Investigating The Possible Role Of Regulatory T Cells In Suppressing DTwP And Measles-Vaccine Induced Responses In 9 Month-Old Gambian Infants

Thesis

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Investigating the possible role of regulatory T cells in suppressing DTwP and measles-vaccine induced responses in 9 month-old Gambian infants

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Thesis submitted to Open University, U.K. in fulfilment of the requirements for the Doctor of Philosophy in the field of Life Sciences

2018

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ABSTRACT

The majority of deaths in children under five are attributed to infections, with neonates and young infants being the most susceptible. The immune system of the newborn is adapted to protect the fetus from infections and avoid harmful inflammation, characterized by limited Th1 immunity and skewed towards Th2 and immunoregulatory responses. This results in high susceptibility to infections and impaired responses to vaccines. Regulatory T cells (Tregs) control pro-inflammatory responses and are therefore important in maintaining a fine balance of immune responses during infections. Tregs are high and functional in neonates, but their precise role in controlling vaccine immunogenicity is not known.

I hypothesised that circulating Tregs at vaccination can limit subsequent vaccine-induced cellular and humoral responses, and that functional Tregs are induced by vaccination in infants. The work in this thesis involves the characterization of Tregs before and after measles and Diphtheria, Tetanus and whole cell pertussis combined (DTwP) vaccination, and association with functional readouts, vaccine-specific humoral and cellular responses. I found that CD4+FOXP3+CD127lo Tregs significantly declined overtime, with this subset and the CD4+CD25hiFOXP3+ Treg subsets inversely correlating with the measles antibodies following vaccination. This supports a functional role for Tregs in controlling the humoral response to measles but not for DTwP vaccination. We also observe an induction of CD4+IL-10+ T cells following measles vaccination. Further evaluation of these cells showed that they were mainly IL-4- and IFN-γ-, excluding the possibility that these may be Th1 or Th2 cells, and suggesting that these may be Tr1 cells induced as part of a homeostatic control mechanism following MV. Further evaluation of the role of Tregs in vaccine immunogenicity in infants is warranted.
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As I write this thesis, we the people of the Gambia are living in political crisis with uncertainty about the future. I dedicate this thesis to the ‘struggle’ and pray for peace to be sustained. 

#GambiaHasDecided

To my daughter Ramou, for the times I could not make it to your school events or help with your homework, I am sorry. Thank you for understanding. This is for you.
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LIST OF ABBREVIATIONS

AA- amino acid
ABC- ATP-binding cassette
ADCC- antibody-dependent cellular cytotoxicity
AHR- aryl hydrocarbon receptor
AID- activation-induced cystidine deaminase
AIDS- acquired immune deficiency syndrome
AIRE- autoimmune regulatory gene
APC- antigen presenting cells
APS-1- autoimmune polyglandular syndrome type 1
BCG- Bacille Calmette-Guerin
BCR- B cell receptor
BFA- Brefeldin A
Bcl6- B-cell lymphoma 6
CB- cord blood
CCL- chemokine ligand
CCR7 - C-C chemokine receptor 7
CDR - complementary determining region
CH - constant region of the heavy chain
CRF - case report form
CTLA-4 - cytotoxic T lymphocyte antigen 4
DC - dendritic cell
DLN - draining lymph node
DMSO - Dimethyl sulfoxide
dsRNA - double stranded RNA
DTP - Diphtheria, Tetanus and pertussis combined vaccines
DTaP - Diphtheria, Tetanus and acellular pertussis combined vaccines
DTwP - Diphtheria, Tetanus and whole cell pertussis combined vaccines
Dtx - Diphtheria toxoid
EDTA - ethylene diamine tetraacetic acid
ELISA - Enzyme-linked immunosorbent assay
EPI - expanded programme on immunisation
ER - endoplasmic reticulum
Fab - Fragments of antigen binding
Fc - Fragment of crystallisable
FCS - fetal calf serum
FMO - fluorescence minus one
GATA3 - Gata-binding protein 3
GC - germinal centre
GITR - glucocorticoid induced tumour necrosis factor receptor
HIV- human immunodeficiency virus
HepB- hepatitis B vaccine
Hib- *Haemophilus influenzae* type b vaccine
HLA- human leukocyte antigen
HSP- heat shock protein
IDDM- insulin dependent diabetes mellitus
IDO- indoleamine 2,3-dioxygenase
IFN- interferon
IL- interleukin
Iono- ionomycin
Ig- immunoglobulin
ITAM- immunoreceptor tyrosine-based activation motif
ITIM- immunoreceptor tyrosine-based inhibitory motif
LAG3-lymophocyte activation gene 3
Mab- maternal antibody
MHC- major histocompatibility complex
MRC- Medical Research Council
MV- measles vaccine
MVA - modified vaccinia Ankara
NK- natural killer
NLR- nod-like receptor nucleotide-binding oligomerization domain-like receptors
OPV- oral polio vaccine
PAMP- pathogen-associated molecular patterns
PBMC- peripheral blood mononuclear cells
PBS- Phosphate Buffered Saline
PCV- pneumococcal conjugate vaccine
PMA- Phorbol 12-myristate 13-acetate
PMN- polymorphonuclear cells
PPD- purified protein derivative
PRR-pattern recognition receptors
Ptx- Pertussis toxoid
RIG- retinoic acid inducible-gene-Like Receptor
ROR- retinoid-related orphan nuclear receptor
SCC- scientific coordinating committee
SEB- staphylococcus enterotoxin B
SFU- spot forming unit
SLAM- signalling lymphocyte activation molecule
SLO- secondary lymphoid organs
ssRNA- single stranded RNA
SSA- sub-Saharan Africa
STAT4- Signal transducer and activator of transcription 4
TAP- transporter associated with antigen processing
TCR- T cell receptor
TGF-transforming growth factor
TLR- toll-like receptor
TNF- tumour necrosis factor
TTx- tetanus toxoid
Tbet- T box transcription factor
Tfh- follicular helper T cells

Th- T-helper

TRA- tissue-restricted antigens

Treg- regulatory T cells

VPD- vaccine preventable diseases

WHO- World Health Organization

WT- wild type

YF- yellow fever
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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 THE HUMAN IMMUNE SYSTEM

The immune system is the body’s defense against pathogens and other harmful agents. It is continually adjusting to our environment to ensure that we are protected from a plethora of infectious agents that we encounter. The immune system is specialized to distinguish between harmful and harmless antigens, induce strong effector functions so as to remove pathogens as well as establish lasting immunological memory so as to provide protection upon reinfection. It can be sub-divided into two main defence systems, which possess qualities that are interconnected and highly dependent on each other.

1.1.1 Innate immunity

The skin and mucosal surfaces form physical barriers and act as the body’s first line of host defence upon encountering a foreign agent. When a pathogen successfully passes through these barriers, innate immunity is induced rapidly to prevent the spread of infection and eliminate the pathogen.

Cells of the innate immune system include the macrophages, dendritic cells, neutrophils, basophils, eosinophils, monocytes, natural killer (NK) cells, \( \gamma \delta \) T cells and mast cells. The complement system is also an important component of the innate immune response. The
pattern recognition receptors (PRRs) on innate immune cells are able to recognize conserved pathogen-associated molecular patterns (PAMPs) expressed by pathogens, which allows for broad specificity against infectious agents. There are several different types of PRRs which act as sensors for pathogens on the innate immune cells. These include the nod-like receptors (NLR), toll-like receptors (TLRs), retinoic acid-induced gene (RIG)-1-like receptors and C-type lectin receptors (Santis, Lopez-Cabrera et al. 1994, Medzhitov and Janeway 1997, Medzhitov, Preston-Hurlburt et al. 1997).

The most widely studied of the PRRs are the TLRs, which are found in both vertebrates and invertebrates. They were first discovered in drosophila and then later in mammals (Medzhitov, Preston-Hurlburt et al. 1997). Signalling through them induces the production of pro-inflammatory cytokines, type 1 interferons and chemokines by innate immune cells, which is vital in clearing the pathogen and the recruitment of other immune cells. The type of pathogen detected depends on the location of the TLRs on the innate cells. TLRs that are expressed on the surface such as TLRs 1, 2, 4, 5 and 6 recognize components of extracellular pathogens such as lipopolysaccharide (LPS) and flagellin, whereas intracellular receptors in the endosomes such as TLRs 3, 7, 8 and 9 recognize components of intracellular pathogens such as double stranded RNA (dsRNA), single stranded RNA (ssRNA), DNA and bacterial CpG oligodeoxynucleotides respectively (Iwasaki and Medzhitov 2015).

There is new emerging evidence that innate immune cells (for example macrophages, monocytes and NK cells) undergo long-term epigenetic changes, which result in enhanced responsiveness following secondary stimulation by vaccination or infection (Netea, Joosten et al. 2016). For example, following Bacille Calmette-Guerin (BCG) vaccination, an epigenetic
modification of the NOD2 receptor on macrophages leads to enhanced innate responses (Kleinnijenhuis, Quintin et al. 2012). This concept is known as ‘trained immunity’ (Netea, Quintin et al. 2011). Another concept is that of LPS-induced tolerance, which occurs when TLR4 engages with LPS causing immune cell function to be inhibited, and is also thought to be mediated by epigenetic mechanisms (Dobrovolskaia and Vogel 2002). Both the processes of LPS-induced tolerance and trained immunity can lead to non-specific immune responses upon secondary stimulation causing either diminished or enhanced cytokine responses respectively, therefore introducing the concept of immunological memory in the human innate immune system (Ifrim, Quintin et al. 2014).

The innate immune system acts as a link to the adaptive immune system through the activation of antigen presenting cells (APCs) and polarizing cytokines and chemokines which subsequently stimulate adaptive immune responses (Kawai and Akira 2010).

1.1.3 Adaptive immunity

The T and B lymphocytes form the basis of the adaptive immune system, which is induced following the innate immune response. Activated APCs move on to the draining lymph node (DLN) to present antigens to the naïve T cells of the adaptive arm of the immune system (Boer, Joosten et al. 2015).

1.1.4 Antigen processing and presentation

The major histocompatibility complex (MHC) molecules, sometimes called human leukocyte antigens (HLAs) are important in the development of the human immune response as they allow for the recognition of peptides by the T cells. Peptides bound to MHC class II molecules
are recognized by CD4+ T cells, whereas the CD8+ T cells recognize peptides bound to MHC class I molecules. The T cell receptor (TCR) on the T lymphocytes recognizes these antigenic peptides when they are presented in an MHC:peptide complex by APCs. Generally, CD8+ T cells recognize and eliminate intracellular antigens that are non-self; whereas CD4+ T cells recognize extracellular epitopes. Two alpha-helices overlaying a beta-sheet make up a peptide binding groove in the MHC molecules. In MHC I molecules, the groove is closed-end and approximately 8-10 amino acid long; whilst that of the MHC II molecules is open-ended and relatively longer with about 13-22 amino acid residues (Sercarz and Maverakis 2003).

