B: CCR9 expression on circulating $CD4^+$ T cells was measured at different time points. There was no significant difference in expression of the gut homing marker on CD4 cells in participants given ATRA alone.

C

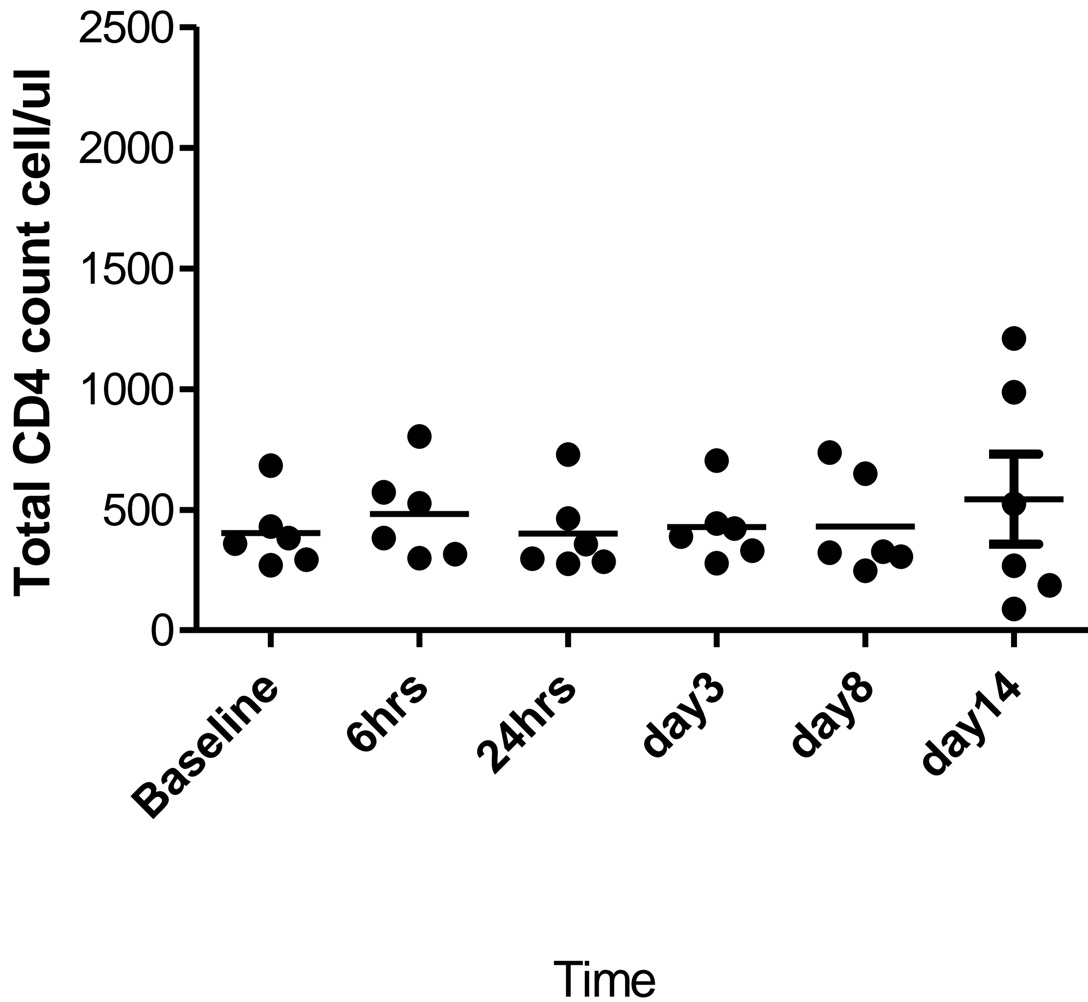


Figure 5-3C: Total CD4 count was measured at different time-points over 14days. There was no significant increase in the CD4 count in the participants that had received ATRA alone.

These data suggest that ATRA treatment during oral vaccination with Vivotif enhances expression of gut homing markers in a time dependent manner and confirms that 14 days is an appropriate interval over which to assess responses.

5.1.4 Expression of activation markers on CD4+ T cells

Three activation markers were also analyzed and results revealed that overall, expression of HLA-DR was more (P=0.04) on all the CD4 cells (Figure 5-4A). When I analysed the gut homing T cell subsets, I found that the activation marker expression was comparable (Figure 5-4A-C)

A

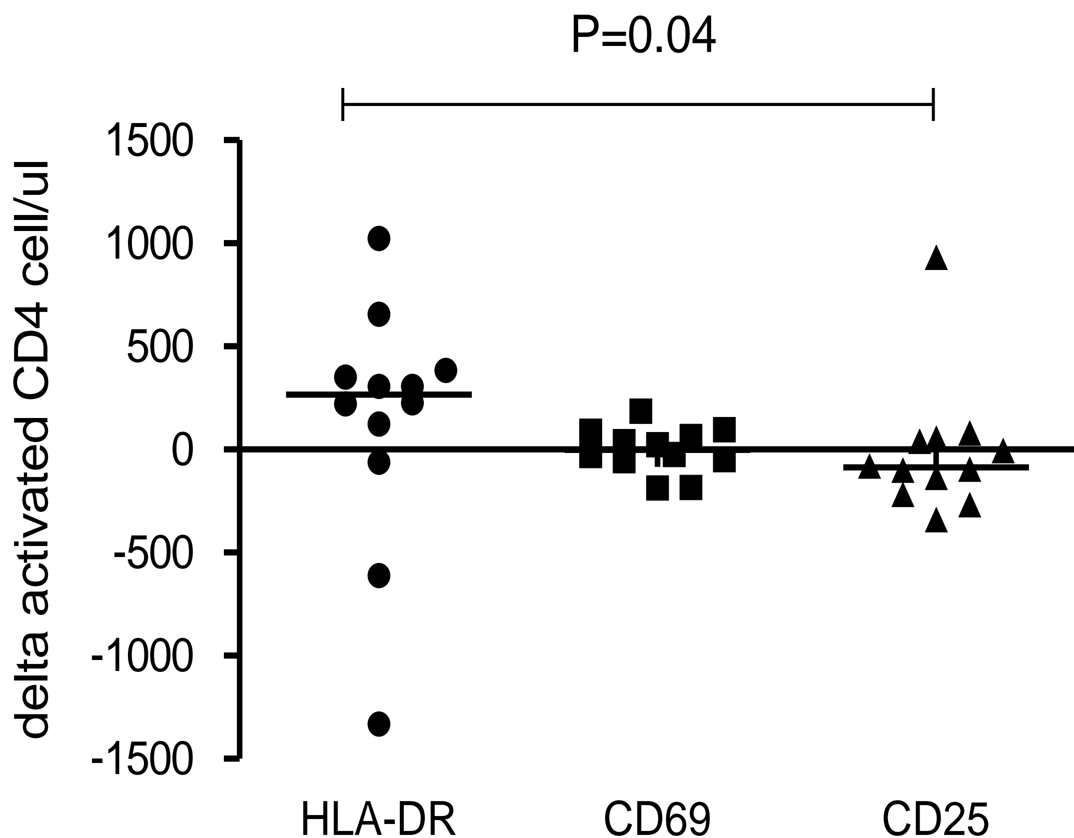


Figure 5-4A: Expression of HLA-DR, CD69 and CD25 on $CD4^+$ T cells from peripheral blood during Vivotif vaccination. HLA-DR expression on total CD4 cells was significantly higher (P=0.04) than the other activation markers.

B

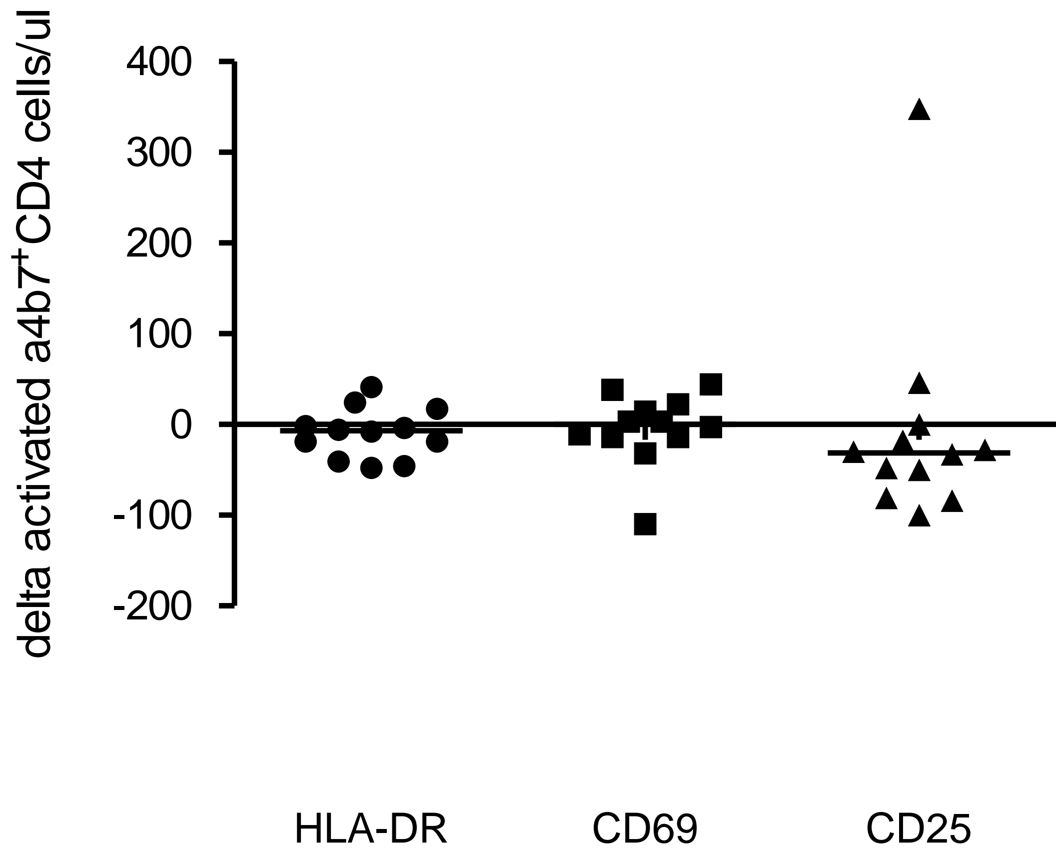


Figure 5-4B: Expression of HLA-DR, CD69 and CD25 on CD4^{+} T cells from peripheral blood during Vivotif vaccination. The was no significant difference in activation markers on the gut homing CD4 cells

C

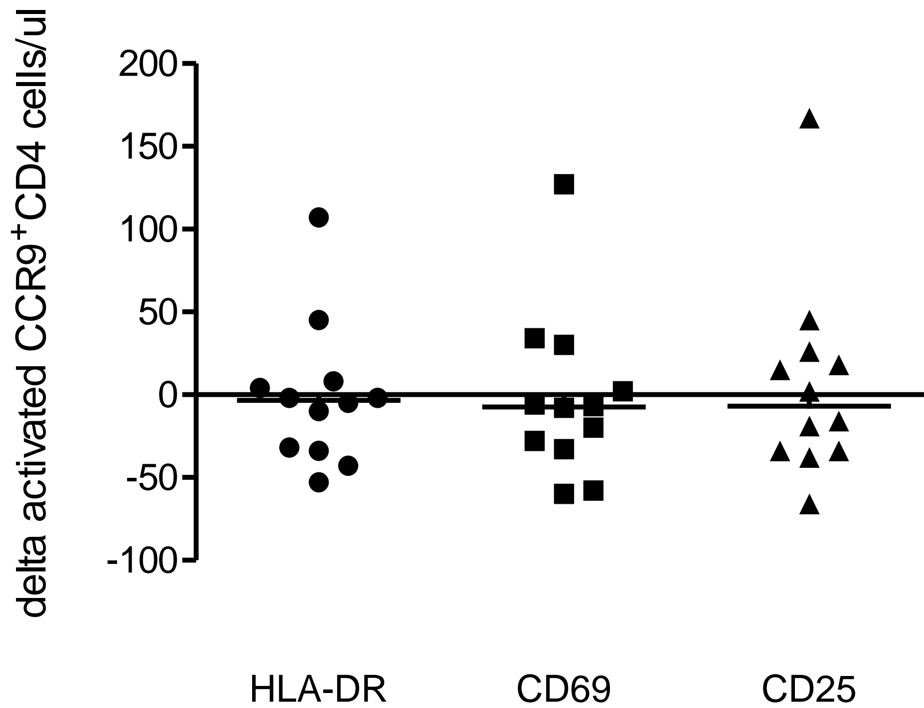


Figure 5-4C: Expression of HLA-DR, CD69 and CD25 on CD4$^+$ T cells from pheripheral blood during Vivotif vaccination. The was no significant difference in activation markers on the gut homing CD4 cells

5.2 ATRA effect on gut homing marker expression on circulating CD4$^+$ lymphocytes during Rotarix and polio vaccination

FACS analysis of expression of gut homing markers at baseline and 14 days post vaccination in participants that received Rotarix or polio vaccine simultaneously with 10mg ATRA and those that received vaccine alone was done. All the vaccine groups showed no significant differences in either α4β7 or CCR9 gut marker expression on the CD4$^+$ T cells (Figure 5-5).

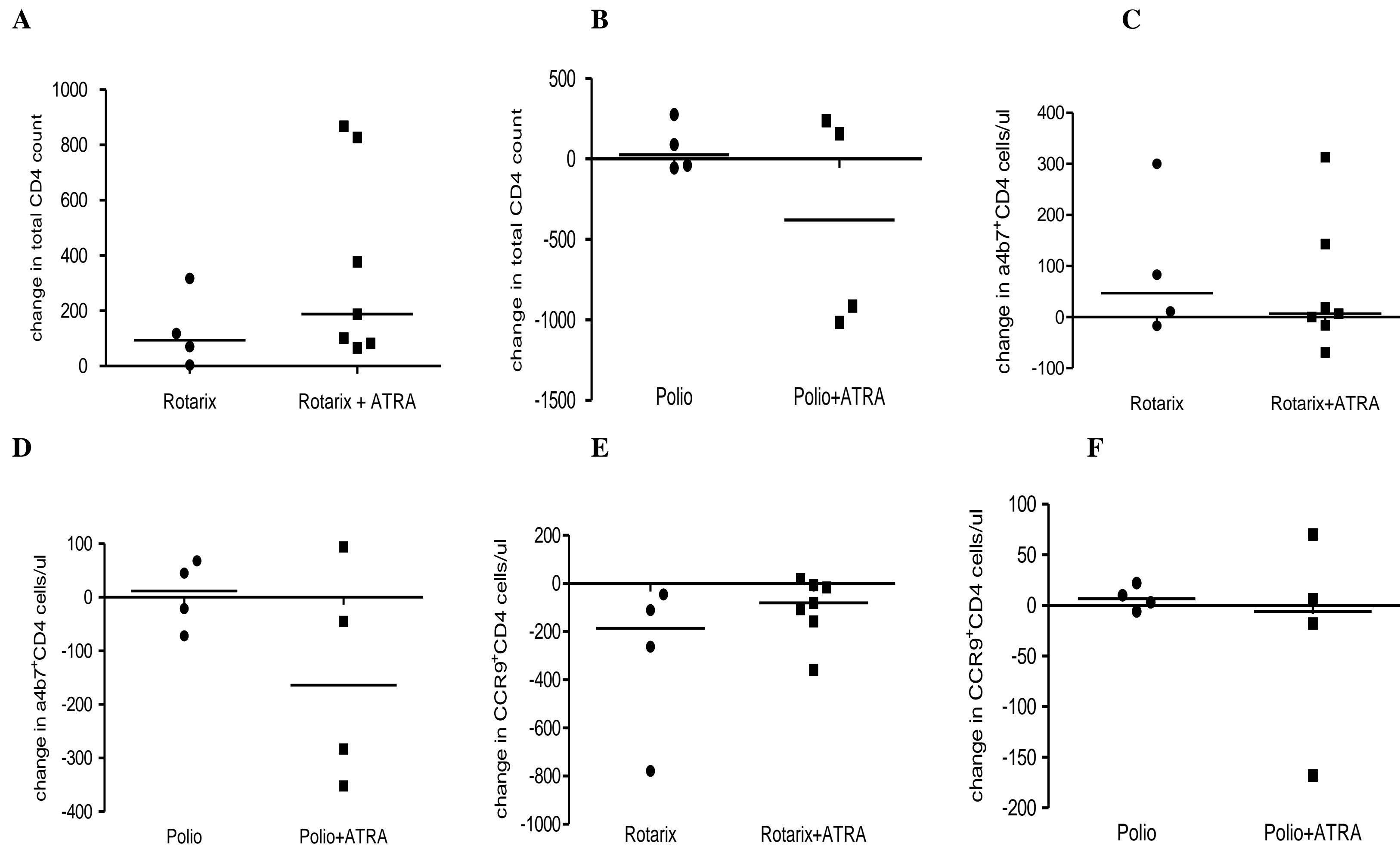


Figure 5-5: Effect of ATRA on circulating CD4 cells and gut marker expression in Rotarix and polio vaccine recipients. There was no difference in total CD4 count (A, B) and expression of either α4β7 (C, D) or CCR9 (E, F) between the vaccine plus ATRA and vaccine only groups.

5.2.1 ATRA effect on gut homing marker expression on circulating $CD8^+$ lymphocytes during Rotarix and polio vaccination

CD8 cells mediate most antiviral responses through Class I presentation so I analysed CD8 subsets. FACS analysis of expression of gut homing markers on $CD8^+$ T cells at baseline and 14 days post vaccination in participants that received Rotarix or polio vaccine simultaneously with 10mg ATRA and those that received vaccine alone was done (Figure 5-6A-F). Interesting, data showed a significant increase (P=0.04) in total CD8 cells in participants that were vaccinated with Rotarix alongside ATRA (Figure 5-6A). However, there was no significant difference in gut homing marker expression for participants given either of the two vaccines (Rotarix or polio) with or without ATRA (Figure 5-6 B, C, D, E and F).

A

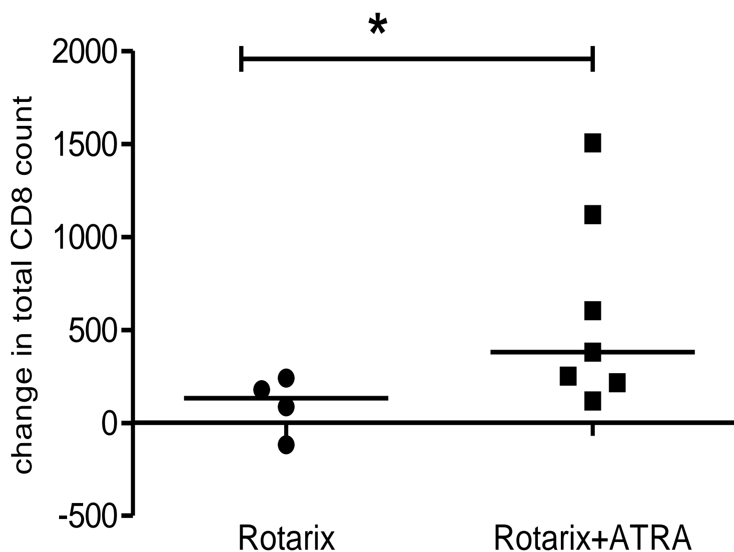


Figure 5-6A: Effect of ATRA on circulating CD8 cells in Rotarix vaccine recipients. Participants who received Rotarix vaccine with ATRA showed a significant increase (*P=0.04) in total $CD8^+$ T cells.

B

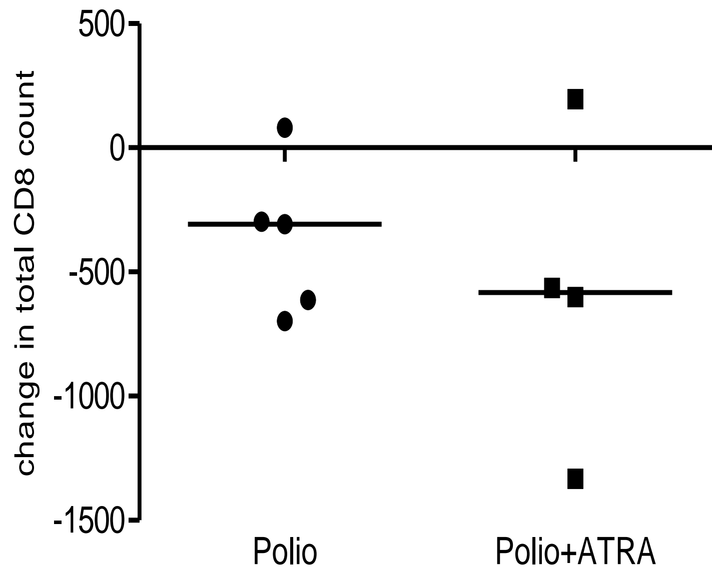


Figure 5-6B: Effect of ATRA on circulating CD8 cells in polio vaccine recipients. No significant difference in total CD8 count was seen between the two groups.

C

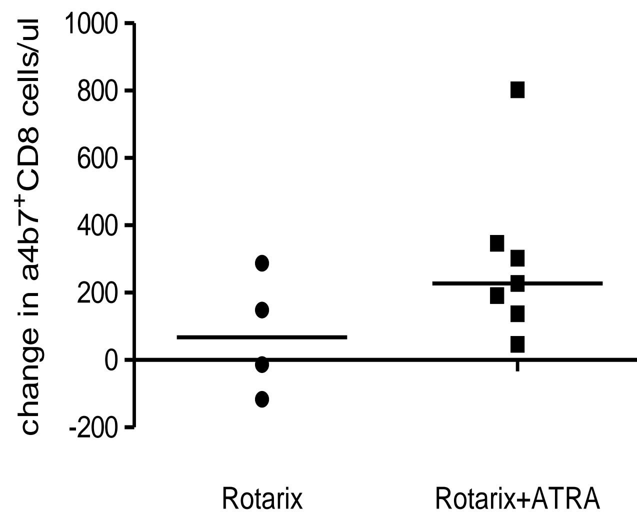


Figure 5-6C: Effect of ATRA on α4β7+CD8 cells in Rotarix vaccine recipients. No difference in expression of α4β7 was seen.

D

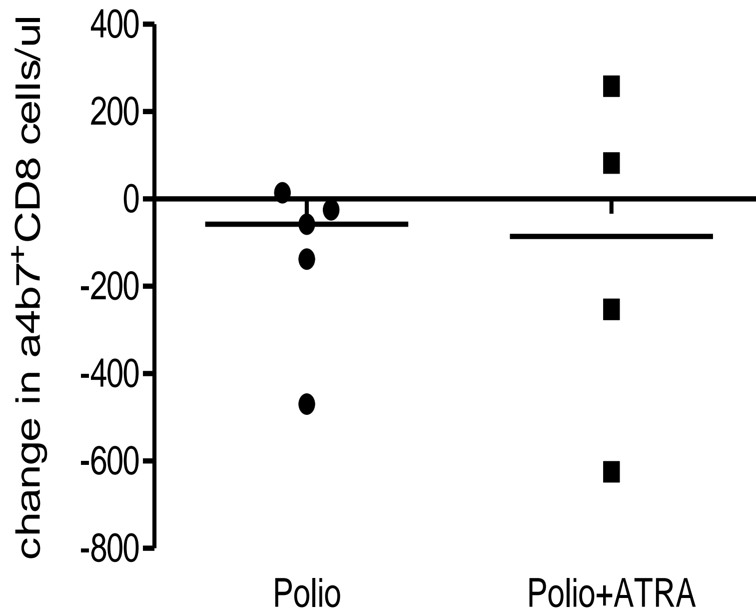


Figure 5-6D: Effect of ATRA on circulating CD8 cells in polio vaccine recipients. No difference in expression of α4β7 was seen.

E

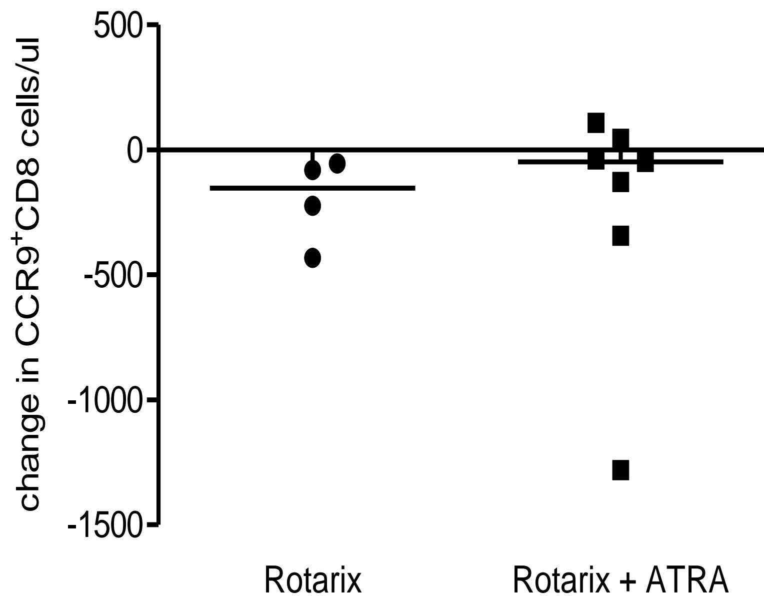


Figure 5-6E: Effect of ATRA on circulating CD8 cells in Rotarix vaccine recipients. No difference in expression of CCR9 was seen.

F

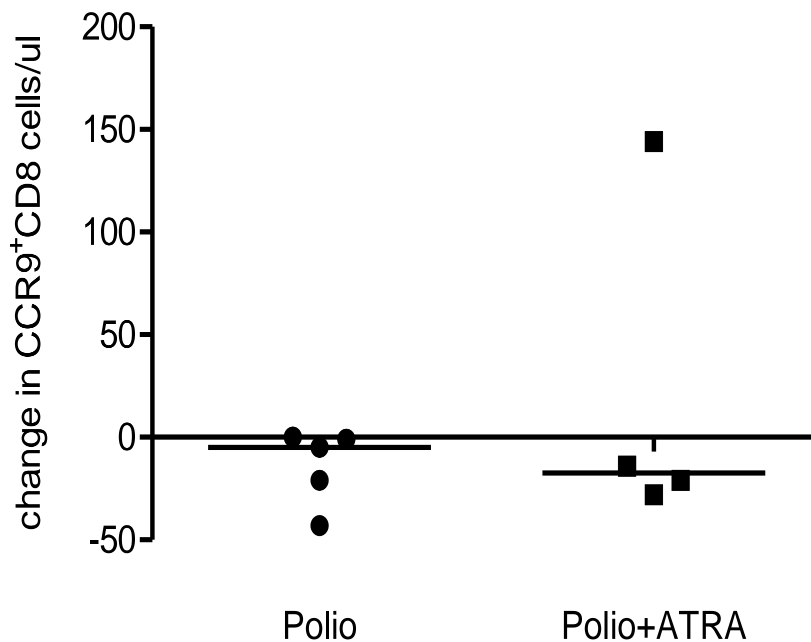


Figure 5-6F: Effect of ATRA on circulating CD8 cells in polio vaccine recipients. No difference in expression of CCR9 was seen.

5.3 ATRA effect is vaccine dependent

In order to investigate whether the effect of ATRA was vaccine dependent, I compared groups that received vaccine together with ATRA to the groups that received ATRA alone at baseline and day 14 post vaccinations. I found that there was a significant increase in expression of α4β7 on activated $CD4^{+}$T cells in the Vivotif plus ATRA group (*P=0.049) and the Rotarix plus ATRA group (*P=0.022) (Figure 5-7A). Analysing expression of α4β7 on the activated $CD8^{+}$T cells (Figure 5-7B) revealed a significant increase of the marker in the Vivotif plus ATRA (* P=0.028) and Rotarix plus ATRA (**P=0.002) groups when compared to those that received only ATRA. CCR9 expression was significantly increased (*P=0.02) only on the activated $CD4^{+}$T cells in the Vivotif plus ATRA group (Figure 5-7C). There

was no difference in CCR9 expression on the activated CD8$^+$ T cells for any of the groups (Figure 5-7D).

A

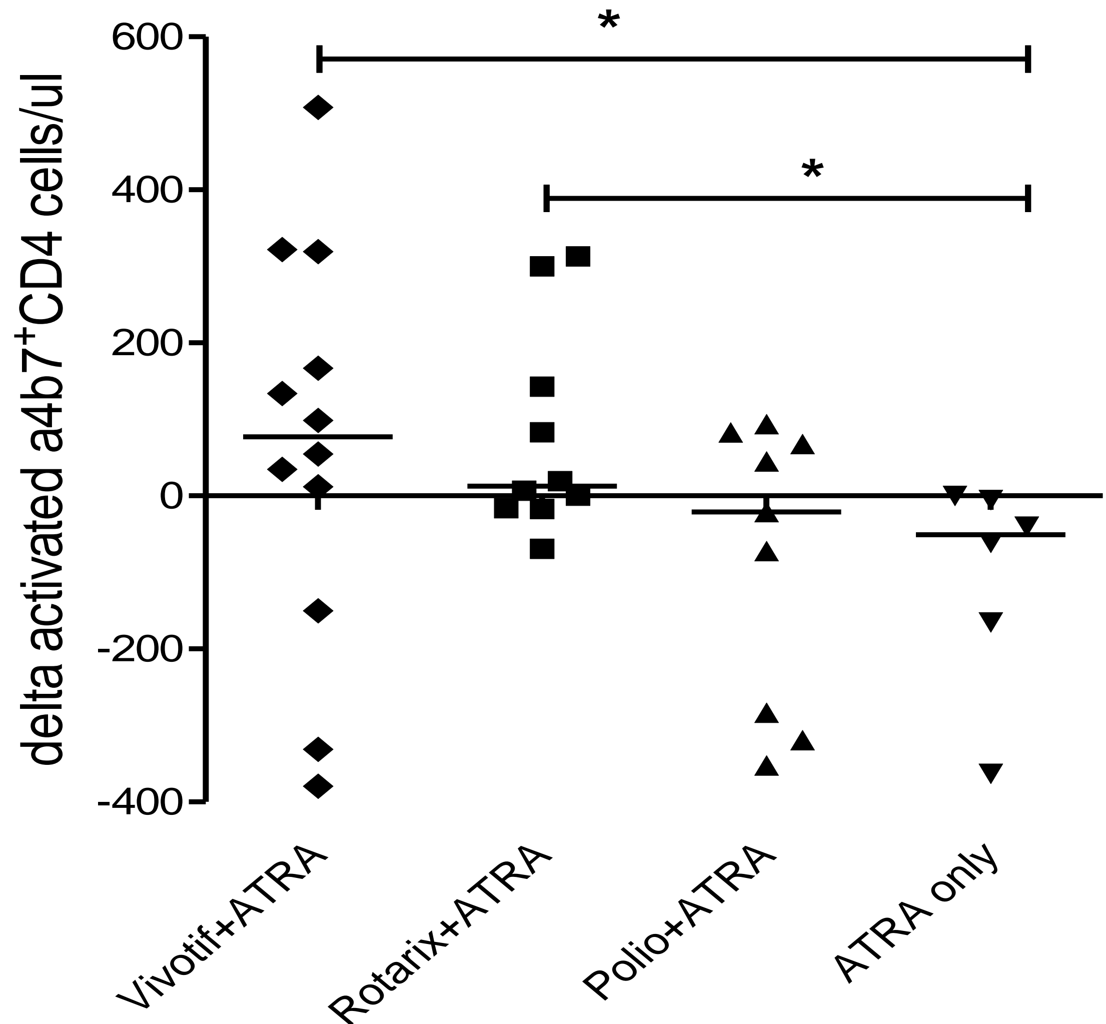


Figure 5-7A: Summary of changes in α4β7 expression on activated CD4$^+$ T cells in volunteers given one of 3 vaccines (Vivotif, Rotarix or polio) together with ATRA and those given ATRA alone. Change in α4β7 expression on activated CD4$^+$ T cells was significantly higher in participants that received Vivotif with ATRA (*P=0.049) and Rotarix with ATRA (*P=0.022).