T cells do not recognize folded proteins hence antigens are broken down and processed into peptides before they can bind to the MHC molecules. MHC-II molecules generally bind peptides generated by lysosomal proteolysis in the endocytic and phagocytic pathways. Generally, MHC-I bind peptides are generated by proteasomal proteolysis in the endoplasmic reticulum (ER) after the peptides are translocated from the cytosol. Translocation from the cytosol is done by a member of the ATP-binding cassette (ABC) family of transporters; the transporter associated with antigen processing (TAP). Being a heterodimeric protein, it has two subunits, known as TAP1 and TAP2, which are encoded by closely linked genes in the MHC molecule. To a lesser extent, exogenous antigens can also be loaded on MHC-1 through the process of cross-presentation (Blum, Wearsch et al. 2013). Transport pathways such as receptor mediated endocytosis, macropinocytosis and phagocytosis allow APCs to take up extracellular antigens for cross-presentation. The DCs, BCRs, Fc Receptors, CLRs and HSP receptors mediate endocytosis of extracellular antigens; whilst macrophages prefer phagocytosis of the antigens (Fehres, Unger et al. 2014).
1.1.5 T cell activation

The diverse repertoire of antigen receptors produced from gene rearrangement found on the T cells, allows for broad specificity and diversity. Genes encode for multiple V, D and J segments of the α and β chains of the T cells, allowing a very high level of diversity. To ensure that the response is targeted towards a specific pathogen, the process of T cells being activated is tightly regulated and requires a number of signals.

In order to survive, naïve T cells need to interact constantly with self-MHC via the TCR/CD3/CD4 or CD8 complex (Morris and Allen 2012) as they recirculate through the SLO. CCL21 engaging with CCR7 on their surface allows the naïve T cells to be carried through the lymph (Gatto and Brink 2010). The recognition and discrimination of self and non-self antigens is the first step required. The activation of T cells involves the engagement of the TCR of CD4 and CD8 T cells to the MHC class II and MHC class I molecules on the APCs respectively (first signal); co-stimulation with co-stimulatory molecules (second signal) and receiving stimuli from cytokines in the environment (third signal).

Expressed on APCs during inflammation, the CD80/86 and CD40 Co-stimulatory molecules bind to CD28 and CD40L on T cells respectively, allowing for the stabilization of the TCR activation signals (Howland, Ausubel et al. 2000). Binding with PRRs such as the TLRs and cytokines in the microenvironment further results in APCs to express co-stimulatory cytokines and produce cytokines. Once activated, T cells have been shown to produce IL-2 and express the high affinity IL-2 α chain receptor, CD25 thereby promoting proliferation (Smith-Garvin, Koretzky et al. 2009) (Smith-Garvin, 2009). Furthermore, the T cells can
differentiate into different subsets, and the expression of specific transcription factors ultimately determines the subset the T cell differentiates into.

1.1.6 T cell lineage development

The cytokine stimuli from APCs and T cells in the local environment is important in determining the transcription factor expressed and T cell subset/lineage a naïve T cell becomes. For instance, type 1 macrophages are pro-inflammatory and produce IL-23 and IL-12 to enhance Th-1 immunity, whereas type-2 macrophages induce regulatory T cells by producing IL-10 (Verreck, de Boer et al. 2004).

In the presence of interferon-γ (IFN-γ) and interleukin-12 (IL-12), naïve T cells express the transcription factor T box transcription factor (T-bet) and differentiate into the Th1 lineage via the transcription factor signal transducer and activator of transcription 4 STAT4. The Th1 cells are responsible for the production of the Th1 pro-inflammatory cytokines such as IFN-γ, IL-2 and tumour necrosis factor (TNF) (reviewed by (Annunziato, Cosmi et al. 2009)). These pro-inflammatory cytokines have been shown to protect against intracellular pathogens, but have also been implicated in causing immune pathology (Glimcher and Murphy 2000). Naïve T cells differentiate into Th2 cells in the presence of IL-2 and IL-4 resulting in the expression of the transcription factor GATA-binding protein 3 (GATA3). The Th2 cells produce cytokines such as IL-4, IL-5, IL-9, IL-10 and IL-13, causing an increase in eosinophils, activation of mast cells and the production of IgE. The Th2 response protects against extracellular parasites but also plays a pathogenic role in allergic disorders and helminths infection (Coffman 2010).
In the presence of IL-1 and IL-23 in humans, there is expression of the retinoic acid-related orphan receptor-y (RORyt) (Ivanov, McKenzie et al. 2006) resulting in the differentiation into Th17 cells which produce IL-6, IL-17, IL-20, IL-21, IL-22, TNF and chemokine ligand 20 (CCL20). Th17 cells protect against extracellular bacteria and fungi, but have also been shown to be responsible for some autoimmune disorders (reviewed by (Louten, Boniface et al. 2009). Furthermore, naïve T cells that express aryl hydrocarbon receptor (AHR) in the presence of TNF and IL-6 become Th22 cells, a CD4 lineage shown to play a role in skin homeostasis and pathology (Azizi, Yazdani et al. 2015). Another described lineage is that of the follicular helper T cells (Tfh), which mediate T helper cell function in aiding antibody production and are essential in the development of the B cell germinal centre and production antibodies with high-affinity and memory B cells. Bcl6 is an essential transcription factor for the differentiation of naïve T cells into Tfh. More recently, Tfh have been implicated in autoimmune diseases and cancer immunity (Crotty 2014).

1.1.7. Generation of memory T cells

The TCR on the T lymphocytes recognise these antigenic peptides when they are presented in the form of an MHC:peptide complex by APCs. The immunoglobulins on the surface of the B cells act as B cell receptor (BCR.) When the adaptive immune response is induced, there is rapid clonal proliferation and differentiation of the activated T and B cells into cells with the required function to eliminate the pathogen. The cells now express the required chemokine receptors and adhesion molecules allowing them to migrate to the infected site.

Once the antigen is eliminated, the immune response is suppressed through the contraction
phase and most of the expanded conventional effectors undergo apoptosis leaving behind subsets of memory cells which persist and are capable of mounting a quicker and stronger response upon re-counter with the same antigen (Pennock, White et al. 2013). The size of the pool of the memory cells is directly affected by that of the naïve CD4 precursor pool. Using surface markers such as CCR7 and CD45RA, the T cells can be subdivided into four main functional populations with distinct homing capacity and effector function. CD45RA and CCR7 expression can be used to distinguish between functional T cell subsets; CD45RA+CCR7+ naïve T cells, CD45RA+CCR7- TEMRA cells, CD45RA-CCR7+ central memory cells, and CD45RA-CCR7+ effector memory T cells (Sallusto, Lenig et al. 1999, Tosello, Odunsi et al. 2008). Since being described, intermediate subsets have also been suggested to exist in-vivo by multiple studies. Generally, data suggests that the generation of memory T cells may involve several mechanisms influenced by the inflammatory environment and the effector lineage. For instance IL-21, IL-15, IL-7 and Bcl-6/Eomes seem to drive T cells towards a memory phenotype; whilst cytokines and factors such as IL-12, IL-2, Blimp-1/Tbet towards effectors and terminally differentiated cells. If exposed to these factors for too long, the T cells may experience exhaustion. (Gasper, Tejera et al. 2014). T cell exhaustion leads to dysfunctional T cells resulting in the inadequate control and clearance of infections which can lead to chronic infection or cancers (Wherry 2011).

1.1.8 Development of long-lived memory B cells

B cells can be activated by Thymus-dependent (T-dependent) or thymus-independent (T-independent) antigens. The T-dependent antigens require T cell help to provide the accessory signal in order to be activated. Antigens that are bound to surface immunoglobulin are internalized, bound to MHC-II molecules and returned to the surface to bind to the T
helper cells thereby providing accessory signals to the B cell (Janeway 2001). T-independent antigens can induce antibody responses without T cell help. The B cell can be induced by directly recognizing microbial antigens or through the binding of repeated epitopes on the pathogen binding to the B cell thereby causing crosslinking of the BCRs (Janeway 2001).

Activated B and T cells migrate to the B cell-T cell border of the secondary lymphoid organs (SLO) where they interact. Effective communication between the two allows the B cells to receive CD4 T cell help. B cells also express CXCR5 which allows them to interact with follicular DCs (Cyster 2010). Both B and T cells begin to express the transcription factor BCL-6 at the B cell-T cell border, causing them to migrate to the outer follicles where the B cells start to proliferate and differentiate into short-lived plasma cells ultimately giving rise to the extra-follicular foci, and GC-independent memory B cells (Kurosaki, Kometani et al. 2015). Some of the activated B cells, retaining the expression of CXCR5, go back to the follicle where they form the Germinal Centre (GC) following rapid proliferation. The formation of the GC is driven by BCL-6 expression (Gatto and Brink 2010, Shlomchik and Weisel 2012).
The germinal centre can be divided into light and dark zones (Figure 1.1), with the former being the site of B cell selection. BCRs with a strong affinity for antigen (presented by the follicular DCs) result in the internalization of the antigen and it being presented via MHC II molecules to Tfh which send signals for the GC B cells to migrate to the dark zone, thereby driving proliferation. In the dark zone, activation-induced cystidine deaminase (AID) driven somatic hypermutation occurs (Preite, Baumjohann et al. 2015). This process is the introduction of random point mutations on the V region of the Immunoglobulin further diversifying the BCR, and altering the affinity of the BCR for antigen (Shlomchik and Weisel 2012). BCRs with weak affinity to the antigen undergo apoptosis, whilst those with medium affinity return to the light zone where antigen-driven affinity maturation takes place with the help of follicular DCs and Tfh cells (Dempsey 2016).

B cells with immunoglobulins that have high affinity for their specific antigen are preferentially selected and undergo isotype/class switching through intrachromosomal deletional recombination events, thereby forming higher-affinity pathogen-specific antibodies (Stavnezer, Guikema et al. 2008). These matured B cells differentiate and exit the germinal centre as either memory B cells or long-lived plasma cells (Shlomchik and Weisel 2012).
Figure 1. 1: B cells in the germinal centre

The generation of somatically mutated high-affinity memory B cells and plasma cells

(Taken from (De Silva and Klein 2015).)
The differentiation into either cell type is driven by affinity of the BCR with plasma cells having BCR with higher affinity, and influenced by the presence of IL-21 and the expression of the transcription factor Blimp-1 (Shlomchik and Weisel 2012). In contrast, memory B cells have lower affinity BCRs. To further distinguish the two, phenotypic markers have been used; memory B cells circulate through the SLO are mainly defined as CD27+IgD-, whilst plasma cells reside in the bone marrow and are defined as CD138+CD19-CD20-CD21- (Eibel, Kraus et al. 2014).

Protection through the humoral response is obtained from sustained protective antibodies produced by long-lived plasma cells (primary humoral response), and memory B cells (reactive humoral response). If the pre-existing antibody titres are not sufficient, the secondary/reactive humoral response is induce, with the memory B cells becoming reactivated to produce antibodies (Bortnick and Allman 2013).

### 1.1.9 Antibody structure and function

Antibodies otherwise known as Immunoglobulins are heterodimeric proteins consisting of four polypeptide subunits; two identical heavy (H) and two identical light (L) chains of differing molecular weight. Functionally, these chains can be separated into variable and constant regions. The light and heavy chains are composed of one constant and one variable region (Schroeder and Cavacini 2010). The variable region contains the antigen binding sites whilst the constant region, which is identical in immunoglobulins (Ig) of the same isotope, is involved in binding to Fragment of crystallisable (Fc) receptors on effector cells and activation of the complement system. The variable segment can be further divided into complementary...
determining regions (CDRs) based on variability in the sequences; and form the antigen binding site (Schroeder and Cavacini 2010) (Figure 1.2). To allow for the specificity required and immunoglobulin diversity, the variable region of the immunoglobulin undergoes a series of mechanisms such as gene rearrangement, following which it undergoes somatic hypermutation once it is exposed to the antigen so as to allow for affinity maturation (Shlomchik and Weisel 2012).

Figure 1. 2: Two-dimensional representation of the immunoglobulin structure

H= heavy chain, L= light chain, N= amino terminus, C= carboxyl terminus, s-s= disulphide bridge. (Taken from (Schroeder and Cavacini 2010)
Gene rearrangement allows for an antibody repertoire of more than $10^{16}$ different immunoglobulins to be generated through combinatorial joining of the individual VDJ gene segments and of the different association between the light and heavy chain regions; combined with somatic variation in the CDR3 region of the immunoglobulin (Schroeder and Cavacini 2010). Once the immunoglobulin is exposed to an antigen, the genes from the variable segment undergo hypermutation. Each Ig has a CH2 domain acting to stabilize the antibody, an N and C-terminus, and disulphide bonds that hold together the light and heavy chains. Each Ig can be separated into 2 Fab (Fragments of antigen binding) and 1Fc fragments (Smith, Brewer et al. 2004). The mechanisms used by antibodies to protect the host include opsonisation, agglutination, antibody-dependent cellular cytotoxicity (ADCC), neutralization and activation of the complement system (Forthal 2014)

The Fab and Fc fragments enable the effector mechanisms of the antibody. For instance, opsonisation occurs when the Fab portion of the Ig binds to the epitope on the antigen of the pathogen. The (Fc) region is exposed which is recognised by the Fc receptor (FcR) on the phagocyte. The binding of the Ig to the Fc receptors expressed on immune cells links the cellular to the humoral component of the immune response. In addition to the classical functions of the FcR in classical functions of immunoglobulins, the engagement of Fc receptors can induce a signal-transduction cascades thereby resulting in direct antimicrobial activity (Casadevall and Pirofski 2011). A vast number of Fc receptors matching the number of different Immunoglobulin isotypes and subclasses have been described. The most widely studied are the Fc receptors that bind to IgG, known as FcγR are expressed on hematopoietic cells, as well other cells such as endothelial cells (van der Poel, Spaapen et al. 2011). IgG binds to the membrane proximal extracellular domain of FcγR (Schroeder and Cavacini 2010).
The end result of the interaction of antibody and antigen with FcγR tends to be a balancing act between inhibitory and stimulatory signals. Signalling through the receptor is regulated by the immunoreceptor tyrosine-based activation motifs (ITAMs) and immunoreceptor tyrosine-based inhibitory motifs (ITIMs). FcRs with ITAMs induce cell activation, and effector functions such as endocytosis, phagocytosis and ADCC, whilst those with ITIMs have an inhibitory effect on cell activation (Nimmerjahn and Ravetch 2008). CD89 is the FcR for IgA and is expressed on myeloid cells such as polymorphonuclear cells (PMN), some DCs and monocytes, Unlike IgG, IgA binds to membrane distal EC1 domain of FcγR (Schroeder and Cavacini 2010).

Similar to opsonisation, the Ig can also form a bridge between the target cells and Fc receptor on effector cells such as NK cells, which in turn release cytotoxins leading to the lysis and eventual destruction of the target cell. Another mechanism is agglutination where the aggregation of several antibody-antigen complexes causes a cluster to form thereby inactivating the pathogens, and making it easily detectable by macrophages (Schroeder and Cavacini 2010). The Ig can also neutralise viruses and toxins by interfering with its interaction with the host. It can attach to the viral epitope and to the active site on toxins thereby interfering with viral infectivity or toxin-mediated toxicity (Casadevall and Pirofski 2011). The Ig can coat the antigen and bind to the FcR of effector cells such as NK cells causing them to destroy the targeted pathogen, this is known as ADCC. Antibodies can also activate the complement system as an additional key effector function (Rosales, Rangel-Rivas et al. 2013).
1.1.10 Properties of adaptive immunity in infancy

1.1.10.1 Infant T cell immunity

The adaptive immune system of the neonate at birth is characterized by minimal immunological memory, with most of their T cells being of a naïve phenotype (Levy 2007). To prevent the mother from developing Th1 alloreactivity to her fetus, there are high levels of the immunosuppressive factors tumour growth factor-β (TGF-β), progesterone and prostaglandin E2 in utero (Philbin and Levy, 2009), and poor innate Th1 support (Langrish, Buddle et al. 2002), thus resulting in an intrinsically skewed Th2-type immunity in the newborn. Nevertheless, adult-like Th1 responses have been induced in infants following BCG vaccination (Marchant, Goetghebuer et al. 1999), thus showing that neonates have the capacity to mount a robust Th1 immune response. However, BCG vaccination at birth results in a Th17 biased mycobacterial response when compared to receiving BCG at 4½ months of age (Burl, Adetifa et al. 2010), showing that there is an early Th17 bias despite ability to prime an early Th1 response.

Furthermore, infants have been shown to have relatively lower numbers of effector memory T cells using the CD45RA-CD45RO+ marker (Adkins 2007). This unique Th2/Th17 polarized responses combined with the limited number of effector memory cells therefore makes them more susceptible to infections within the first year of life.
1.1.10.2 Infant B cell immunity

There are some key differences between humoral responses in young infants to that of adults. In young infants, humoral responses are delayed in onset, of shorter duration, and achieve lower peak levels with lower affinity than in adults (Basha, Surendran et al. 2014). Acquisition of IgG antibodies transplacentally from mothers provides newborn infants with protection against infections they are exposed to in early life. Therefore, to protect young infants who are yet to be vaccinated, maternal immunisation of pregnant mothers to protect against pertussis, influenza and tetanus infections is routinely done in some parts of the world, as the transfer of maternal antibodies (MAbs) may confer protection during the critical period before some of the childhood vaccines are administered (Steedman, Kampmann et al. 2016).

Levels of MAbs usually wane over the first 6 months of life, with absent or minimal levels after the first year of life (Edwards 2015). For instance, the duration of protection from measles by MAbs in infants is approximately 3.3 months (Waaijenborg, Hahne et al. 2013). Inhibition of humoral responses to vaccines administered in infancy by MAbs has been described in several studies, with even low levels of decaying antibodies shown to interfere with vaccine-induced responses (Jones, Naidoo et al. 2011). MAb have been shown to interfere with humoral responses to live vaccines such as measles (Albrecht, Ennis et al. 1977) and oral poliomyelitis vaccines; and non-live vaccines including pertussis (Burstyn, Baraff et al. 1983, Englund, Anderson et al. 1995), tetanus and diphtheria toxoids (Bjorkholm, Granstrom et al. 1995), Hib conjugate vaccine (Claesson, Schneerson et al. 1989, Daum, Siber et al. 1991) and Hepatitis A vaccine (Kanra, Yalcin et al. 2000). Although MAb inhibition may
occur, humoral responses generated may still be protective (Jones, Pollock et al. 2014). MAb induced interference is not thought to occur with T cell responses (Siegrist 2003).

The distribution of the IgG isotypes in young children are different to that of adults, with levels of IgG1 and 2 only peaking at around 3 to 4 years of age, and IgG4 at 4 to 6 years of age. Conversely, levels of IgG3 are shown to be stable from birth. The other immunoglobulin subclasses (IgA, IgD, IgE, IgM) cannot pass through the maternal-placenta interface and are thus not present at birth and develop in the first few weeks after birth (Ngamphaiboon, 1998).

1.2 CENTRAL AND PERIPHERAL T CELL TOLERANCE

Recombination of the TCR genes allows for the specificity and diversity of the TCR in thymocytes, however, thymic selection is required to remove self-reactive thymocytes (Xing and Hogquist 2012). The two main mechanisms of central tolerance (in the thymus) are clonal deletion and clonal diversion. Another mechanism known as receptor editing has been described in which secondary TCR gene rearrangement results in the changing TCR specificities in thymocytes with a high affinity for self-peptide-MHC (Santori, Arsov et al. 2002). These thymocytes escape clonal deletion and diversion. Thymocytes are selected based on self-reactivity, with TCRs that do not bind to MHC-self peptide ligands dying by neglect. The remaining thymocytes undergo a process of positive and negative selection. Thymocytes with TCRs that bind to self-MHC with low affinity undergo positive selection;
survive, differentiate into single positive CD4 and CD8 T cells and become effector and memory T cells. In contrast, there is selection against thymocytes with TCRs that bind with an affinity that is too strong; these undergo negative selection (Morris and Allen 2012, Brownlie and Zamoyska 2013).