B

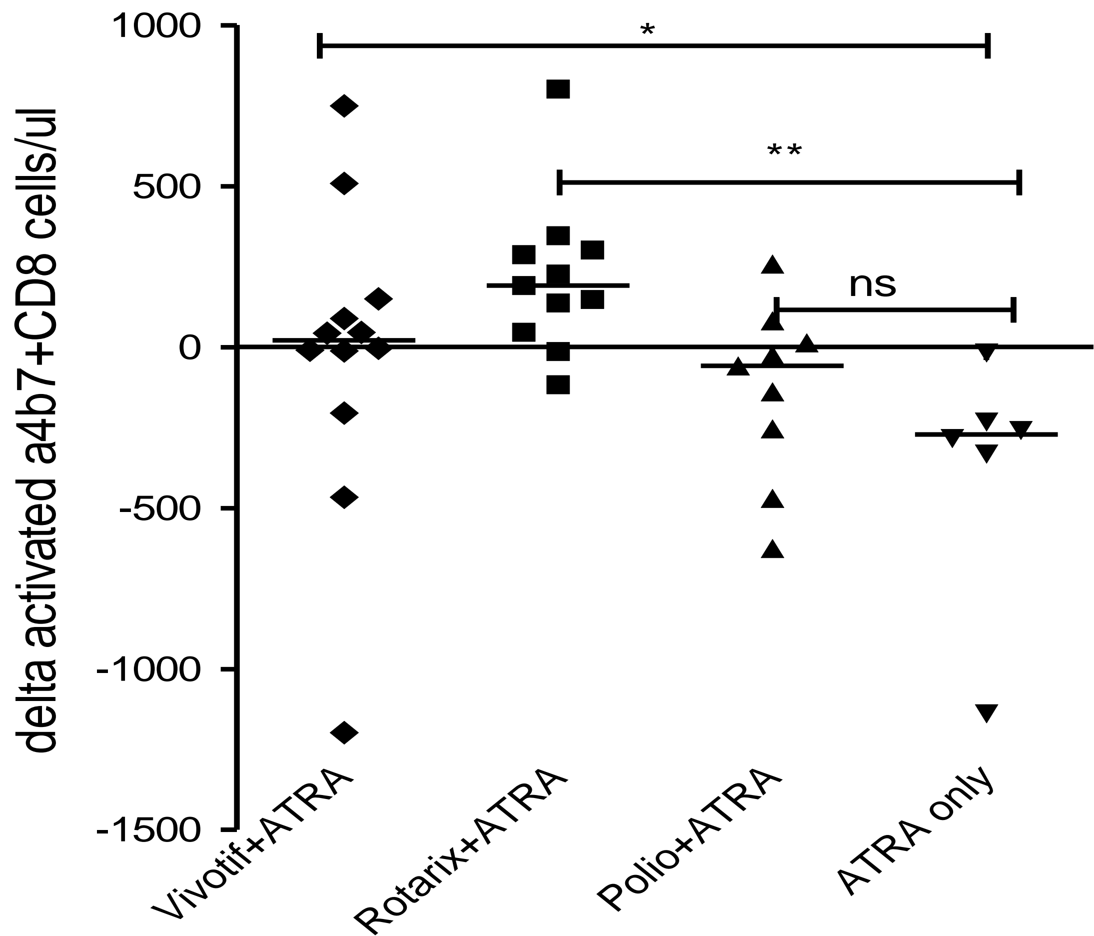


Figure 5-7B: Summary of changes in α4β7 expression on activated $CD8^+$ T cells in volunteers given one of 3 vaccines (Vivotif, Rotarix or polio) together with ATRA and those given ATRA alone. Change in α4β7 expression on activated $CD8^+$ T cells was also significantly higher in the group that received Vivotif with ATRA (*P=0.028) and Rotarix with ATRA (**P=0.002).

C

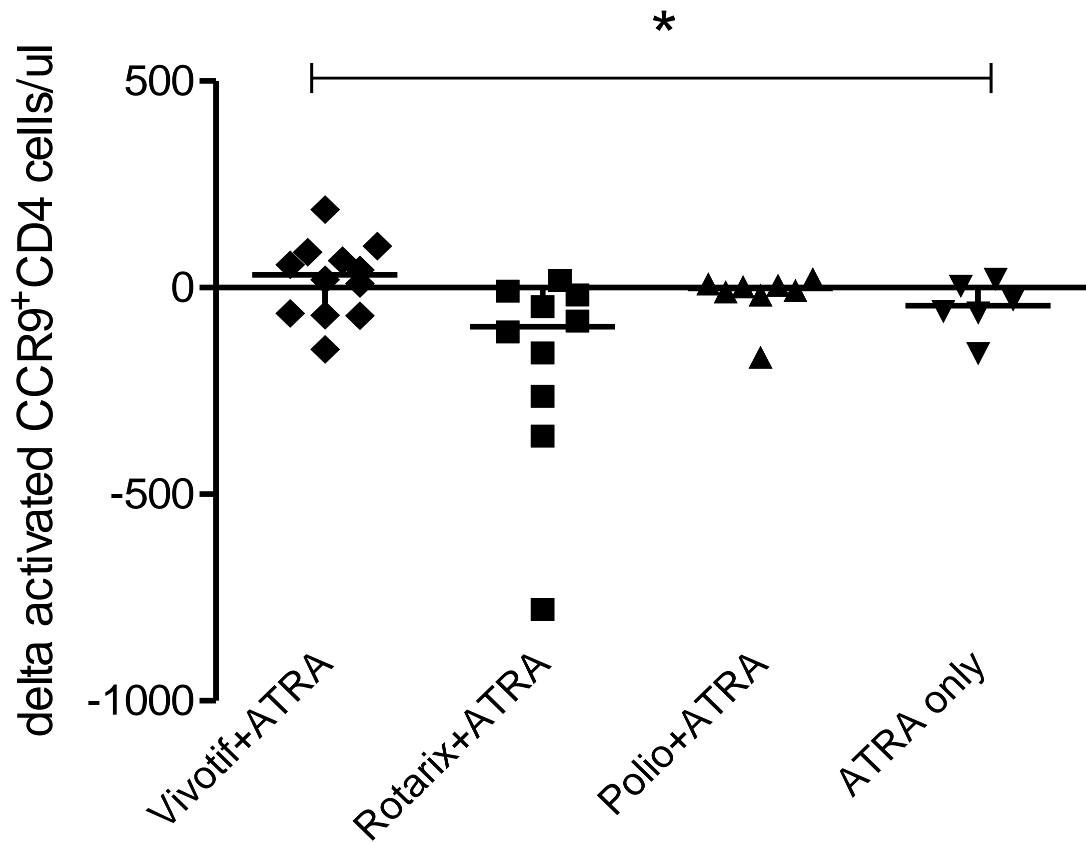


Figure 5-7C: Summary of changes in CCR9 expression on activated $CD4^+$ T cells in volunteers given one of 3 vaccines (Vivotif, Rotarix or polio) together with ATRA and those given ATRA alone. Change in CCR9 expression on activated $CD4^+$ T cells was significantly higher in participants that received Vivotif with ATRA (*P=0.02).

D

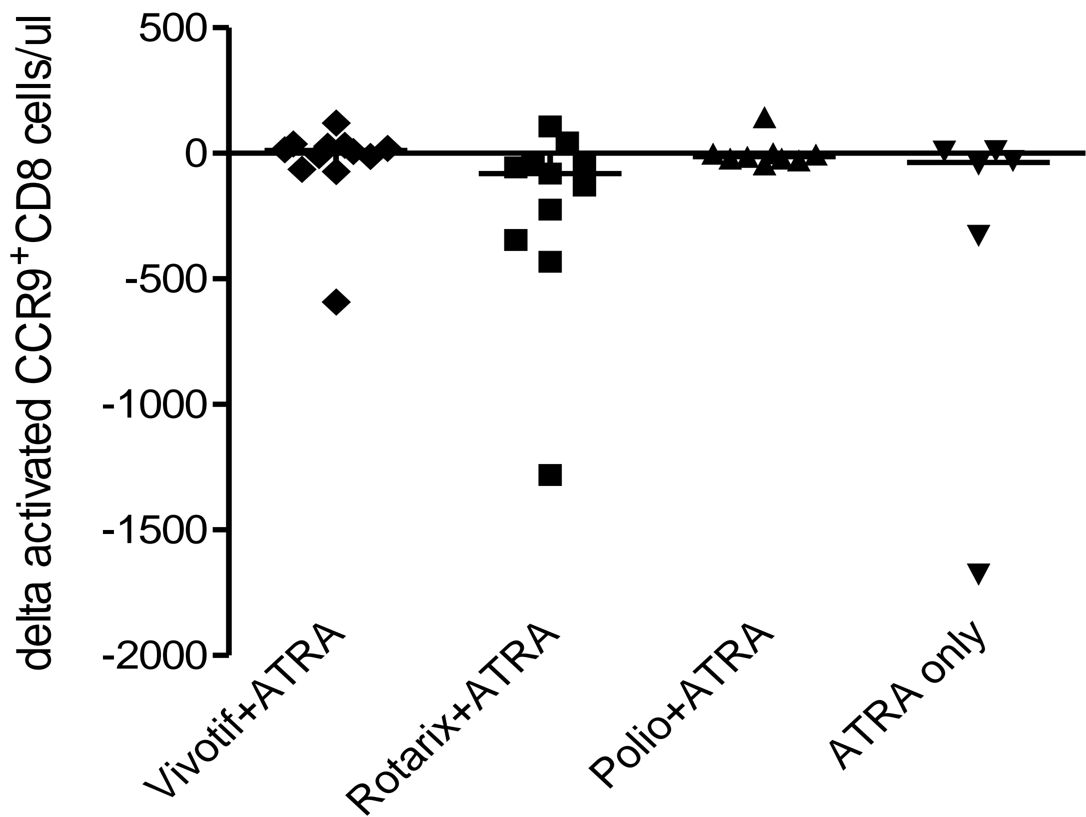


Figure 5-7D: Summary of changes in CCR9 expression on activated $CD8^+$ T cells in volunteers given one of 3 vaccines (Vivotif, Rotarix or polio) together with ATRA and those given ATRA alone. There was no significant difference in CCR9 expression on the activated $CD8^+$ T cells for any of the groups.

CHAPTER 6

6.0 RESULTS II

6.1 Effects of ATRA on gut IgA and IgG responses to oral vaccines

Whole gut lavage fluid samples were used to test for ATRA effects on gut IgA and IgG antibody responses to vaccine-specific antigens between the day of vaccination and day 14. Antibody titres to Vivotif, Dukoral and Rotarix were measured as change in log titres in response to vaccination given with or without 10mg ATRA daily for 8 days. IgA antibody titres in whole gut fluid against Vivotif LPS antigen were significantly increased (Figure 6-1A) in the group that received Vivotif vaccine alongside ATRA but there was no significant change in IgA responses to Dukoral (whether LPS or CTB) antigens or Rotarix antigens (Figure 6-1 B, C, D). The IgG antibody titres in Vivotif and Dukoral recipients were also measured. Data showed no significant difference in the vaccine groups (Figure 6-1E, F).

I went on to examine serum from Vivotif, Dukoral, Rotarix and polio vaccine recipients. There was no significant change in the levels of vaccine-specific serum IgA and IgG in any of the vaccine groups (Figure 6-2). Together, these data suggest that the ATRA effect is mediated in the mucosal compartment when given with Vivotif vaccine and does not alter the responses to the other oral vaccines tested.

A

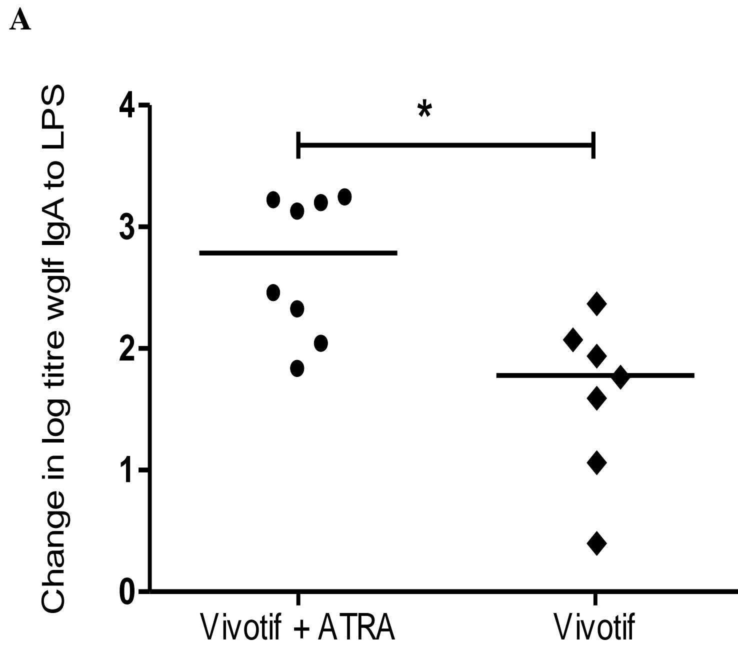
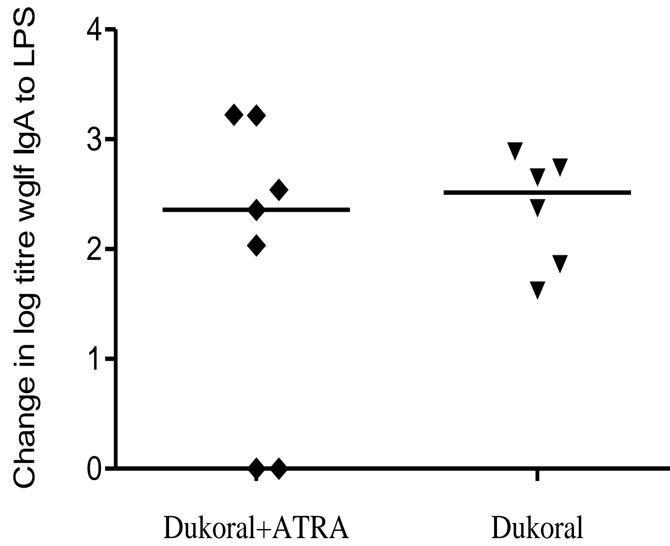


Figure 6-1A: Change in specific IgA in whole gut fluid from vaccine recipients as measured at baseline and day 14 post vaccinations. Antigen-specific IgA responses to Vivotif were measured in whole gut fluid (WGLF). Specific IgA in wglf against Vivotif LPS was increased (**P*=0.01) in Vivotif recipients who received ATRA.

B



C

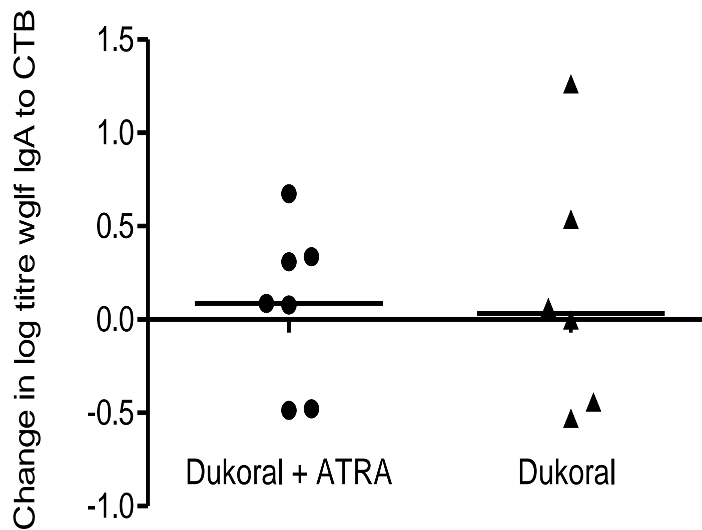


Figure 6-1B and C: Change in specific IgA in whole gut fluid from vaccine recipients as measured at baseline and day 14 post vaccinations. Antigen-specific IgA responses to Vivotif and Dukoral LPS, CTB and Rotarix antigen were measured in whole gut fluid (WGLF). No change was seen with (**B**) Dukoral LPS and (**C**) Dukoral CTB. Responses are shown measured as change in log titres.

D

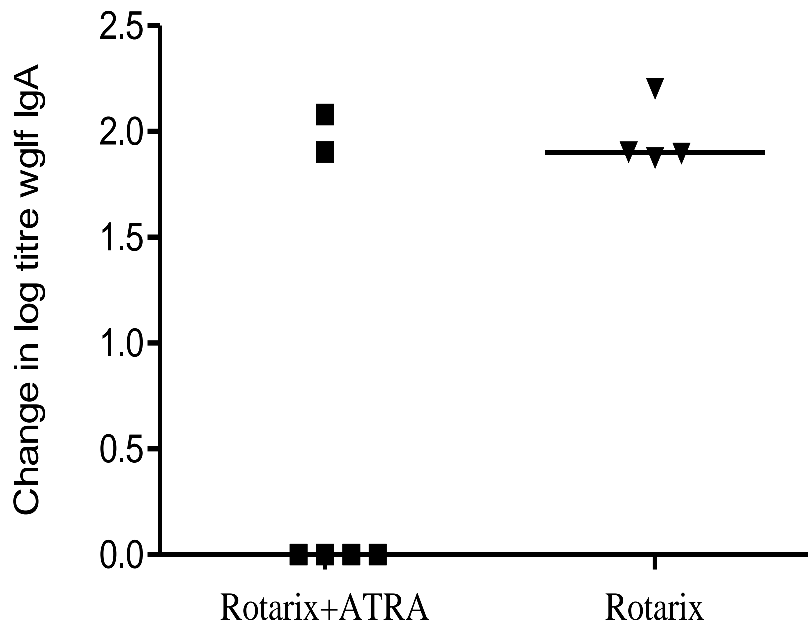
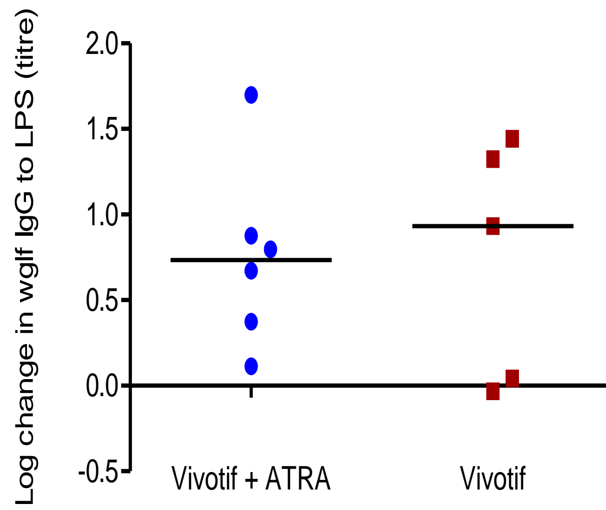


Figure 6-1D: Change in specific IgA in whole gut fluid from vaccine recipients as measured at baseline and day 14 post vaccinations. Antigen-specific IgA responses to Rotarix antigen were measured in whole gut fluid (WGLF). There was no difference between the two vaccine groups.

E



F

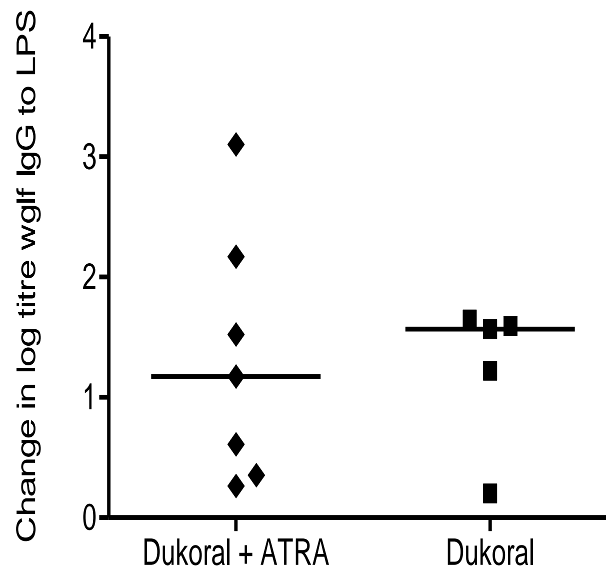


Figure 6-1E and F: Summary of changes in wglf IgG in response to vaccine antigens. No significant difference in wglf IgG to (E) Vivotif LPS or (F) Dukoral LPS was seen in the participants given vaccine plus ATRA or those given vaccine alone.

6.2 Effects of ATRA on serum IgA and IgG response to oral vaccines

Serum from vaccine recipients was also analysed for IgA and IgG responses to vaccine antigens. I found that there was no significant difference between the vaccine plus ATRA recipients and those that got the vaccine alone for all vaccines tested.

A

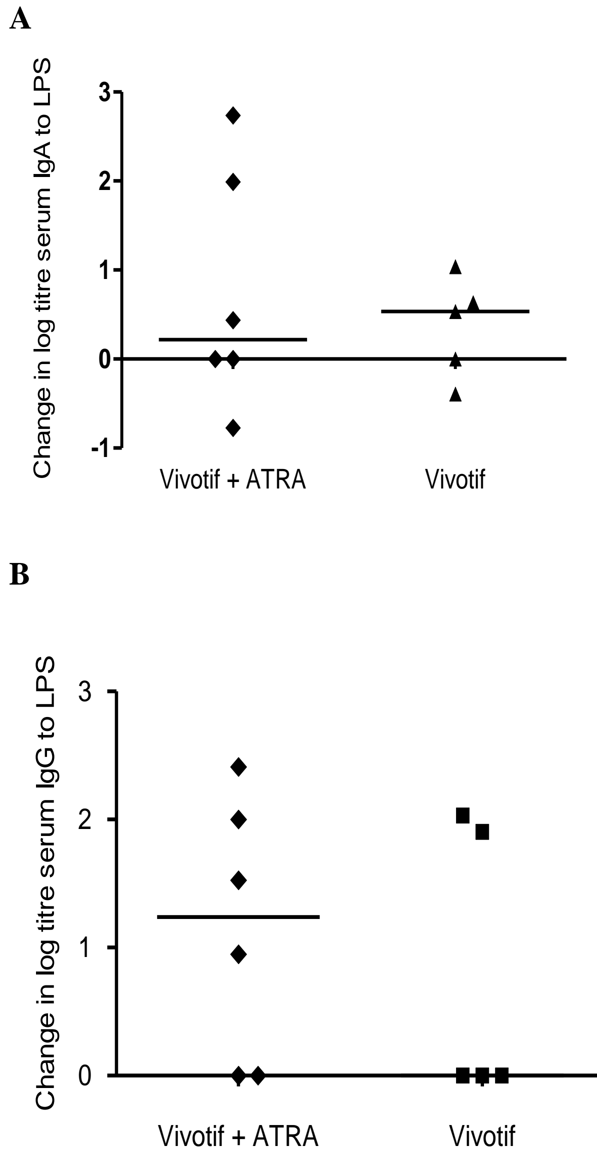


Figure 6-2A and B: Summary of changes in serum IgA and IgG in response to vaccine antigens. There was no significant difference in either (A) serum IgA or (B) IgG to Vivotif LPS.

C

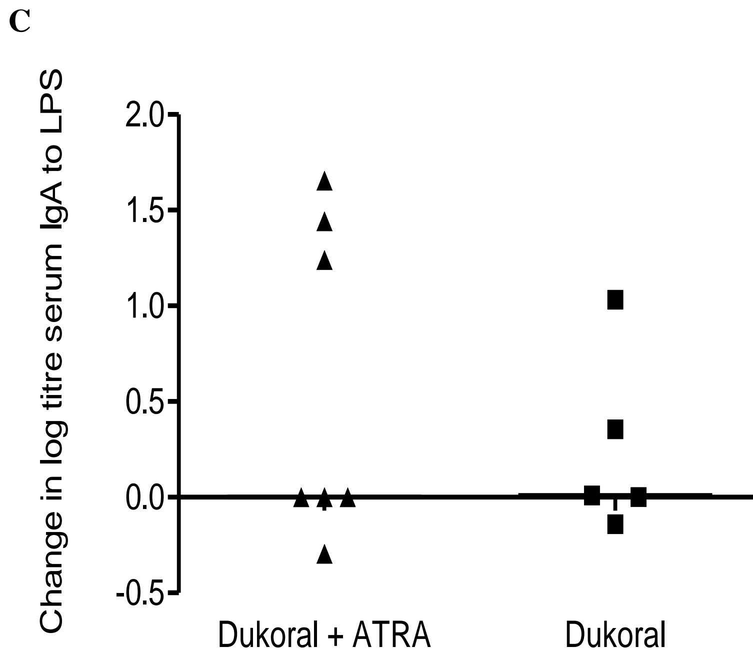


Figure 6-2C: Summary of changes in serum IgA and IgG and wglf IgG in response to vaccine antigens. No significant difference in serum IgA responses to Dukoral LPS.

D

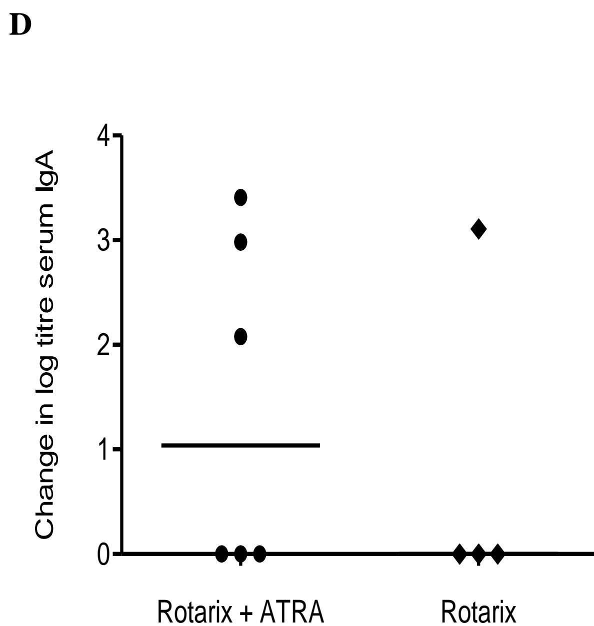


Figure 6-2D: Summary of changes in serum IgA and IgG and wglf IgG in response to vaccine antigens. No significant difference in serum IgA responses to Rotarix vaccine antigens.

E

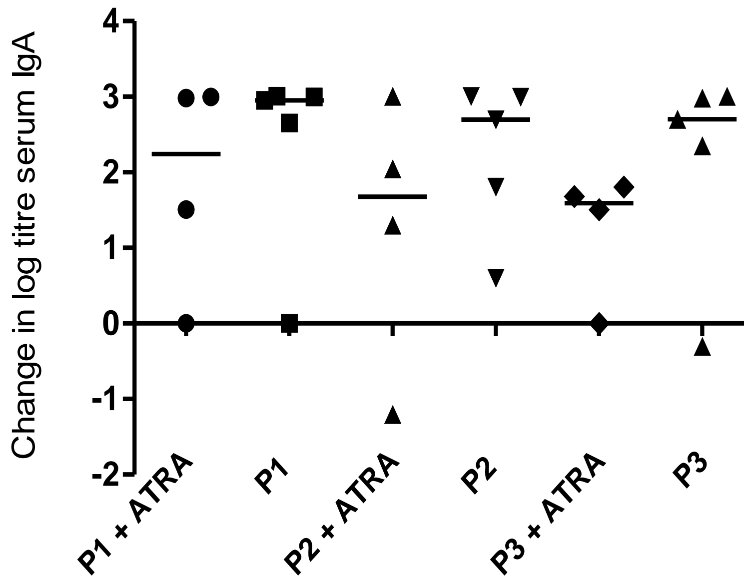


Figure 6-2E: Summary of changes in serum IgA and IgG and wglf IgG in response to polio vaccine antigens. There was no significant difference in serum IgA responses to polio vaccine antigens. Trivalent polio vaccine (OPV) was given and antibodies against the 3 polio antigens (P1: polio antigen 1, P2: polio antigen 2, P3: polio antigens 3) were measured

6.3 Effect of reduced ATRA dose on specific IgA response to Vivotif

When tested whether a single dose of ATRA would have the same effect as the full 8 days dosage, data showed that the effects of ATRA on gut IgA responses were not significant in the two ATRA dose regimes (Figure 6-3).

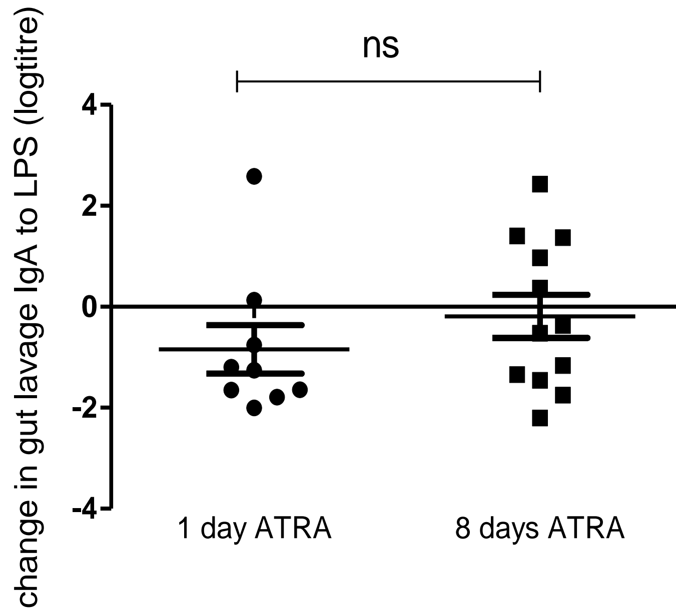


Figure 6-3: Effect of ATRA dose on specific IgA response to Vivotif vaccine LPS in gut lavage. All participants received Vivotif plus ATRA. There was no difference in gut IgA response (P=0.27) between the two ATRA dose regimes.