For both clonal deletion and diversion to occur, TCR engagement is also required. Thymocytes presenting TCRs with high affinity to MHC self-peptide complexes present a potential threat and undergo clonal deletion or clonal diversion; with deletion occurring with stronger signals and diversion with weaker signals (Xing and Hogquist 2012). Expression of pro-apoptotic proteins such as Bim and Nur77 lead to apoptosis, whilst the presence of the cytokines such as TGF-β and IL-2 prevents apoptosis and leads to diversion resulting in the differentiation of Tregs (Baldwin and Hogquist 2007). In the thymus, positive selection of Tregs occurs in the cortex, whilst negative selection of autoreactive T cells occur in the medulla (Xing and Hogquist 2012). In the medulla of the thymus, the negative selection of self-reactive T cells occurs through the expression of self-antigens such as tissue-restricted antigens (TRAs) on the mTEC. This expression is mediated by the autoimmune regulatory gene (AIRE), which also promotes the positive selection of Tregs. Mutations in AIRE results in autoimmune polyglandular syndrome type 1 (APS-1). More recently, another transcription factor has been identified FEZF2 which also regulates TRAs and is vital in suppression of autoimmune responses (Takaba and Takayanagi 2017).

Although mechanisms ensuring central tolerance are efficient, additional checks have to be in place to eliminate other self-reactive T cells. In the periphery, antigens such as developmental and food antigens are encountered for the first time. Some lymphocytes also
encounter self-cognate antigens for the first time.

Secondary signals are provided by co-stimulatory molecules on the T cells to ensure T cell tolerance by inducing hyporesponsiveness/anergy in the self-reactive T cells, and thereby prevent autoimmunity. Important costimulatory molecules, which are important in maintaining T cell tolerance, include PD-1 (and its ligands PD-L1 and PD-L2) and CTLA-4. PD-1 ligation with PD-L1 and PD-L2 limits and inhibits the activation and expansion of thymocytes with TCRs with high affinity to MHC self peptide (Probst, McCoy et al. 2005). PD-1 has also been shown to be involved in the conversion of naïve T cells to Tregs. CTLA-4 binds to co-stimulatory molecules in the B7 family, transducing an immunoregulatory signal (Buchbinder and Desai 2016).

### 1.3 REGULATORY T CELLS AND THEIR MECHANISMS OF ACTION

Humans have evolved several mechanisms to ensure the development of protective immunity, whilst limiting immune damage as a result of an over exuberant or uncontrolled immune response (Sakaguchi 2000, Belkaid, Piccirillo et al. 2002, Mills and McGuirk 2004, Belkaid 2007). Regulatory T cells (Tregs) were first described about three decades ago as suppressor T cells (Gershon, Cohen et al. 1972) mainly using murine models. Recent advances in their molecular and phenotypic characterization have resulted in a resurgence of interest in this T cell subset over the past decade. The importance of Tregs was first identified when thymectomy performed on 3 day-old mice resulted in the production of autoantibodies and autoimmunity. The subsequent transfer of thymic cells from adult mice to the mice that had
undergone thymectomy reversed the development of autoimmunity and immune pathology (Sakaguchi, Takahashi et al. 1982).

Tregs can suppress different stages of T cell activity, and there is evidence that they can suppress T cell proliferation, differentiation and effector T cell functions such as cytokine production, cytolytic activity and antibody production either directly or indirectly via the CD4 helper T cells (Figure 1.3) (Sojka, Huang et al. 2008). Although there is compelling evidence for suppression of Th1 and Th2 responses by Tregs, their role in limiting Th17 responses is contentious. Indeed, rather than a regulatory effect, Tregs and Th17 cells are shown to have a reciprocal relationship with the immune system polarizing towards one subset or the other (Chen and Oppenheim 2014). While Tregs have been shown to be beneficial in maintaining immune homeostasis and tolerance, some studies show that they can limit the favourable effector responses needed for sterilizing immunity, thus allowing pathogens to persist in the host (Kao, Zhang et al. 2010).
Figure removed for copyright reasons

Figure 1. 3: The modulation of immune responses by Tregs

There is evidence that Tregs suppress effector T cell proliferation, differentiation and terminal effector T cell function. (Taken from (Sojka, Huang et al. 2008).)

More recently, a consensus nomenclature for FOXP3+ Tregs has been introduced by a group of experts in the field, since previous terms were to some extent considered ‘inaccurate and ambiguous’ (Abbas, Benoist et al. 2013). Rather than natural Tregs (nTregs), FOXP3+ Tregs originating from the thymus are now known thymus-derived Tregs (tTregs); and the previous term inducible Tregs (iTregs) used for Tregs for those that differentiate in the periphery are now called peripherally derived Tregs (pTreg). The pTregs possess identical characteristics to the tTregs, hence both subsets are described below.

1.4 THYMUS DERIVED AND PERIPHERAL CD4+FOXP3+ TREGS

The discovery of the defining marker transcription factor FOXP3 invigorated the Treg field. FOXP3 is described as the master regulator of Tregs, vital not only for the development and function, but also Treg homeostasis (Fontenot, Gavin et al. 2003, Hori, Nomura et al. 2003).
The development of a severe fatal disorder associated with FOXP3 mutations in humans known as immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome (Bennett and Ochs 2001, Gambineri, Torgerson et al. 2003) further highlights the importance of FOXP3 and Tregs \textit{in-vivo}. First described in the early 1980s, it usually associated with an early onset of severe diarrhoea, dermatitis, insulin dependent diabetes mellitus (IDDM) and ‘failure to thrive’ (Powell, Buist et al. 1982). Most of the children who do not receive any treatment die before their second birthday, mainly from complications from ‘failure to thrive’ and sepsis. Only males are affected, while females who carry the gene remain healthy (reviewed by (Barzaghi, Passerini et al. 2012) depicting an X-linked inheritance pattern.

1.4.1 Phenotype of human CD4$^+$FOXP3$^+$ Tregs

Belonging to the forkhead family of transcription factors, FOXP3 remains critical for the development and function of tTregs and pTregs. Studies have shown that 1-5\% of CD4$^+$ T cells express FOXP3 in humans (Mercer and Unutmaz 2009). Although transiently, activated conventional T cells have also been shown to express FOXP3 (Wang, Ioan-Facsinay et al. 2007, Kmieciak, Gowda et al. 2009). Nevertheless, it still remains the most defining marker of Treg cells. Being the most widely studied Treg subset, these suppressive CD4$^+$FOXP3$^+$ Tregs were initially described in mice as a subset of CD4$^+$ T cells constitutively expressing the interleukin-2 (IL-2) receptor alpha-chain (CD25) (Sakaguchi, Sakaguchi et al. 1995). It was later shown in humans that cells with a similar phenotype and function as seen in mice had the ability to suppress the proliferation of other CD4$^+$ and CD8$^+$ T cells when co-cultured and stimulated with a specific antigen in the presence of IL-2 (Takahashi, Kuniyasu et al. 1998, Thornton and Shevach 1998). FOXP3 was later identified as the transcription factor, critical
for the development and function of Tregs in humans (Fontenot, Gavin et al. 2003, Hori, Nomura et al. 2003).

Defining the precise phenotype of tTregs and pTregs in humans has proven to be difficult. For example, CD25 expression is transient and is also a marker of T cell activation (Hatakeyama, Tsudo et al. 1989), and a more reliable marker of the CD4+FOXP3+ Tregs is thought to be the CD25hi subset in humans (reviewed by Schmetterer, Neunkirchner et al. 2012). Nevertheless, within the total FOXP3+ Tregs in humans, at least 50% are CD25lo/-, indicating that some of CD4+FOXP3+ Tregs may be missed when human Tregs are solely identified using CD25hi expression (Chen, Subleski et al. 2010). It has therefore been proposed to use a combination of CD25+/hi with other markers for the identification of functional Tregs in humans (Chen and Oppenheim 2011).

Liu et al. showed that FOXP3 expression inversely correlates with expression of the IL-7 receptor CD127 in humans, with FOXP3+ Tregs tending to express low levels (Liu, Putnam et al. 2006, Seddiki, Santner-Nanan et al. 2006). As a result, CD127lo is used as an additional marker of functional Tregs in human studies. Human CD4+FOXP3+ Tregs frequently express high levels of the co-inhibitory receptor cytotoxic T lymphocyte antigen-4 (CTLA-4) (Sansom and Walker 2006) and glucocorticoid inducing tumour necrosis factor receptor (GITR) (McHugh, Whitters et al. 2002), but neither are considered reliable markers of human Tregs.

More recent studies have shown that CD45RA expression can be used to distinguish naive thymus-derived Tregs (nTregs) or resting tTregs (rTregs) (CD45RA+FOXP3lo) from the memory activated subset (CD45RA-FOXP3hi) (Miyara, Yoshioka et al. 2009). They found that
the CD45RA-FOXP3hi activated Tregs rapidly die, whereas the CD45RA+FOXP3lo resting Tregs (rTregs) proliferate and convert into activated Tregs (aTregs). From their microarray analysis, they also saw that aTregs were more involved in the transcription of IL-10 and less involved in the transcription of TGF-β as compared to the rTregs. Further subdivision of the memory Tregs into central memory (Treg<sub>CM</sub>) and effector memory (Treg<sub>EM</sub>) is based on the expression of chemokine receptor 7 (CCR7) (Sallusto, Lenig et al. 1999, Tosello, Odunsi et al. 2008). Another phenotypic marker of FOXP3+ Tregs is the co-expression of the ectoenzymes CD39 and CD73, which activate the A2A receptor on the effector T cells and production of adenosine which results in effector T cell suppression (Deaglio, Dwyer et al. 2007).

More recently, the subpopulation of CD4<sup>+</sup>FOXP3<sup>+</sup> Tregs expressing tumour necrosis factor receptor 2 (TNFR2) has been found to be a highly active and immunosuppressive subset (Minigo, Woodberry et al. 2009, Govindaraj, Madondo et al. 2014). The cytokine TNF, has been shown to induce a negative feedback response on proinflammatory response by causing the expansion of Tregs through the TNFR2 receptor (Chen, Baumel et al. 2007). More so, the TNFR2 receptor is constitutively expressed on tTregs (Anunziato, Cosmi et al. 2002). Combining TNFR2 with other Treg markers, suppressive Tregs have been identified in humans in the CD25lo/- subsets (Chen and Oppenheim 2010). In addition, vaccine adjuvants which induce TNF are thought to stabilize the expression of FOXP3 on the TNFR2+ Tregs (Chen and Oppenheim 2011). As TNFR2+ Tregs have been described as a functionally suppressive subset in adults, whether the same is true for TNFR2+ Tregs in neonates and infants is yet to be explored.
1.4.2 Mechanisms of CD4⁺FOXP3⁺ Treg mediated suppression

Suppression of the proliferation and activation of a multitude of immune cell types including T cells, NK and NKT cells, monocytes, macrophages, B cells, DC and eosinophils by CD4⁺FOXP3⁺ Tregs has been described. Tregs employ a variety of mechanisms to mediate this suppression, but are also flexible in that they can adapt the mechanism according to their local environment (Wing and Sakaguchi 2012).