6.4 FACS analysis on the effect of reduced ATRA dose on gut homing marker expression

When tested whether a single dose of ATRA would have the same effect as the full 8 days dosage on gut homing maker expression, data showed that the difference between the two ATRA dose regimes was non-significant (Figure 6-4).

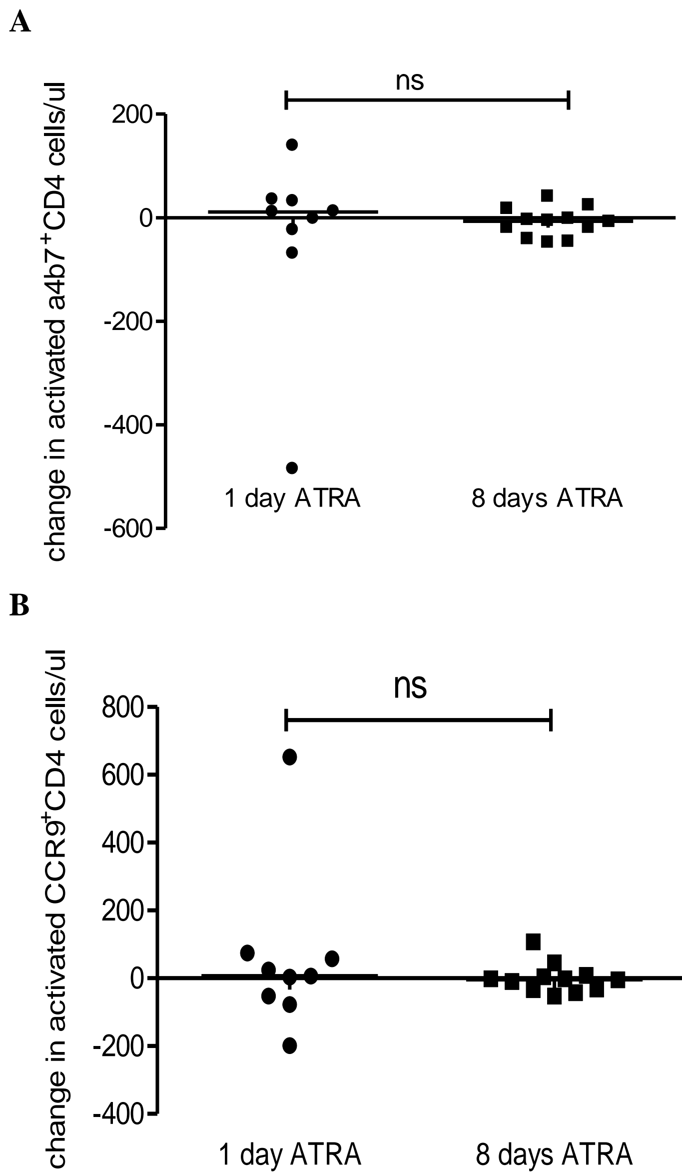


Figure 6-4: Effect of ATRA dose on gut homing markers on lymphocytes. All participants received Vivotif plus ATRA. There was no significant difference in (A) α4β7 (P=0.52) or (B) CCR9 (P=0.62) between the two ATRA dose regimes.

Chapter 7

7.0 RESULTS III: Correlation between gut homing profiles of circulating lymphocytes and gut IgA responses

Having established that Vivotif plus ATRA resulted in an increased expression in α4β7 and CCR9 at 14 days post immunisation, I explored whether expression of the gut homing markers α4β7 integrin and CCR9 could be correlated by analysing their expression on day 0 and day 14 after Vivotif vaccination. My data revealed a strong correlation ($\rho=0.83$; $P < 0.0001$) of these gut homing markers on $CD4^{+}$ T cells (Figure 7-1A) and the same was true for $CD8^{+}$ T cells (Figure 7-1B) but only when ATRA is given with vaccine. The gut markers on the T cells were either increased or decreased at the same time, and therefore defined those individuals with a coordinated increase as ‘’positive responders’’ and those with coordinated decrease as ‘’negative-responders’’. This coordinated increase in both gut markers was observed in 57% (12/21) of volunteers that received Vivotif and ATRA simultaneously; this effect was not seen with those that received Vivotif alone (Figure 7-1.1A), ATRA alone (Figure 7-1.1B), Rotarix (Figure 7-1.2) or polio (Figure 7-1.3) vaccinees. Our data therefore suggest that the coordinated gut homing response to ATRA is unique to Vivotif.

7.1 Correlation between α4β7 and CCR9 on circulating activated CD4+ and CD8+ lymphocytes in Vivotif plus ATRA recipients

A

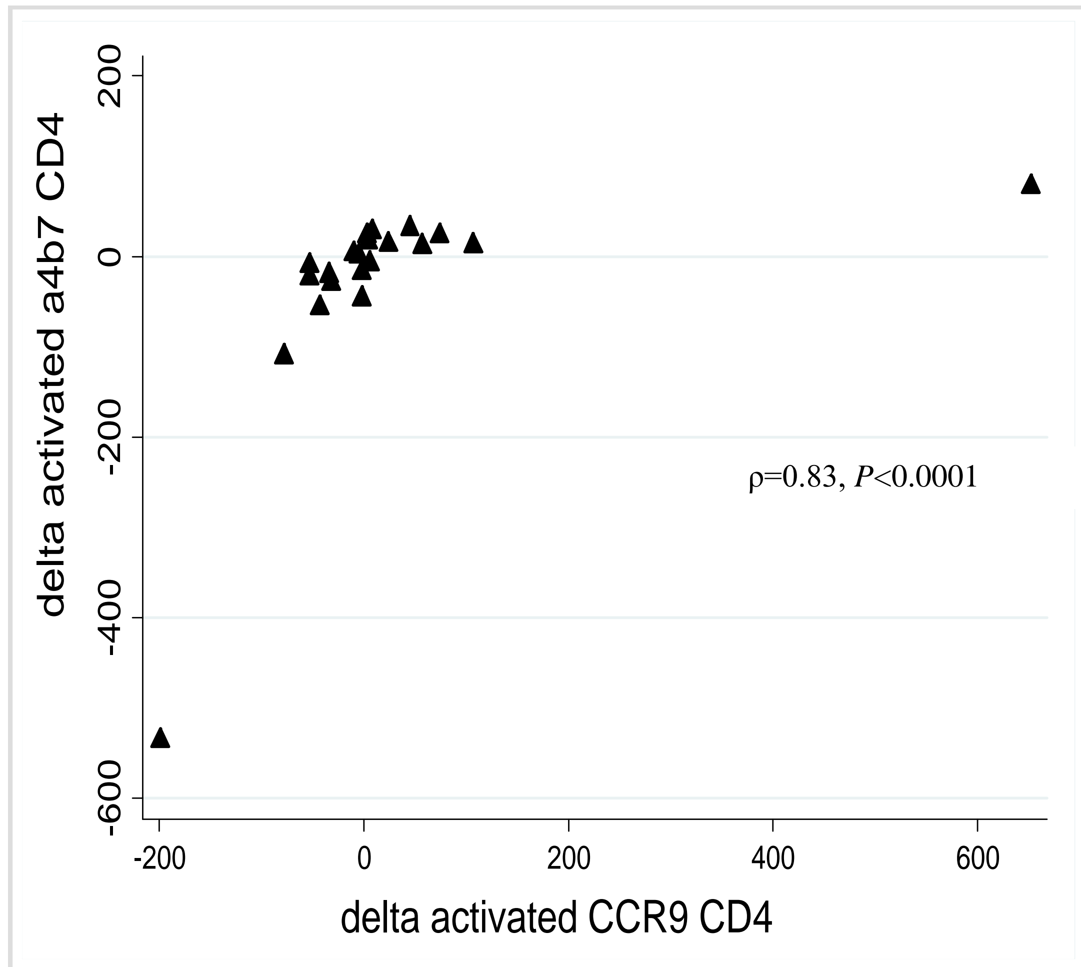


Figure 7-1A: ATRA induces coordinated expression of α4β7-integrin and CCR9 on activated CD4 T cells in Vivotif recipients who also were given ATRA. Change in α4β7 and CCR9 gut markers was closely correlated on activated $CD4^+$ T cells ($\rho=0.83$; $P < 0.0001$) in participants that received Vivotif with ATRA.

B

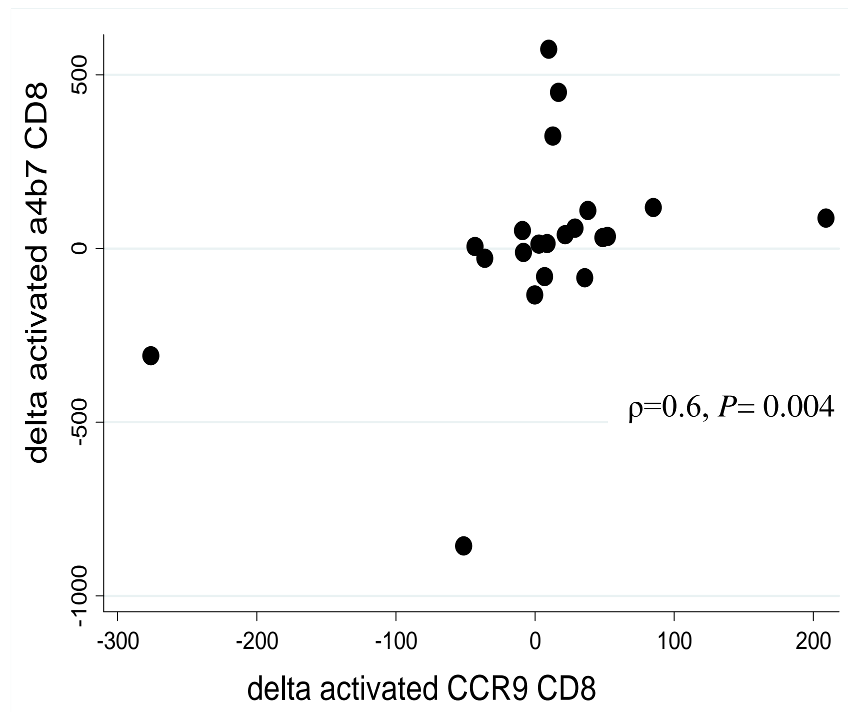


Figure 7-1B: Change in α4β7 and CCR9 gut markers was closely correlated on activated CD8⁺ T cells (ρ=0.6; P=0.004) in participants that received Vivotif with ATRA.

*Note that none of the volunteers that received ATRA and Vivotif had increased expression of only one of the homing markers.

7.1.1 Correlation between α4β7 and CCR9 on circulating activated CD4+ lymphocytes in recipients of Vivotif without ATRA and those given ATRA only

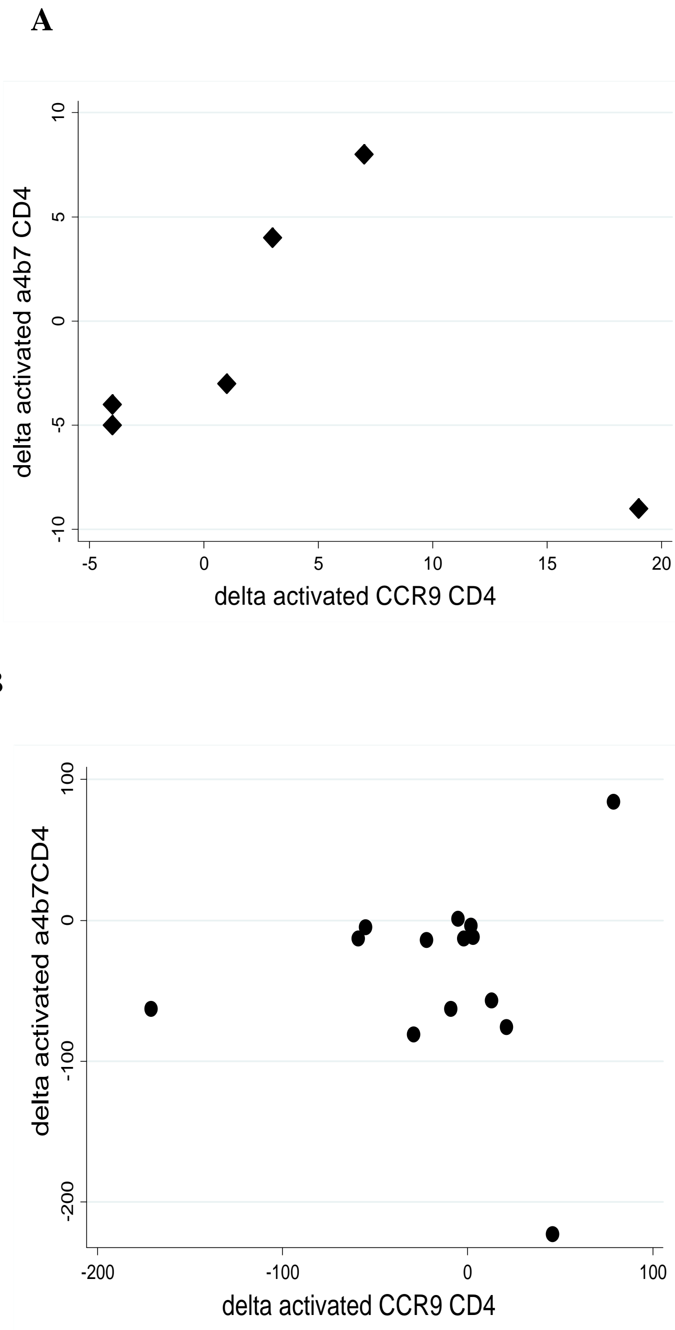
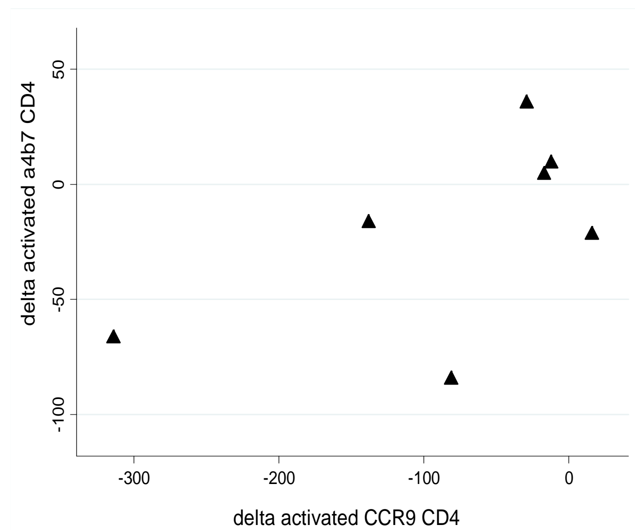


Figure 7-1.1: ATRA had no significant effect on gut marker expression on the participants that received (**A**) Vivotif alone (ρ=0.12; P=0.83) or (**B**) ATRA alone (ρ=0.07; P=0.82).