Both IL-2 and CTLA-4-dependent mechanisms are thought to occur, with CD25 and CTLA-4 knockout mice having a similar phenotype to Foxp3 deficient mice (Wing and Sakaguchi 2012). The constitutive expression of CD25 by CD4⁺FOXP3⁺ Tregs is thought to allow them to consume the available IL-2 in the local environment, thus depriving effector T cells and leading to effector cell death (de la Rosa, Rutz et al. 2004). Down-modulation of CD28 signalling by CTLA-4-expressing tTregs (Walker 2013), as well as the reduced co-stimulatory capacity of CD80/86 on the dendritic cells (DCs) can suppress T cell responses (Wing, Yamaguchi et al. 2011) (Figure 1.4).

Production of soluble inhibitory factors, including either membrane bound or released immunosuppressive cytokines IL-10, TGF-β and IL-35, is also implicated in Treg function (Collison, Workman et al. 2007). Additionally, there is evidence that the inhibitory factor adenosine can be generated in high concentrations by FOXP3⁺ Tregs (Mandapathil, Hilldorfer et al. 2010). Binding to the A2a receptor on immune cells activates an immune-inhibitory loop (Sitkovsky and Ohta 2005). CD39 causes a breakdown of adenosine triphosphate (ATP), which then results in the inhibition of proliferation and cytokine production by the T cells (Raskovalova, Huang et al. 2005).
Figure 1. 4: Simple representation of the proposed four main modes of action of Tregs

Tregs have been proposed to exert their suppression on the effector T cells by (A) the production of inhibitory cytokines, (B) direct induction of apoptosis of target cells, (C) disrupting the metabolic processes of the target cell, and (D) modulating the function of the APCs (taken from (Vignali, Collison et al. 2008). Treg = regulatory T cell, Teff = effector T cell, IL-2= interleukin 2, TGF= transforming growth factor, IDO= indoleamine 2,3-dioxygenase, APC= antigen presenting cells, CTLA-4 = cytotoxic T lymphocyte antigen 4 (Taken from (Vignali, Collison et al. 2008) .

1.5 TYPE 1 REGULATORY T CELLS (TR1)

Unlike the CD4+ Tregs expressing CD25 and/or FOXP3, the Tr1 Tregs are a unique subset that do not rely on the expression of high levels of either marker in order to function (Levings and Roncarolo 2000). Antigenic stimulation in the presence of IL-10 results in their activation (Groux, O’Garra et al. 1997, Vieira, Christensen et al. 2004). Specific markers for Tr1 cells include lymphocyte-activation gene 3 (LAG3) and CD49b (Gagliani, Magnani et al. 2013).
Secretion of IL-10 has been shown to be the main mechanism by which Tr1 cells mediate suppression, with the IL-10 being either free or membrane bound. Furthermore, this unique subset has also been shown to produce high levels of the immunosuppressive cytokine TGF-β, some IL-5, low levels of IFN-γ and IL-2, but no IL-4 (Groux, O'Garra et al. 1997). Effector T cell proliferation/activation is suppressed both directly and indirectly via a modulation of APC function (Roncarolo, Gregori et al. 2006). Other mechanisms of action shown to be used by the Tr1 cells includes production of granzyme B and perforin (Gregori, Tomasoni et al. 2010) causing Treg-induced cytolysis (Gregori, Goudy et al. 2012).

1.6 T HELPER 3 (TH3) TREG CELLS

Earlier studies investigating oral tolerance resulted in the first identification of another unique subset of TGF-β producing Tregs, the Th3 cells (Gol-Ara, Jadidi-Niaragh et al. 2012). They have been shown to suppress the proliferation and activation of Th1 cells and suppress the development of autoimmunity in the mouse model of multiple sclerosis (MS) (Chen, Kuchroo et al. 1994). Activation in the periphery occurs upon encounter with a specific antigen, followed by suppression via the production of the inhibitory cytokine TGF-β. Evidence shows that Th3 cells may also play a role in controlling autoimmunity and allergy in humans (Andersson, Olsson et al. 2002, Perez-Machado, Ashwood et al. 2003), however their role in maintaining immune tolerance in humans is yet to be defined.
1.7 CD8+ TREG CELLS

While fewer studies have been done on CD8+ Tregs, there is increasing evidence that subsets of CD8 T cells may also play important immunoregulatory roles, and that impaired CD8+ Treg function may result in autoimmunity (Hu, Ikizawa et al. 2004, Lu, Kim et al. 2008, Liu, Lan et al. 2014) (reviewed by Liu et al., 2014). The most widely described CD8+ Treg subset is the CD25+CD28- subset (Ciubotariu, Colovai et al. 1998, Filaci, Fravega et al. 2004). Some additional markers include CD122, CTLA-4, GITR, CD38 and CD103 (Uss, Rowshani et al. 2006, Simone, Zicca et al. 2008, Smith and Kumar 2008, Liu, Lan et al. 2014). Variable combinations of these markers have been used to describe different CD8+ Treg subsets (Suzuki, Jagger et al. 2012). Unlike CD4+ Tregs, FOXP3 expression in CD8+ Tregs seems to represent an activation marker rather than a defining CD8+ Treg marker, as in some studies FOXP3+CD8+ T cells have been shown to be minimally suppressive (Mayer, Floess et al. 2011). However, a novel subset of CD8+ T cells with the phenotype CD8+CD45RA+CCR7+FOXP3+ cells have been shown to have some immunosuppressive capacity (Suzuki, Jagger et al. 2012). Similar mechanisms of suppression as the CD4+ Tregs have been reported for CD8+ Tregs (reviewed by Suzuki, Konya et al. 2008) (Uss, Rowshani et al. 2006, Simone, Zicca et al. 2008, Smith and Kumar 2008, Liu, Lan et al. 2014). In our study, we will only be investigating the role of CD4+ Tregs in vaccine immunogenicity.

1.8 TREG SUPPRESSION ASSAYS

In vitro co-culture suppression assays were first developed in 1998 using the murine model (Takahashi, Kuniyasu et al. 1998, Thornton and Shevach 1998). The assay is used to study the functionality of Tregs by investigating their capacity to suppress either T cell proliferation or...
pro-inflammatory immune responses such as cytokine production. Treg cells are added either
directly to effector cells, or are added to a transmembrane compartment, which does not
allow cell-to-cell contact but allows the passage of soluble suppressive factors. Both studies
showed that the CD4+CD25+ population in mice were anergic *in vitro* and suppressed the
production of IL-2 by CD4+CD25- effector cells. The mechanism of suppression of these cells
was shown to be cell-to-cell contact dependent.

In murine studies, Tregs derived from spleen can be used since they provide high numbers
required for co-culture assays. In humans, peripheral Tregs are usually isolated from blood
samples. Earlier studies showed that 10-15% of murine CD4+ T cells are CD25+ (Thornton and
Shevach 1998), however Baecher-Allan et al show that in human peripheral blood, only 1-3%
of CD4+ T cells with the highest expression of the CD25 surface marker (CD25hi) exhibit
regulatory capacity (Baecher-Allan, Brown et al. 2001). This makes conducting *in vitro*
suppression assays in humans, particularly in young infants, more challenging. Due to ethical
constraints, large volumes of blood cannot be collected from infants and suppression assays
require large volumes for Treg isolation. It is therefore not surprising that most studies in
neonates and young infants use cord blood (CB) to investigate the function of Tregs, as it is
easier to collect large volumes of CB than peripheral blood in early life.

1.9 TREGS IN INFANTS, AND THEIR PHENOTYPIC AND FUNCTIONAL DIFFERENCES AS
COMPARED TO ADULTS

Studies in infants usually use neonatal cord blood (CB) to analyse the Tregs in comparison to
those in adults. Interestingly, FOXP3+ Tregs have been found in much higher levels at birth as
compared to adults, whether defined as CD4+CD25+CD127lo (Nettenstrom, Alderson et al. 2013) or CD4+CD25+FOXP3+ (Flanagan, Halliday et al. 2010). Indeed, preterm neonates have been shown to have the highest levels (Luciano, Arbona-Ramirez et al. 2014). By contrast, a study comparing CD4+CD25+CD127lo Tregs at different age groups observed higher Treg frequencies in adults compared to that in neonatal cord blood: 6.10% in CB; 7.22% in adults aged 20–25 years; and 7.5% in adults over the age of 60 years (Santner-Nanan, Seddiki et al. 2008). Yet another study found similar numbers of FOXP3+ cells in neonates compared to their mothers, as well as lesser CD4+CD25bright cells (Ly, Ruiz-Perez et al. 2009). The reason for these discrepancies is not known, but methodological factors may have a role to play as the phenotypic Treg markers used varied.

Several studies have shown that CB Tregs predominantly express CD45RA+CD45RO- naïve phenotype (Kanegane, Miyawaki et al. 1991, Wing, Ekmark et al. 2002, Takahata, Nomura et al. 2004, Ly, Ruiz-Perez et al. 2009, Flanagan, Halliday et al. 2010). In addition, CB Tregs are mostly CD27+ and express lower levels of CD95/Fas, suggesting that they are at an earlier differentiation state and have a lower apoptotic potential than their mothers (Flanagan, Halliday et al. 2010). The chemokine receptor CCR6, which characterizes Th17 and Th22-like Tregs, is expressed at lower levels by CB Tregs than their matched mothers (Duhen, Duhen et al. 2012).

With regards to the functionality of newborn CB Tregs, several studies have shown that they are highly functional, suppressing both T cell proliferation and IFN-γ production (Wing, Ekmark et al. 2002, Godfrey, Spoden et al. 2005). CD4+CD25+ CB Tregs exhibited stronger immunosuppressive capacity than adult blood Tregs following two cycles of polyclonal
stimulation (Fan, Yang et al. 2012). By contrast, Mayer et al found that although CB CD4+CD25hi cells failed to suppress T cell proliferation upon TCR activation, freshly purified adult Tregs did (Mayer, Bannert et al. 2012). However, these CB Tregs became strongly suppressive after antigen-specific stimulation. This is further supported by a recent study showing that CB Tregs expand more easily than Tregs from adult blood (Lin, Lu et al. 2014). In the same study, they also observed that CB Tregs are better suppressors than adult peripheral blood Tregs.

Taken together, these studies suggest that there are distinct phenotypic and functional differences between infant and adult Tregs. However, characterization of the Tregs is not consistent, as different phenotypic markers have been used in these studies. Additionally, using CB may not necessarily portray what is occurring in the periphery. Nevertheless, Tregs seem to be present in higher frequencies in CB than in adults, are more naïve and less differentiated, and highly suppressive, all supporting an active immunoregulatory role in early life.

1.10 IMMUNOSUPPRESSIVE IMMUNE ENVIRONMENT AT BIRTH

At birth, the body of the newborn undergoes a dramatic transition as it is suddenly exposed to a vast range of pathogens after being sheltered in the sterile intrauterine environment. All the organ systems following this transition then gradually mature with age (Levy 2007)). The immune system of the foetus is adapted so as to avoid pro-inflammatory responses, which can potentially harm it and lead to spontaneous abortion (Makhseed, Raghupathy et al. 2001).
Plasma from neonates and infants contains a number of immunoregulatory factors, shown to be higher in the plasma of the newborn than in adults (Belderbos, Levy et al. 2013), which serve to maintain Th2 polarization. Levels of plasma adenosine, for instance, have been shown to be high in newborns and infants (Pettengill, Robson et al. 2013). Adenosine is an endogenous purine metabolite, which possesses immunosuppressive properties, and as a result is currently being studied in cancer models (Vijayan, Young et al. 2017). It causes mononuclear cells to produce cAMP, which in turn inhibits TLR-stimulated production of pro-inflammatory cytokines and results in skewing toward IL-10 and Th17 cytokine production (Levy, Coughlin et al. 2006, Philbin, Dowling et al. 2012). Through their A3 adenosine receptors (Hasko and Pacher 2008), monocytes in newborns and infants have increased sensitivity to these effects of adenosine.

The immunosuppressive cytokine IL-10 is another one of these immunosuppressive factors shown to be in high levels in neonatal cord blood (De Wit, Olislagers et al. 2004, Belderbos, van Bleek et al. 2009, Nguyen, Leuridan et al. 2010). IL-10 acts at a number of stages of the immune response, aiding in the control of inflammation by inhibiting the production of pro-inflammatory cytokines and chemokines by various immune cells including the monocytes, macrophages and dendritic cells (DCs). This causes a reciprocal increase in IL-10 production by various T cell subsets. Whilst suppressing both Th1, and the more recently described ‘Th1+Th17’ cells. By two years of age, the amount of IL-10 being produced upon TLR activation has been shown to decline to levels comparable to that of adults (Corbett, Blimkie et al. 2010).
The immune system of the neonate and young infant is therefore unique with a generally depressed proinflammatory/Th1 cell-polarizing function (Dowling and Levy 2014), therefore limiting pro-inflammatory innate and adaptive immunity resulting in the high susceptibility to infections and impaired responses to certain vaccines (Ndure and Flanagan 2014).

1.11 HIGH BURDEN OF INFECTIOUS DISEASES IN INFANCY

There has been a considerable reduction in child mortality rates in the past decade globally, however, infectious diseases such as pneumonia, diarrhoea, malaria, measles and HIV/AIDS (human immunodeficiency virus / acquired immune deficiency syndrome (HIV/AIDS) continue claims lives and are the five leading infectious causes of under-5 deaths (Figure 1.5). In 2015 alone, 5.94 million children under the age of five are estimated to have died, with infectious diseases claiming many of the lives (Liu, Oza et al. 2017). The majority of deaths occurred during infancy, with the risk highest in the neonatal period (the first 28 days of life) when 45% of all under-five deaths occurred. Sub-Saharan Africa has the highest childhood mortality rate, where a child under the age of five is fifteen times more likely to die than in the developed parts of the world (Lozano, Wang et al. 2011).

Pneumonia remains the main cause of death in children under the age of 5 worldwide, causing 12.8% of all post-neonatal child deaths and 15.5% of deaths within the neonatal period in 2015. Diarrheal diseases are the second most frequent, accounting for 8.6% of post-neonatal childhood deaths worldwide in the same year (Liu, Oza et al. 2017). In 2013, 1·8 million new cases of HIV infection and 1·3 million HIV deaths were reported globally (Murray, Ortblad et al. 2014). Since 1990, there has been an increase in malaria cases and deaths
worldwide peaking in 2003 and 2004 respectively with 232 million cases and 1.2 million deaths. From 2004 however, the number of child deaths as a result of malaria infection has decreased gradually. Nevertheless, we are still observing a high number of child deaths from malaria infection especially in the sub-Saharan Africa region as the World Health Organization (WHO) reports that 70% of all malaria deaths in high malaria transmission areas occur in children under the age of five, with a child dying every 2 minutes from complications of malaria infection (W.H.O. 2016).

Figure 1. 5: Global causes of under-5 mortality
(Source (Liu, Oza et al. 2017)

1.12 IMPORTANCE OF VACCINATION IN PREVENTING CHILDHOOD INFECTIONS

Vaccination is widely considered to be one of the most important public health interventions of the 20th Century. Benjamin Jesty gave birth to the concept of ‘vaccination’, named after the word ‘vacca’ for cow, when he showed that inoculating with cowpox protected against smallpox (Weiss and Esparza 2015). A global smallpox vaccination campaign later led to its eradication globally in 1988. Before the last case of
smallpox was reported, the W.H.O went on to develop a vaccine program to combat infections from six major pathogens; namely *Mycobacterium tuberculosis* (TB), poliomyelitis (polio), *Corynebacterium diphtheriae* (diphtheria), *Clostridium tetani* (tetanus), *Bordetella pertussis* and the measles virus. This later led to the inception of the Expanded Programme on Immunization (EPI) in 1974 (W.H.O 2014).

<table>
<thead>
<tr>
<th>Vaccinations</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCG/ OPV/ HepB</td>
<td>Birth</td>
</tr>
<tr>
<td>DTwP-Hib-HepB/PCV-13/OPV/RV</td>
<td>2 months</td>
</tr>
<tr>
<td>DTwP-Hib-HepB/PCV-13/OPV/RV</td>
<td>3 months</td>
</tr>
<tr>
<td>DTwP-Hib-HepB/PCV-13/OPV/RV</td>
<td>4 months</td>
</tr>
<tr>
<td>Measles/Yellow Fever/OPV</td>
<td>9 months</td>
</tr>
</tbody>
</table>

Table 1.1: The current Expanded Programme on Immunisation (EPI) schedule in The Gambia

*BCG = Bacille Calmette Guerin, OPV = oral polio vaccine, HepB = Hepatitis B vaccine, RV = rotavirus vaccine, Hib = haemophilus influenzae, PCV = pneumococcal conjugate vaccine*

To date, vaccinating against infectious diseases saves millions of lives annually worldwide, however lives are still being lost from vaccine preventable diseases (VPD) especially in young children. This further underscores the need for more effective delivery of vaccines in the first year of life.

In the EPI schedule of the Gambia, infants receive the BCG, Hepatitis B (HepB) and oral poliovirus vaccines (OPV) at birth. At 2, 3 and 4 months of age, they are given the pentavalent vaccine which is a combination of 5 vaccines; the combined diphtheria, tetanus and whole-cell pertussis vaccine (DTwP), the *Haemophilus influenzae* type b (Hib)
and HepB vaccines. They also receive the rotavirus (RV), OPV and the 13-valent pneumococcal conjugate vaccine (PCV-13) alongside the pentavalent vaccine. At 9 months of age, infants receive MV, the OPV and yellow fever (YF) vaccines (Table 1.1).

A poor response to certain vaccines in neonates and young children have been described and this has been attributed to interference from the presence of maternally acquired antibodies and the adaptation of the infant immune system (reviewed by (Gervassi and Horton 2014). Although the measles vaccine (MV) and the combined diphtheria, tetanus and pertussis (DTP) vaccines have been used since the inception of the EPI, we are still seeing children who have received these vaccines not being protected (Figure 1.5). In some young children, sub-optimal responses to MV have been observed, especially in the low and middle-income countries (LMIC) (Kumar, Johnson et al. 1998, Bautista-Lopez, Vaisberg et al. 2001, Gans, Yasukawa et al. 2004).

1.13 IMMUNE RESPONSE TO MEASLES VACCINE

Measles vaccine (MV) was first developed in the 1950s using an attenuated form of the wild-type virus cultured in human and chicken embryo fibroblasts (reviewed by (Katz, Enders et al. 1962). The Edmonston strain is the most common strain of the virus used in the live MV worldwide. Despite the availability of this highly effective vaccine, measles still caused around 1.2% of all post-neonatal deaths globally in 2015 (Figure 1.5). Measles virus-induced immunosuppression is the main cause of death associated with infection characterized by lymphopenia, lack of cell proliferation and elevated levels of IL-10 (reviewed by (Moss, Ryon et al. 2002, Griffin 2010).
The role of the humoral response as a correlate of protection against measles is well documented. Neutralizing antibodies against the hemagglutinin and fusion glycoproteins (shown in Figure 1.6) present on the surface of the measles virus are thought to provide protection following MV vaccination (Griffin and Oldstone 2009). Neutralizing measles IgG titres correlate with protection (Chen, Markowitz et al. 1990), including maternal measles IgG which has also been shown to protect neonates and young infants against infection (reviewed by Plotkin 2010). Neutralizing antibody titres of ≥120 mIU/ml have been shown to be protective against disease.
Figure 1.6: Structure of the measles virus

The negative strand RNA is covered by an envelope, which contains the ‘H’ (haemagglutinin) and ‘F’ (fusion) transmembrane proteins. These proteins are involved in initiating infection. (Source: www.microbiologybytes.com).

Studies have also highlighted the importance of the cell-mediated immune (CMI) response as an additional measure of measles vaccine efficacy (Ward, Boulianne et al. 1995). Later studies on wild-type measles infection further highlighted the role of the CMI response, particularly the CD8+ T cell response, in the clearance of the virus, recovery from infection and the establishment of long-term protection (van Els and Nanan 2002, Permar, Klumpp et al. 2004, Griffin, Lin et al. 2012). Rather than the B cells, studies show that it is the depletion of CD8 T cells in monkeys that is associated with persistent MV infection (Permar, Klumpp et al. 2003, Permar, Klumpp et al. 2004). In humans, persistent infection and increased severity of disease is described in T-cell deficient measles patients (Kaplan, Daum et al. 1992, Griffin, Ward et al. 1994, Jaye, Magnusen et al. 1998); whereas agammaglobulinaemia patients have the ability to clear the infection from the measles virus (Naniche 2009) further supporting the
importance of the CMI response in measles virus infection and vaccination. The production of IFN-γ has been described as the mechanism used by the CD8 T cells to clear measles infection, and is also one of pro-inflammatory cytokines induced following measles vaccination.