7.1.2 Correlation between α4β7 and CCR9 on circulating activated CD4+ lymphocytes in Rotarix plus ATRA recipients

When tested whether the gut homing markers in Rotarix vaccine recipients correlated, data showed that there was no correlation seen in both vaccine groups (Figure 7-1.2).

A



B

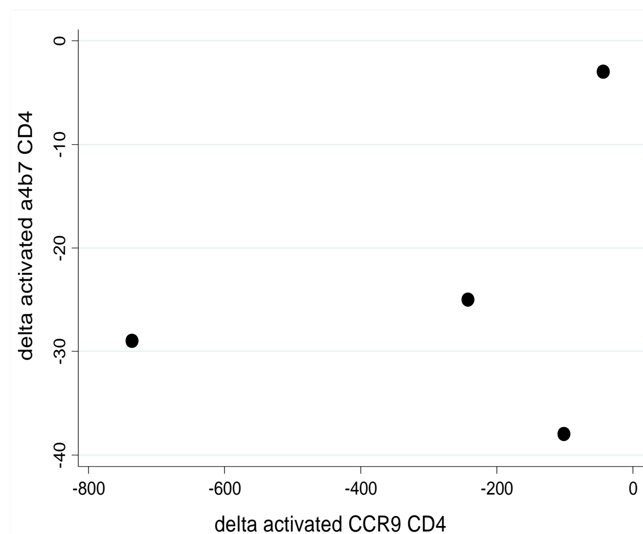
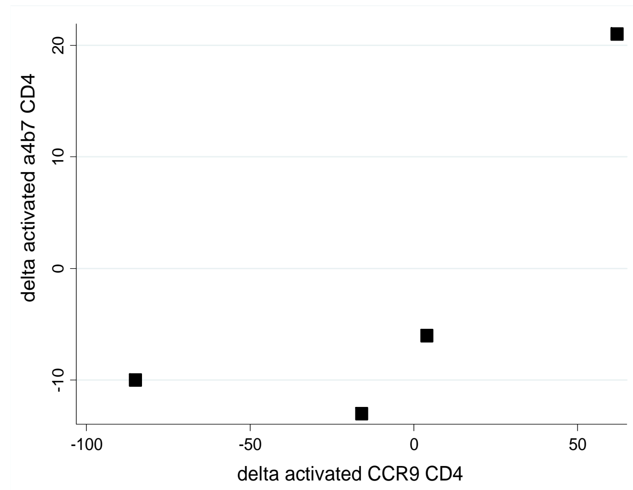


Figure 7-1.2: ATRA does not induce coordinated expression of α4β7 and CCR9 on activated $CD4^+$ T cells in Rotarix vaccine recipients. No correlations between change in α4β7-integrin and CCR9 expression was seen in **(A)** Rotarix + ATRA recipients or those that got **(B)** Rotarix only.

7.1.3 Correlation between α4β7 and CCR9 on circulating activated CD4+ lymphocytes in polio plus ATRA recipients

When tested whether the gut homing markers in polio vaccine recipients correlated, data showed that there was no correlation seen in both vaccine groups (Figure 7-1.3).

A



B

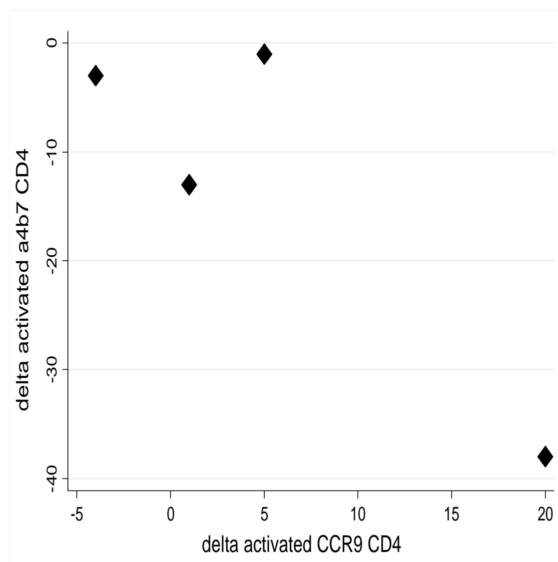
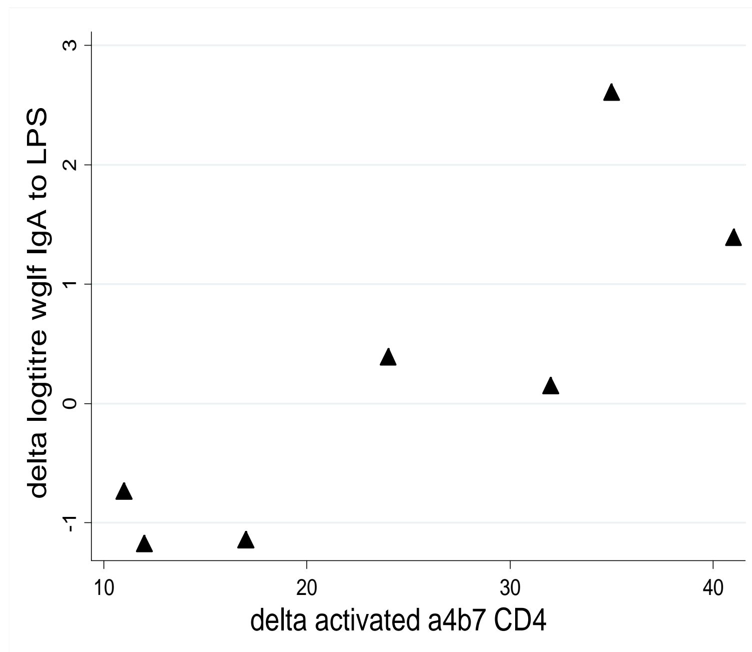


Figure 7-1.3: ATRA does not induce coordinated expression of α4β7 and CCR9 on activated $CD4^+$ T cells in polio vaccine recipients. No correlations between change in α4β7-integrin and CCR9 expression was seen in **(A)** polio+ ATRA recipients or **(B)** polio alone.

7.2 Correlation between gut homing marker profile and IgA response to Vivotif in the positive responders

I next asked whether the increase in gut homing marker expression is correlated to the IgA response to Vivotif. The change in α4β7 marker expression on activated CD4 cells was strongly associated (ρ=0.82; P=0.02) with an increase in specific IgA response to Vivotif LPS in WGLF (Figure 7-2A), whereas increase in specific IgA response showed very little change in CCR9 expression (Figure 7-2B). This effect was not seen on the CD8 lymphocytes

A



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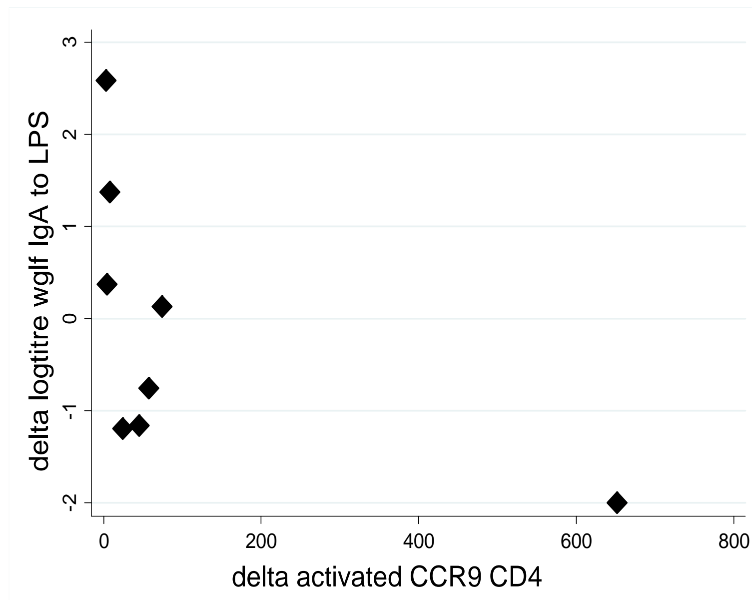
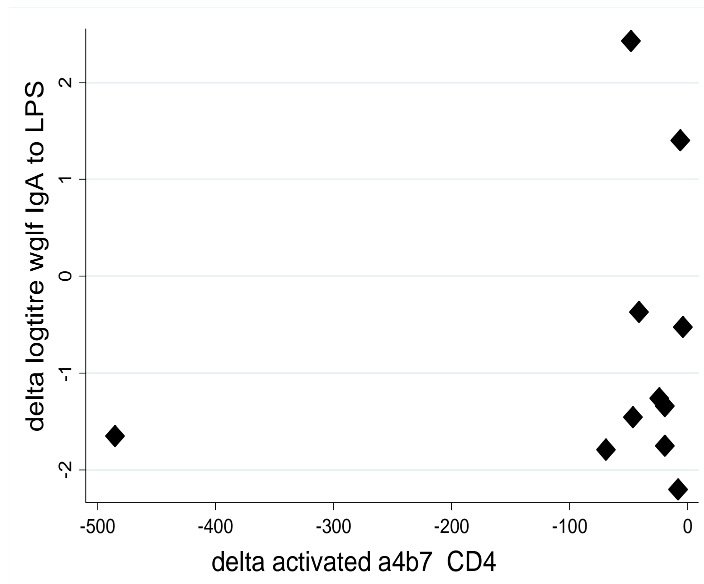


Figure 7-2: (**A**) The positive responders who received Vivotif plus ATRA showed a strong correlation (ρ=0.82; P=0.02) of change in α4β7 on activated $CD4^+$ T cells to change in gut IgA response to Vivotif LPS while (**B**) CCR9 expression showed a negative association (ρ= -0.74; P=0.04).

7.2.1 Correlation between gut homing marker profile and IgA response to Vivotif in the negative responders

In the participants whose gut homing markers were both decreased, the change in α4β7 marker expression on activated CD4 cells showed no correlation to specific IgA response to Vivotif (Figure 7-2.1 A). The same was true for CCR9 expression (Figure 7-2.1B)

A



B

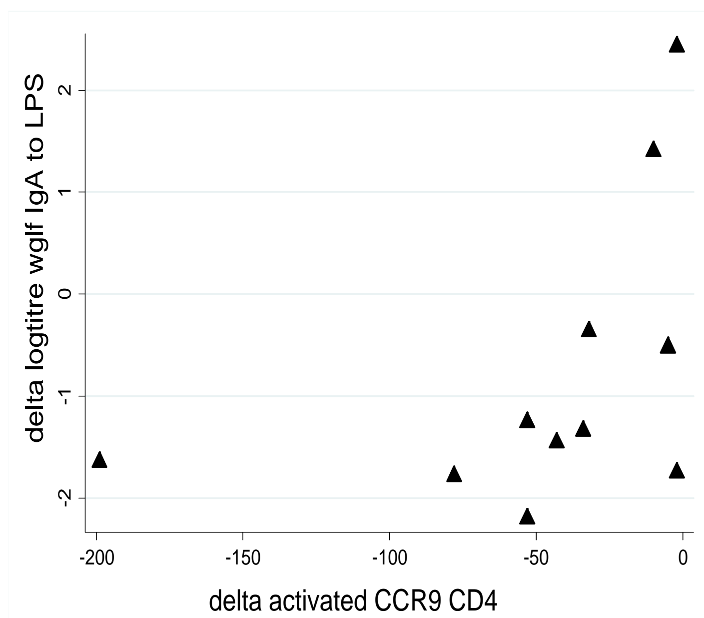
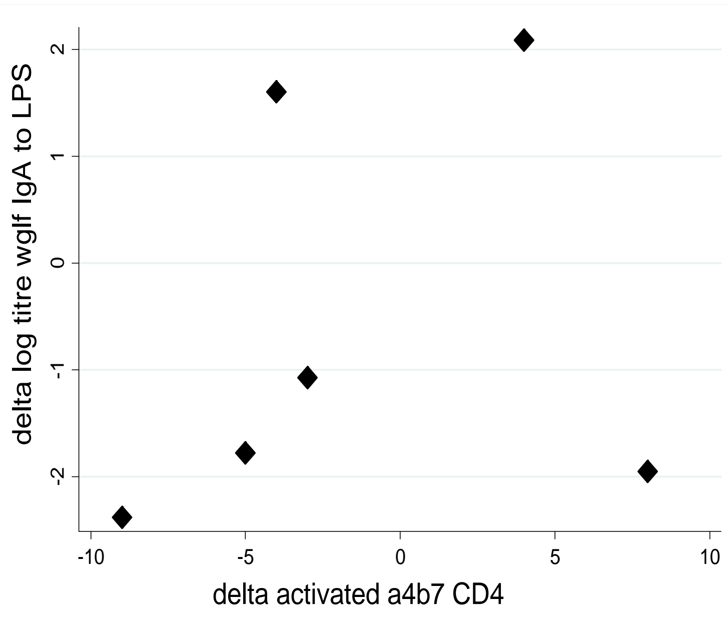


Figure 7-2.1: Negative responders who also received Vivotif plus ATRA but decreased gut marker expression showed no correlation in (**A**) $\alpha 4\beta 7$ (ρ=0.31, P=0.2) or **(B**) CCR9 (ρ=0.33, P= 0.1) to change in specific gut IgA response to Vivotif LPS.

7.2.2 Correlation between gut homing marker profile and IgA response in the participants that received Vivotif only

A



B

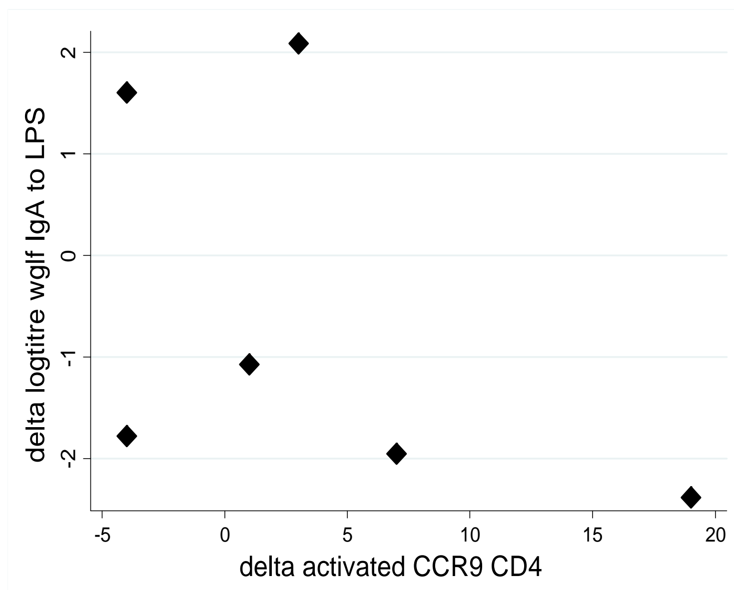


Figure 7-2.2: Participants given Vivotif alone showed no correlation of (A) α4β7 or (B) CCR9 to gut IgA response to Vivotif LPS.