A T-cell response is elicited the first time MV is given and is sustained; yet subsequent doses do not seem to enhance the response but do boost the concentrations and avidity of MV-induced antibodies (Njie-Jobe, Nyamweya et al. 2012, Gans 2013). The presence of maternal antibodies (MAb) in young children when the vaccine is initially given may greatly interfere with the initial humoral response (Siegrist 2003). Proposed mechanisms of inhibition of vaccine immune responses by Mab include neutralization of the vaccine and inhibition of the B-cells. The T cell mediated immune response is not similarly affected by the presence of MAb (Niewiesk 2014).

The two measles virus surface receptors used by the measles virus to gain entry into the host cells are CD46 and CD150/SLAM (signalling lymphocyte-activation molecule). CD150/SLAM is selectively expressed on B and T cells whilst CD46 is expressed on all nucleated cells. Wild-type measles virus strains use CD150/SLAM as a cellular receptor (Tatsuo, Ono et al. 2000), while vaccine strains such as the Edmonston strain can use both SLAM and CD46 as a receptor (Naniche, Varior-Krishnan et al. 1993).
1.14 INFANT IMMUNE RESPONSE TO DTP VACCINATION

Studies investigating the immune response following DTP vaccination have classically analysed for the induction of antibodies to diphtheria toxoid (Dtx), tetanus toxoid (Ttx) and pertussis toxoid (Ptx), and this has been clearly documented. Antitoxins levels of 0.01 μg/mL after the administration of the diphtheria and tetanus vaccines both has been shown to provide some protection, with levels of ≥0.1 providing complete protection against disease (Livorsi, Eaton et al. 2010). However, there have been rare reported cases of infection despite the presence of high levels of antitoxin Abs following vaccination. The infections were described as mild; and attributed to the poor diffusion of the antitoxins to the sites of infection (de Moraes-Pinto, Oruamabo et al. 1995). There is no standard agreed level of antitoxin which provides protection following pertussis vaccination. However, using an IgG-PT (pertussis toxin) titre of 20EU/ml, the proportion of children with levels higher than this benchmark was almost halved in children who had not received a booster dose of the acellular Pertussis vaccine compared to those who did (Carollo, Pandolfi et al. 2014).

Although the humoral response plays a vital role in protecting against these infections, as with many other bacterial infections, the role of the cell-mediated immune response is thought to be important in combating these infections. Unlike antibody levels which have also been shown to decline following DTP vaccination, the CMI response was shown to last longer (Cassone, Ausiello et al. 1997).

The pertussis vaccine comes either as a whole cell formulation (wP) or acellular (aP). It is given in combination with other vaccines, including diphtheria and tetanus toxoids as the DTP vaccine, or DTP combined with Hib and hepatitis B vaccine as the pentavalent vaccine.
The whole-cell vaccine has been associated with a high rate of adverse reactions following vaccination (Cody, Baraff et al. 1981, Long, Deforest et al. 1990). This prompted the development of the acellular vaccine, which is more purified and causes less adverse events. The wP vaccine is widely used as part of the EPI schedule in the low and middle-income countries including The Gambia, as it is relatively inexpensive and highly effective. In The Gambia, wP is administered as part of the Pentavalent vaccine at 2, 3 and 4 months of age (Table 1.1). In the high income countries, DTwP has been replaced with DTaP due to the high level of reactogenicity to DTwP described above.

There is evidence that the two vaccines induce different types of immune responses, with DTwP associated predominantly with a Th1 response, whereas with DTaP vaccination, there is an induction of both Th1 and Th2 cytokines (Ausiello, Urbani et al. 1997). DTwP has also been shown to be more immunogenic, and has been described as being ‘self-adjuvanted’ as it contains the TLR4 agonist bacterial lipopolysaccharide (LPS). Immunity induced by DTaP is shorter-lived and less robust (Slifka and Amanna 2014).

Resurgence of interest in pertussis infection research has occurred due to recent outbreaks of pertussis infections in western countries. A major outbreak in the US in 2010 reported over 9,000 cases, which is the highest number reported in 60 years (Winter, Harriman et al. 2012). In 27 European countries, more than 27,000 cases were reported which equalled an incidence rate of 4.9 per 100,000 (Zepp, Heininger et al. 2011). In 2012, the UK reported 10 deaths attributed to pertussis infection, and all of the deaths occurred in infants (Billingsley 2012). Increasing vaccine refusal in industrialised countries will also have played a role. The re-emergence may also be as a result of genetic changes in the circulating strains of the
bacteria and the waning immunity to the bacteria as protection only lasts between 5-8 years, which would explain the shift from school-going children to adults who then transmit it to the very young children yet to be vaccinated (Chiappini, Stival et al. 2013). Infants under 6 months of age are the worst affected as 90% of all pertussis deaths occur in this age group (Munoz and Englund 2011). Chiappini et al suggested possible reasons for this re-emergence of pertussis as a result of changes in the reporting procedures and improved laboratory methods.

In murine studies, there is evidence for a possible role of Tregs in pertussis infection. Mills et al., reported the presence of Tr1 Treg cells specific for B. Pertussis antigens FHA and pertactin in the lungs of mice acutely infected with B Pertussis (McGuirk, McCann et al. 2002).

### 1.15 SEX DIFFERENCES IN IMMUNITY TO VACCINES

Sex differences in immunity to vaccines has been reported for almost all the commercially available vaccines, including BCG, measles, rubella, hepatitis B, diphtheria, tetanus, influenza, rabies and yellow fever vaccines (Flanagan 2015, Klein and Flanagan 2016) (Table 1.2). Differences in clinical outcomes, reactogenicity / adverse reactions and CMI responses following vaccination have also been described. Generally females report more frequent and severe local and systemic reactions to viral and bacterial vaccines than their male counterparts (Cook 2008), possibly depicting stronger inflammatory responses among females as compared to males. The immune response following vaccination with viral and bacterial vaccines also resulted in generally higher antibody levels in females compared to males (Klein, Jedlicka et al. 2010). Moreover, dose-response studies using the influenza vaccine showed that females had comparable antibody levels when vaccinated with half the
dose of the vaccine compared to males receiving the full dose (Giefing-Kroll, Berger et al. 2015). Although still not fully understood, a number of immunological mechanisms may account for the sex differences in the vaccine responses observed in young children.

Table 1. 2: Sex-differential responses to certain vaccines in different age groups

(Taken from (Klein and Flanagan 2016)

Infant males have higher levels of testosterone and females have higher levels of oestrogens in the first year of life (Flanagan 2015) (Figure 1.7). This is the age when the majority of all childhood vaccines are administered. Since multiple immune cells express receptors for sex hormones, it will inevitably lead to sex-based disparities in vaccine-induced immunity. More
so, multiple immune response genes are expressed on the X-chromosome, further accounting for sex differences in immunity (reviewed in (Flanagan, van Crevel et al. 2013, Klein, Marriott et al. 2015)

Figure 1. 7: Levels of sex hormones in males and females in the first year of life

(Taken from Flanagan 2015)
1.16 HETEROLOGOUS EFFECTS OF VACCINES

It is now generally accepted that vaccines have non-targeted heterologous effects aside from their targeted effect of inducing vaccine-specific immunity (Fine, Williams et al. 2009, Klein, Jedlicka et al. 2010, Shann, Nohynek et al. 2010, Flanagan, van Crevel et al. 2013, Jensen, Ndure et al. 2015). For example, BCG has been shown to have beneficial effects on child survival that cannot be attributed to protection from TB alone (reviewed by Flanagan, van Crevel et al. 2013). This effect can be observed in a randomized trial conducted on BCG vaccinated low birth weight (LBW) neonates where there was a 17% reduction on all-cause mortality (Aaby, Roth et al. 2011). Being intrinsically ‘self-adjuvanted’, prior BCG vaccination has also been shown to enhance antibody and CMI responses to some EPI vaccines (Ota, Vekemans et al. 2002).

The early studies describing sex differential and heterologous effects of vaccines were observational epidemiological studies (Aaby, Martins et al. 2010, Aaby, Benn et al. 2012, Benn and Aaby 2012). Immunological studies are now emerging which show sex differences heterologous vaccine effects in young children. In our group, a recent study showed that measles vaccine enhanced innate immunity in males but not females, whereas DTwP vaccine caused diminished cellular and innate immunity in females but not males (Noho-Konteh, Adetifa et al. 2016). Whether Tregs play a role in the heterologous responses to certain vaccines seen in young children is not clear.
The role of Tregs in infectious diseases has been widely studied. They have been shown to be beneficial to the host by limiting immunopathology caused by the immune response to pathogens, there is also evidence that they can cause the pathogen to survive in the host by suppressing protective immune responses and preventing sterilizing immunity (Belkaid, Piccirillo et al. 2002). Meanwhile, there is a paucity of studies investigating the role they play in controlling or limiting vaccine immunogenicity. Considering that fact that Tregs are induced by natural infections in order to regulate the inflammatory response and prevent immune pathology, it makes sense that vaccines would also induce Tregs, particularly the live attenuated vaccines. Indeed, murine studies have shown that Tregs can be induced by vaccination with BCG (Lacan, Dang et al. 2013). Although not the focus of our research, recent studies have also shown that human CD8+ Tregs are induced following BCG vaccination (Boer, van Meijgaarden et al. 2014, Boer, Joosten et al. 2015).

Some studies also suggest that Tregs may interfere with the generation of vaccine-induced immunity. The DEREG mouse is an elegant model for investigating how the immune response is altered in the absence of Tregs, since they can be depleted at any time point during an immune response in these mice using diphtheria toxin (Lahl and Sparwasser 2011). An augmented anti-tumour response to vaccination against an established melanoma was seen following Treg depletion (Klages, Mayer et al. 2010), and short-term depletion greatly increased vaccine-induced immunity against a solid tumour by promoting NK cells and CD8+ T cell activation and IFN-γ production (Matarollo, Steegh et al. 2013).

The use of anti-CD25 monoclonal Ab to deplete the CD25+ cells when administering BCG and
HepB vaccines in mice greatly enhanced the vaccine-induced response compared to when the vaccine was given alone (Moore, Gallimore et al. 2005). Antigen-specific Tregs induced by environmental mycobacteria have been shown to suppress Th1 immune responses, thus altering the response to BCG vaccination in mice (Ho, Wei et al. 2010). Pre-existing Tregs and the subsequent vaccine response were also seen to negatively correlate in this study, supporting an immunoregulatory role.