The increase in $\alpha4\beta7$-integrin in the positive responders showed a strong correlation to specific IgA response to Vivotif. This was not reflected in the negative responders or those who only received Vivotif alone. Therefore the coordinated effect of ATRA on gut marker expression may result in an increase in IgA response

Chapter 8

RESULTS IV

In an attempt to define possible explanations for positive and negative responses to ATRA when given with Vivotif, I set out to investigate whether baseline serum retinol, HIV status played a role in immune response to ATRA. I also did some *in vitro* work to investigate the effect of different concentrations of ATRA on gut homing marker expression

8.1 Analysis of baseline serum retinol concentration

To test the hypothesis that peripheral blood responses and WGLF responses might be influenced by baseline vitamin A status, I analysed the serum retinol concentration of the participants that were either positive or negative responders to ATRA. The median (IQR) baseline concentration of retinol (Table 8-1) was 1.41µmol/L (1.06-2.48µmol/L) in the positive responders which was lower (P=0.0318) than the negative responders whose median (IQR) baseline level was 2.68µmol/L (1.68-3.29 µmol/L).

Table 8-1 Baseline characteristics of participants in retinol study

	Responders	Non responders	*P*
n	12	9	
Sex	all male	all male	
Age (mean, years)	36	29	
HIV seropositive (*n*)	3	2	
Serum retinol (µmol/L)	1.4 (1.06-2.48)	2.68 (1.68-3.29)	0.03
RBP4 (mg/L)	29 (23-44)	37 (33-43)	0.19

A retinol concentration of 1 µmol/L is equivalent to 28.5µg/dL.
Manufacturer's reference range for RBP4 is 12.7-48.6mg/L
RBP4: Retinol Binding Protein

8.2 Does HIV status affect ATRA responses?

In view of the effects that HIV has on immune responses, it was important to evaluate how HIV status affected ATRA responses. The HIV prevalence in this study cohort of 94 adult men was 21%. It has been shown (Lisulo et al., 2013) previously that HIV had no impact on intestinal IgA responses to Vivotif. Consistent with this, the data in this study revealed no significant difference in vaccine specific IgA responses (Figure 8-2A) and gut homing marker responses (Figure 8-2B and C) between the HIV positive and HIV negative participants.

A

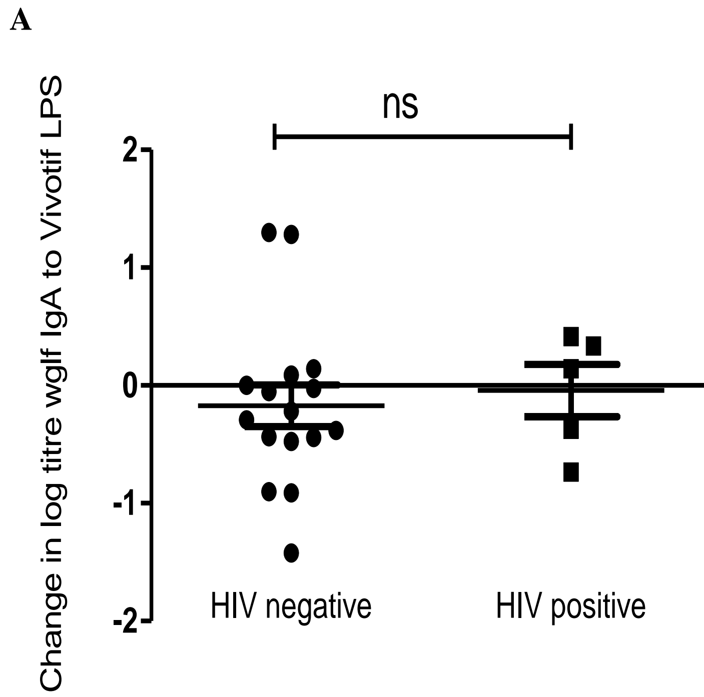
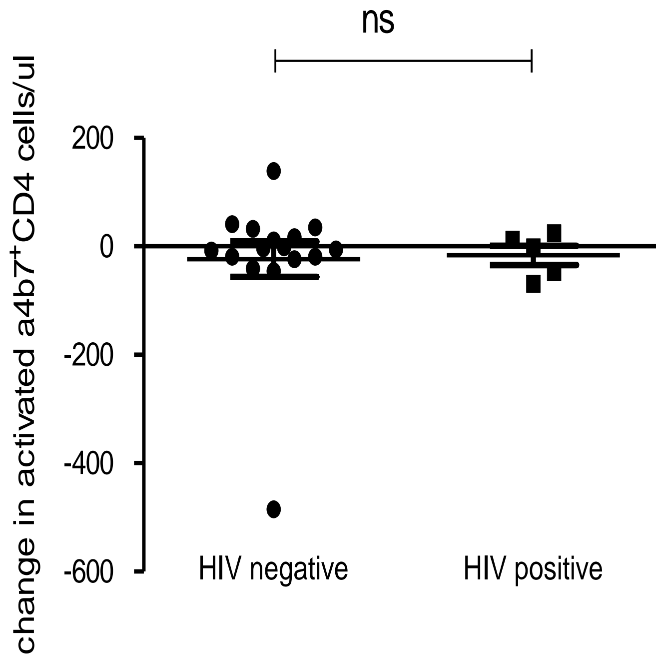


Figure 8-2A: Changes in WGLF IgA response to Vivotif LPS in HIV seronegative and HIV seropositive Vivotif vaccine recipients. No significant difference was seen in WGLF IgA to Vivotif LPS (P=0.34) between the two groups.

B



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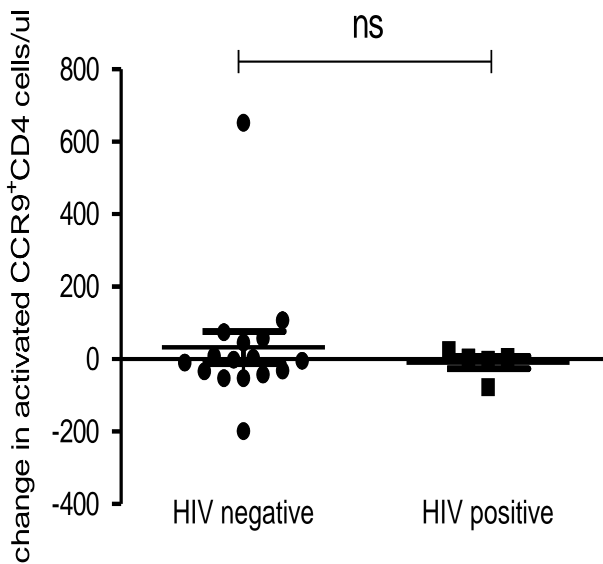
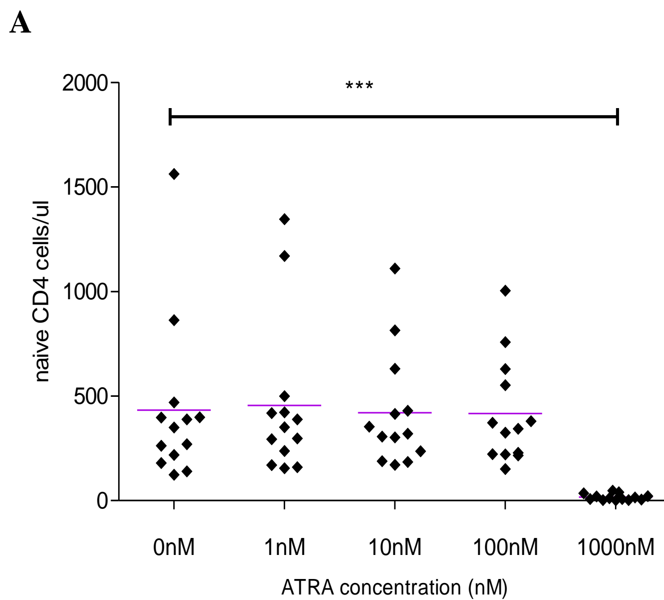


Figure 8-2B and C: Change in expression of α4β7 and CCR9 on CD4 T cells in HIV seronegative and HIV seropositive Vivotif recipients. No significant difference was seen in $\alpha4\beta7^{+}$CD4 (P=0.34) and $CCR9^{+}$CD4 T cells (P=0.97) between the two groups.

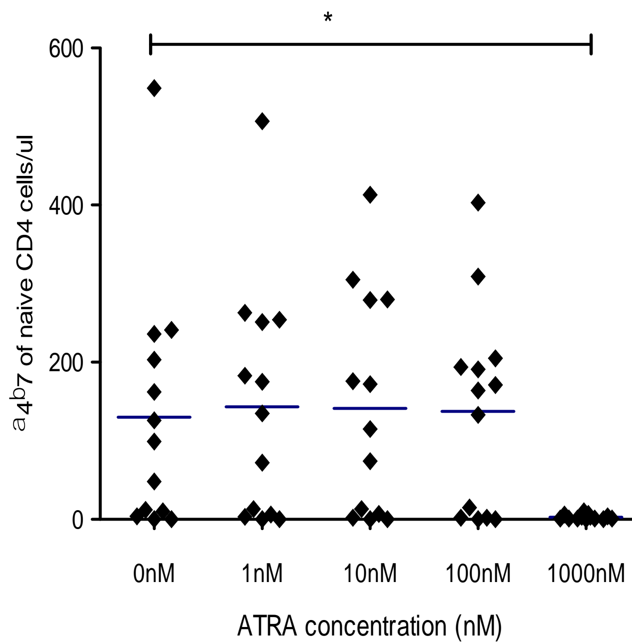
8.3 Effects of various concentrations of ATRA

Different concentrations of ATRA seem to act differently on immune cells. A number of studies (Bidad et al., 2011) carried out *in vitro* have used ATRA at pharmacological concentrations (about 1000nM) while others (Iwata et al., 2004) have reported of ATRA significantly enhancing expression of α4β7 at concentrations as low as 0.1nM *in vitro*. I set out to investigate the effect of different concentrations of ATRA on gut homing marker expression and identify the optimal concentration *in vitro.* Data analysed revealed that concentrations greater than 1000nM ATRA caused extensive decrease total CD4 count and in gut homing marker expression (Figure 8.3).

A



B



C

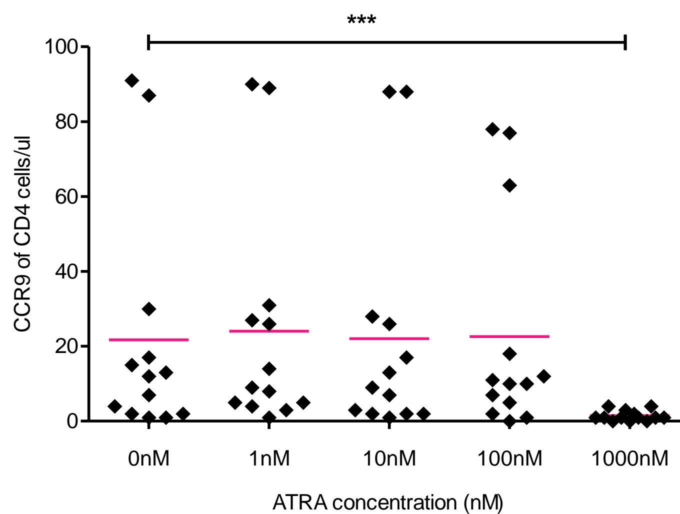


Figure 8-3: The effects of various concentrations of ATRA. A significant fall in (A) total CD4 count cells ($P<0.001$), (B) α4β7 ($P=0.017$) and (C) CCR9 ($P=0.001$) was seen at concentrations greater than 100nM ATRA. Using 1-way ANOVA test

Chapter 9

DISCUSSION

The global burden of diarrhoeal disease and the reduced efficacy of oral vaccines in populations from developing nations have highlighted the need for new strategies for vaccination against diarrhoeal diseases, possibly using oral adjuvants. Here the latter is explored in a population that is faced with high burden of diarrhoea and HIV. This study has revealed three important effects of ATRA on immune response to oral vaccination: firstly, the data suggests that ATRA can increase gut mucosal IgA directed at an oral live attenuated typhoid vaccine consistent with previous findings (Lisulo et al., 2013). This effect was seen only with Vivotif. Secondly, that ATRA is able to enhance gut homing marker expression, α4β7 and CCR9 on circulating activated $CD4^+$ T cells in a coordinated and vaccine-specific fashion. Thirdly, that the α4β7 increase was strongly correlated with the intestinal IgA responses in those with coordinated increase in both gut homing markers.

As previously demonstrated (Lisulo et al., 2013), ATRA is able to enhance specific IgA in WGLF against vaccine antigens suggesting that it could play an adjuvant role when given alongside Vivotif. The study set out to determine whether the adjuvanticity of ATRA could be translated to other oral vaccines, and so evaluated its effect on three other vaccines; Dukoral, Rotarix and polio vaccines, all orally administered with or without 10mg ATRA given daily for 8 days. Note that polio was not tested in WGLF as it requires a Class III facility which was not available in our setting (University Teaching Hospital). The data analysed revealed that ATRA adjuvanticity in this study only applied to Vivotif and not the other oral vaccines suggesting that ATRA effect could be vaccine specific. There may be several contributors to the non-generalisability of ATRA adjuvanticity to the other vaccines.

The most important factor is likely to be the type of immune responses being elicited by the vaccines. Vivotif induces a vigorous T-cell response that favours the production of interferon gamma (IFN-ɣ), tumor necrosis factor-α (TNF-α), interleukin -1 (IL-1) and IL-6 indicative of a Th1-type response probably through Class II antigen presentation (Lundin et al., 2002 and Pasetti et al., 2011). Most responses to viral immunisations are IFN-ɣ dependent through Class I antigen presentation, although some studies (Sartono et al., 2010; Banda et al., 2012; Jensen et al., 2014) have shown the contrary. In one study (Banda et al., 2012), Rotarix vaccine showed no discernible effect on IFN-ɣ and TNF-α response in a considerable number [16/21 (76%)] of vaccinees. Another study (Sartono et al., 2010) showed that OPV was associated with down regulation of cytokine (IFN-ɣ and TNF-α) production when co-administered with BCG. The authors speculated that polio virus may have specific immune modulatory molecules that down-regulate immune responses to antigens to which immune responses are being mounted simultaneously. The inactivated bacterial vaccine Dukoral has also been shown to elicit diminished or skewed CD4+ T cell responses towards development of a Th2- T cell phenotype because of the presence of CTB (Kuchta et al., 2011). Consistent with observations in the present study, others also have evidence that Vivotif, Rotarix and ETEC vaccines elicit different innate immune responses to Rotarix (Kelly, manuscript in preparation). Of the vaccines studied, Vivotif is likely to have been the only vaccine favouring a Th1-type immune response through Class II antigen presentation. I hypothesise here that the nature of the context of antigen presentation, could determine whether ATRA has an effect on vaccination (Brown et al., 2015). The different responses to Vivotif are not merely a consequence of increased sample size as the primary comparisons used similar numbers of volunteers (Figure 5-7 and

6-1). Of note is that the data are presented as the absolute numbers of α4β7+ and CCR9$^+$ T cells, indicating that the selective increase in these T cell subsets may have been responsible for the overall increase in circulating T cells. However, this could not distinguish whether the increase in gut marker expression was on T cells already expressing some degree of the gut homing molecules or whether there was reconfiguration of other T cells to begin expressing these gut homing molecules. This would need further investigation.

This may be the only other study which has evaluated ATRA effects on immune function in humans *in vivo*; all other studies (Iwata et al., 2004, Evans et al., 2013, Kang et al., 2007, Eksteen et al., 2009, Tan et al., 2011) have used *in vitro* systems or animal models rather than studying the effect when ATRA is given to a living volunteer. The *in vivo* data presented here show an increase in α4β7 and CCR9 on activated T cells following oral administration of 10 mg ATRA but only when given with Vivotif. These results are consistent with other studies in animal models (Iwata et al., 2004, Hammerschmidt et al., 2011, Eksteen et al., 2009). On the contrary however, another study (Evans et al., 2013) showed that ATRA treatment of PBMCs isolated from healthy volunteer donors only increased α4β7 expression but had no overall effect on CCR9. This effect however was at much higher concentrations (200 nmol/ l) than were previously found in volunteers receiving 10mg ATRA (10-40 nmol/l) (Lisulo et al., 2013). ATRA increased the expression of α4β7and CCR9, on CD4$^+$ lymphocytes is in a time dependent manner with a significant effect being seen at day 14 post Vivotif vaccination. This however was not the same with Rotarix or polio vaccination. In this study, the restriction of the effect of ATRA on immune response to the context of a particular vaccine is consist with some animal findings

(Tan et al., 2011) although other animal studies have suggested otherwise (Iwata et al., 2004, Evans et al., 2013).

Although the ATRA dependence of T lymphocyte mucosal homing marker expression has been well documented *in vitro* (Iwata et al., 2004, Kaufman et al., 2011, Mora et al., 2003), ATRA had divergent effects on both gut markers (α4β7 and CCR9) at the same time in different individuals. In a subset of the vaccinees, both gut markers were co-ordinately increased while in the others the markers were decreased, indicating that there were 'positive responders' and 'negative responders' to ATRA treatment. ATRA induces the α4β7 and CCR9 expression together in an entirely coordinated expression shift only when given together with an oral vaccine and this phenotypic change on CD4 cells is very strongly correlated with the gut IgA specific response.

These data have also revealed that the positive responders in this particular population had lower serum retinol concentration at baseline compared to the negative responders. However, there was no difference in RBP4 which is another marker of vitamin A status. Of the positive responders, 4/10 (40%) had a clear or borderline vitamin A deficiency but none in the negative responders. Studies have suggested that vitamin A deficiency promotes a Th1 response and elevates pro-inflammatory cytokines (Ross 2012). This may signify that the effect of ATRA is principally seen in people with borderline vitamin A deficiency which favours a Th1 immune response. If confirmed in larger studies, this might explain some of the variations in α4β7 and CCR9 response to ATRA. However, it still leaves a high proportion of this variability unexplained, so the possibility of an immunogenetic predisposition needs to be considered. Surprisingly, it also appears that ATRA has

the same effects on gut homing marker expression and gut IgA responses in HIV infected adults as in HIV uninfected individuals.

Chapter 10

Conclusions

Retinoic acid response is not the same in humans as in animal models despite using doses described to achieve similar concentrations. This study has shown that of the people studied, some were positive responders while others were negative responders to ATRA when given alongside Vivotif vaccine. The adjuvant effect of ATRA including up-regulation of IgA and gut homing marker expression, was only applicable to oral typhoid vaccine which elicits an immune response that favours IFN-ɣ production. The increase in α4β7 was found to be strongly correlated to IgA secretion in gut fluid. This finding could be useful in future vaccination studies where α4β7 can be substituted for IgA as a correlate of protection as gut lavage is a laborious method. This strategy however, needs to be confirmed in a large population-based field trial. The results of this study suggest that ATRA only really impacts on α4β7 in a subset of people with borderline vitamin A deficiency and when given alongside typhoid oral vaccine. In low-income countries where the majority of the populations are vitamin A deficient, this finding could be useful to try and improve vaccine responses. The Zambian population for example is at high risk of vitamin A deficiency as demonstrated by low serum retinol concentrations in 20% of adults living in Ndola (E. Kafwembe, unpubl. Obs.). As the quest to try and improve oral vaccine efficacy in developing countries continues, my findings suggest that ATRA may be useful as an oral adjuvant, but only under certain conditions.

LIMITATIONS

1. For safety reasons, this study was carried out only in men due to the teratogenicity of ATRA (Lee et al., 2012, Adams et al., 2010); and with no clear safe dose, ATRA could not be used in women who might become pregnant during the study. Also, a number of studies (Aaby et al., 2004, Klein et al., 2015) throughout the world confirm differences between males and females in response to vaccination and so, including women in the study may have generated different responses.
2. A further limitation is that I did not access the gut mucosal T cell responses in gut biopsies where I could have also determined if ATRA induces a T cell migration to the intestinal mucosa. Further work is needed to explain the molecular basis of the coordinated regulation of α4β7 and CCR9 on activated CD4 cells and as such may allow for a more refined approach to retinoid enhancement of oral vaccination.
3. The study was done in adults whilst the burden of diarrhoea disease is in children under 5 years. There is therefore need for work to be done in children where increasing evidence suggests of higher burden of infection and reduced vaccine efficacy.
4. The choice of Vivotif vaccine which are enteric coated oral capsules and are normally not recommended in children below 6 years who cannot swallow them. In this age group parenteral vaccines are used.

Recommendations

A number of questions still remain to be answered. It has been clear for several decades that there are distinct differences in the morphology and function of the gut in developed and developing countries. In view of this it would be interesting to access gut mucosal T cell responses in gut biopsies. Also, the variations in the α4β7 and CCR9 expression seen in participants in this study in response to ATRA may be due to immunogenetic predisposition and would need further investigation possibly through some transcriptome analysis. It would be ideal to try and explain the molecular basis of the coordinated regulation of α4β7 and CCR9 and thereby allow for a more refined approach to retinoid enhancement of oral vaccination. Other oral vaccines (ETEC, shigella, amoeba) will soon be available and could also be evaluated. Finally and most importantly, evaluation of effects of ATRA on vaccination in children will be very important and my data which has shown that immunophenotyping can predict gut responses to vaccine might help make this evaluation possible.

Chapter 11

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APPENDICES

APPENDIX I

Publications from the study

APPENDIX II

Information Sheet

We have invited you to participate in a sub study that is part of the ''Retinoic Acid as an oral adjuvant: A phase 2 study''. This study is designed to see if we can answer some more of the questions that the parent study will not focus on but is still in the same line of using a derivative of vitamin A called TRETINOIN [*Vesanoid*] to improve responses to vaccines given by mouth. In a previous study in Misisi the parent study have used vaccines given by mouth, so you may already be familiar with them. We want to continue evaluating Tretinoin as there are exciting findings that it can act as an oral adjuvant which means that it can boost the effect of the vaccine. We hope that this will mean it can boost the protection these vaccines offer against serious infections. As in the previous study, this study will only include men because when given to pregnant women there is a fear that it can endanger the pregnancy. So as to avoid this problem in future we are trying to use a low dose which might not cause these effects in pregnant women, but until we are sure of this we are only studying men. Tretinoin does NOT cause such problems when given to men.

What are we asking you to do?

We want to analyse exactly how the Tretinoin affects the cells we have in the blood to fight off infections. We want to see how the Tretinoin affects the cells in the blood over a period of time. Also we want to look at how the Tretinoin is affecting the cells when they are outside the body. This analysis will be done in the laboratory.

20 men will beasked to give 5ml [1 teaspoon] of blood sample then be vaccinated and asked to take Tretinoin and have 5ml blood sample taken after 3hrs and 6hrs. They will then be asked to come back the next day at the same time (after 24hrs) to

give another 5ml of blood. The last two blood samples will be given on day 3 and day 8.You will be provided with transport and lunch at all these times.

As this is a study of immune responses, we will need to do an HIV test, which we will discuss with you fully and the result of which will be confidential.

What do we do with the samples we take?

Plasma (the liquid part of the blood) will be used for HIV test and for measurements of the systems the body uses to fight off infections (cells and antibodies). You need to know that we intend to store part of every sample for future analysis and to allow quality control checks.

What are the possible benefits to me?

If you agree to participate in this study, you will be given a vaccine which has been designed to offer protection against typhoid a selection which will be made at random. You will also be able to get our help with your health care for you and your family for the period of the study.

What are the possible disadvantages to me?

All participants will need to do an HIV test, but this will help you to safeguard your health, and we will offer full pre-and post- test counseling. Collection of the blood samples at the different timepoints will take up your time and thus we will compensate your expenses for the days you have to take off work.

Confidentiality

Your details will be recorded on a paper form which will be locked away in our offices in the UTH. Your details will be entered on a computer but only in code form with only your ID number and no name. Samples will be stored in the laboratory using a unique number. Any information and results will remain absolutely

confidential, and family members or work colleagues will not be granted access to this information.

The study is voluntary

You do not have to participate in this study if you do not want and even if you refuse, we will still provide the best care we can. If you do agree, you are also free to change your mind at a later date. This research study has been approved by the Biomedical Research Ethics Committee of the University of Zambia and their contact details are given below.

Principal Investigator: Mpala Mwanza Lisulo, Department of Medicine, School of Medicine UNZA (0975954130)

Research Ethics Committee: The Chairperson, REC office, department of Anatomy, Ridgeway campus Lusaka (phone 0211 256067).

TO BE KEPT BY PARTICIPANT

APPENDIX III

Consent record sheet

I confirm that I have understood the information I have been given about the study. I agree to participate in the study. I confirm that I am joining the study of my free will and that I can withdraw at any time without affecting the care available to me. I understand what will be required of me.

Name:

Signed (or thumbprint)

Date:

Signature (or thumbprint) of witness

Name:

Date:

I confirm that I have explained the information fully and answered any questions.

Signed for the study team:

Name:

Date:

APPENDIX IV

Consent record sheet for storage of samples for future use

I confirm that I have understood the information I have been given about the study. I have given permission for my samples to be stored and used for future analysis.

Name:

Signed (or thumbprint)

Date:

Signature (or thumbprint) of witness

Name:

Date:

I confirm that I have explained the information fully and answered any questions.

Signed for the study team:

Name:

Date:

APPENDIX V

Laboratory Protocols

Daily start-up procedure for FACS machine

1. Switch on FACS machine
2. Check fluidics levels: Sheath fluid should be full and empty waste tank then add 10% FACS Clean
3. Log onto FACSuite software
4. Wait for 15 min for FACS lasers to warm up
5. Run the sheath fluid for 15min on high flow rate:
    - Use a 15ml or 50 ml conical and relevant adapter
    - Create an experiment and name it Running sheath fluid (Experiment→New→Manage experiment→right click)
    - Create a tube and adjust flow rate to high
    - Run sheath fluid for 15 minutes under the preview mode
6. Perform a daily clean using FACS clean then distilled water (click cytometer→daily clean)
7. Cytomter→fluidics→purge sheath filter twice using distilled water
8. Cytomter→fluidics→drain and fill flow cell twice using distilled water
9. Run the performance quality control (PQC) using 2 drops of CS&T beads in 500ul sheath fluid
10. Tube and assay settings will update automatically during the PQC
11. Update reference settings on a monthly basis by creating a new experiment with a blank tube , right click the tube→update reference settings→run CS&T and FC beads

Running samples on a worklist

1. Open a new worklist
    - Worklist menu on side left panel→ file → new worklist
2. Add the number of tasks and a daily clean
3. Go to preferences → worklists → FCS → select ‘’export’’ after acquisition
4. Go to loading options → manual
5. To run samples select run all

Creating statistics

After creating the dot plots and gates or quadrants around the interested populations:

1. Right click on gate to make it unique to ensure that the gate remains in the constant location for the particular tube
2. Rename the quadrant:
    - Click on the population hierarchy window
    - Click on: show statistical gates/ populations
    - Click once on the quadrant that you want to rename
3. Create the statistics window and edit populations (medians, % parent etc)

Manual compensation

1. Prepare FC beads according to instructions
2. Set up experiment on FACSVerse and add tubes for each of the fluorochromes being used
3. Select the appropriate tube
4. Draw a dot plot of the FSC-A verses SSC-A. Duplicate dot plots of the fluorochrome in the tube against every other fluorochrome being compensated
5. Place tube containing the appropriate fluorochrome, preview and aquire the sample
6. For each fluorochrome visually compare the plots against every other fluorochrome and determine the plots which need compensation
7. Gate the populations of interest or place quadrants on the selected plots
8. Draw statistics table
9. Edit statistics (select median for the fluorochromes on the x and y axis)
10. Edit population (select gates or quadrants of interest)
11. Right click on the tube and select properties
12. Select compensation and adjust values

		Fluorochrome in tube			
		FITC	PE	PerCP	APC
Filters (voltage)	FITC	**100**	1.00	1.00	0.86
	PE	8.53	**100**	0.85	5.00
	PerCP	0.00	1.00	**100**	9.00
	APC	1.10	10.0	0.75	**100**

NOTE

From the statistics table compare the medians of the negative and positive populations of the fluorochrome not in the tube. The aim is to adjust the false positive until it reaches a value close to that of the negative value. If you increase the compensation value, you increase the number of events that will be excluded therefore the median in the statistics table will reduce and vice versa.

Antibody Titration Protocol (using CD8 FITC as an example)

1. Label 6 tubes with antibody volumes (2-fold serial dilution volumes) starting with the recommended volume (eg : 20ul, 10ul, 5ul, 2.5ul, 1ul, 0.5ul)
2. Add the appropriate antibody volume to each tube
3. Add 100ul whole blood to each tube
4. The first tube will now have a volume of 120ul. Bring the volume of the other tubes to 120ul with PBS
5. Proceed with incubation and lysing as per staining protocol
6. Set up the FACSVerse and gating protocol as required
7. Draw a histogram for the CD8 FITC data
8. Acquire the data using the optimized voltage settings. 10 000 events is sufficient.
9. Compensation is not needed as you are running single stains
10. On the 20ul tube, gate the $CD8^-$ population and $CD8^+$ population on the histogram
11. Display the statistics for these gates
12. Calculate the ration between the CD8 positive and the negative populations. This gives you the separation between the negative and positive populations. Do the same for the other volumes.
13. Insert a scatter chart of volume vs ratio. The best volume is the one with the best separation

Antibody master mix preparation template

No. of samples		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
	Vol of Ab (ul)										
CD3 APC-H7	1	2	3	4	5	6	7	8	9	10	11
CD4 PerCP	1	2	3	4	5	6	7	8	9	10	11
α4 FITC	2.5	5	7.5	10	12.5	15	17.5	20	22.5	25	27.5
β7 APC	1	2	3	4	5	6	7	8	9	10	11
HLA-DR/ CD69/ CD25 PE-Cy7	5	10	15	20	25	30	35	40	45	50	55
CCR9 PE	1	2	3	4	5	6	7	8	9	10	11
CD45RA PE-Cy7	5	10	15	20	25	30	35	40	45	50	55

Volume of master mix to add to each homing panel sample tube: **11.5ul**

LAB PROTOCOL

Effect of ATRA concentration on $CD4^+$ T cell gut homing marker expression

Dissolve 1 capsule of Vesanoid (10mg ATRA) into 3.32ml DMSO to obtain 10mM stock solution

Final concentration nM	1000	100	10 (physiological conc of ATRA)	1	0
Amount of 10mM ATRA soln in 100ul	10	1	0.1	0.01	0

Sample collection

5ml of blood was drawn at baseline

FACS analysis

Put 100ul of blood into a labelled FACS tube. Add the required amount of ATRAvolume from the ATRA stock solution. Incubate at room temperature in the dark for 24hrs then stain the blood with appropriate antibodies in each of the Homing panels following the Lyse wash protocol

Serum

Spin down the remaining blood at 3500rpm for 5min and aliquot the serum. Store at -80°C.