It is difficult to translate these studies into humans as Tregs in mice are neither phenotypically nor functionally identical to that of humans. Furthermore, ethical concerns do not allow for the in vivo depletion of FOXP3+ Tregs in healthy humans. However, in vitro depletion of Tregs in HIV patients vaccinated with dendritic cell (DC)-based vaccines supports data from in-vivo mouse studies. Significantly enhanced vaccine-induced anti-HIV-1-specific polyfunctional T cell responses were observed, thus supporting enhanced vaccine-induced immunity when Tregs are removed (Macatangay, Szajnik et al. 2010). A marked increase in the CD4+CD25hiFOXP3+ Treg numbers following vaccination was also shown, though this increase did not correlate with the effector CD8+ T cell vaccine-induced response. An increase in FOXP3 mRNA expression was also demonstrated in malaria-vaccinated adults (Mwacharo, Dunachie et al. 2009), although this may be attributable to the participants being naturally exposed to the malaria parasite rather than as a result of vaccination per se. Vaccination of malaria-naïve adults with the malaria vaccine candidate RTS,S/AS02A combined with MVA-CS (modified vaccinia virus Ankara encoding the circumsporozoite protein) showed that pre-existing levels of IL-10 and TGF-β inversely correlated with the post-vaccination antibody levels (Dunachie, Berthoud et al. 2010).
Vaccination of healthy adults with candidate TB vaccines suggests that regulatory pathways may be induced following vaccination. Vaccination with MVA85A in BCG-vaccinated adults boosts responses and has been associated with a downregulation of TGF-β levels. They found a correlation between the CD4+CD25hiOXP3+ cells and the TGF levels suggesting that downmodulating the immunoregulatory response may boost vaccine response (Fletcher, Pathan et al. 2008). In healthy African adults vaccinated with M72/AS01, Tregs were induced (Day, Tameris et al. 2013); similarly, Treg induction was also observed following vaccination with modified vaccinia Ankara-85A (MVA85A) (Matsumiya, Stylianou et al. 2013). The latter study also showed an inverse correlation between CTLA-4 and IL-2RA mRNA expression and IFN-γ response very early after vaccination in adults, therefore suggesting a possible role of Tregs in influencing favourable effector T cell responses. In contrast, the same group observed early strong IFN-γ responses in healthy vaccinated infants, and this response did not correlate with CTLA-4 expression. This underscores the need for more evaluation of the influence of Tregs in controlling vaccine immunogenicity in different age groups (Matsumiya, Harris et al. 2014). This is one of the limited number of studies investigating the effect that Tregs have in controlling vaccine immunogenicity in infants. This is important, as it is the age that the majority of childhood vaccines are given.

Previous studies in our group found that BCG vaccination induced FOXP3+ cells in 4½ month old infants, however no association was seen between PPD-specific CD4+CD25+FOXP3+ Tregs or CD4+IL-10+ Tregs, or PPD stimulated total IL-10 production and IFN-γ responses to PPD following vaccination (Burl, Adetifa et al. 2010). Functional Treg assays were not conducted in this study. Akkoc et al. also show that IL-10 production is induced by BCG vaccination in neonates and in vitro depletion of the CD4+CD25+ cells results in reduced IL-10 production in
the PPD-stimulated cultures, suggesting a possible role for Tregs following BCG vaccination in young infants (Akkoc, Aydogan et al. 2010).

The production of immunosuppressive cytokines by Tregs suggest that previously induced Tregs can suppress responses to an unrelated antigen resulting in a ‘bystander’ or ‘heterologous’ suppressive effect, therefore compromising the immune response to unrelated antigens (Joosten and Ottenhoff 2008). This can be observed in helminth infections where Tregs have been shown to be induced, and vaccination in helminth-infected people can compromise the immunogenicity of the BCG and tetanus vaccines (van Riet, Hartgers et al. 2007). However, other mechanisms independent of Treg induction may influence responses in helminth-infected individuals as the response has been shown to be Th2-dominated, thereby modulating the Th1 response. In schistosomiasis infections in humans, for instance, T cell proliferation is suppressed during chronic infection, as well as Th1 cytokine production (Colley and Secor 2014). One of the proposed mechanisms is by IL-10-producing Th2 cells. In addition, TGF-beta dependent mechanism, independent of IL-10 or Tregs have also been described during Helminth infection (Hubner, Shi et al. 2012).

Nevertheless, it is possible that Tregs might be responsible for the immunosuppressive heterologous effects of vaccines such as DTP, but this has never been investigated.

1.18 RATIONALE OF THE THESIS

As discussed in this chapter, the majority of under-five deaths occur within the neonatal and infancy period, many attributable to VPDs. The infant immune system differs from that of the adult immune system, resulting in suboptimal responses to certain vaccines and inability to
clear infections effectively. There is evidence that Tregs are higher in number at birth compared to later in life, even more so in preterm neonates (Luciano, Arbona-Ramirez et al. 2014). Although Tregs are instrumental in preventing an over-exuberant immune response and immune pathology, there is evidence that Tregs can allow for persistent infection by preventing sterilizing immunity by accumulating at the site of infection and preventing the effector T cells from eliminating the pathogen (Belkaid, Piccirillo et al. 2002). It is not known whether Tregs dampen the vaccine-induced response in young children, nor whether they are induced by vaccination. Most vaccine studies predominantly look at the pro-inflammatory responses following vaccination, completely overlooking the regulatory response, which is also vital to better understand vaccine-induced immunity. A number of studies point to role for Tregs in controlling vaccine responses in mice and adult humans, but studies in young children are lacking. There is therefore a need for studies investigating whether Tregs influence vaccine immunogenicity or are induced by vaccination in infants, as this is the age when the majority of childhood vaccines are given.

1.19 HYPOTHESES, AIMS AND OBJECTIVES OF THE THESIS

1. I hypothesised that regulatory T cells present at the time of vaccination dampen vaccine immunogenicity. To explore this hypothesis:

I aimed to

- Determine whether pre-existing Tregs will inversely correlate with vaccine-specific responses postvaccination.
- Determine whether Tregs would significantly correlate with functional effector readouts in our cohort.
Specific Objectives:

- To investigate whether Tregs at the time of vaccination correlate with vaccine-specific humoral and cellular postvaccination responses (Chapter 3).

- To determine the Treg frequencies using classic Treg markers, and measles-specific antibody and cytokine responses before and after measles vaccination (Chapter 5).

- To determine the correlation between pre-existing and post-vaccination Tregs and the measles-specific antibody and CD4/CD8 T cell cytokine responses (Chapters 3 and 5).

2. I hypothesised that Tregs can be induced and phenotype altered by vaccination, with subsequent immunoregulatory effects. To explore this hypothesis:

I aimed to

- Determine whether vaccination will alter Treg frequencies/phenotype and whether this will differ depending on the vaccine administered.

- Explore mechanisms of action used by these Tregs.

Specific Objectives:

- To determine the ex-vivo Treg frequencies before and after vaccination following measles and/or DTwP vaccination (Chapter 3).

- To characterize different Treg phenotypes to determine their functional characteristics before and after measles vaccination (Chapter 6).

- To determine whether adding MACS-isolated Tregs to the PBMCs or the depletion of the CD25hi subset of CD4+ T cells by FACS will suppress cytokine production by the conventional CD4+ T cells (Chapter 4).
• To analyse for expression of the measles receptors by the CD4+ and CD8+ T cells, and investigate their role and that of key cytokines in the suppressive effects of Tregs (Chapter 6).

• To investigate whether Tregs correlate with CD4+ and CD8+ T cell cytokine responses and transcription factors expression when samples are cultured in exogenous cytokines (Chapter 6).
CHAPTER 2: MATERIALS AND METHODS

2.1 STUDY COHORT

To investigate the study hypotheses described in Section 1.19, three different cohorts of infants were used. The first study was nested within a larger study the MV/DTP study which explored the effect of vaccine group on Th1/Th2 cytokine profile and Treg frequencies. Within the MV/DTP study, I designed a sub-study in consultation with Dr Flanagan (Chapter 3). I then recruited a small cohort of 10 infants in December 2011 for the experiments conducted in Chapter 4. Subsequently, 50 donors were then recruited for the experiments in Chapter 5. This cohort was recruited between October 2012 and February 2013. From this cohort of 50, 10 donors at both visits (20 samples) were used for the phenotypic analysis in Part 2 of Chapter 4 and Chapter 6.

All the samples used were collected from the Sukuta Health Centre in The Gambia, West Africa (Figure 2.1 and Figure 2.2). Samples in Chapters 3-5 were processed at The MRC Laboratories in Fajara, The Gambia (Figure 2.2). PBMC samples were sent to Monash University in Melbourne, Australia for the detailed phenotyping done in Chapter 6.

2.2 SUKUTA HEALTH CENTRE STUDY SITE

Sukuta is a peri-urban area in the Kombo district in The Gambia, West Africa (Figure 2.2). The Gambia is a small country in West Africa with a population of 1.8 million, of which 57% live in the urban areas. There is an infant mortality rate of approximately 66.4 per 1,000 live births and an HIV prevalence rate of 1.5% in adults (UNICEF). The Sukuta Health Centre is a
Government Health facility, with an MRC clinic on-site equipped with a team of field workers, qualified nurses and 2 paediatricians. Being quite close to the MRC laboratories in Fajara, it takes around 20-25 minutes to drive from Sukuta to the main laboratories. This allows for samples collected at the health centre and transported to the laboratories to be processed within a maximum of 2 hours.

Figure 2.1: Map of the Gambia, West Africa

This map shows the small West African country of The Gambia, enclosed by Senegal on three sides, and the Atlantic Ocean on the other. (Source: Google Maps)
Figure 2. 2: Map showing directions from MRC at Fajara to the study area in Sukuta, West Coast region, The Gambia

(Source: Google Maps).

2.3 STUDY 1: TREG FREQUENCIES IN MV AND/OR DTWP VACCINATED 9 MONTH-OLD INFANTS

2.3.1 Infant recruitment and informed consent procedure

This study was nested within a bigger trial entitled “Sex Differences in Vaccine-Specific and
Heterologous Immunity following the Administration of Measles and/or DTwP vaccines to nine-month old Gambian Infants. The study was approved by the Medical Research Council (MRC) Scientific Coordinating Committee (SCC), the Joint Gambia Government / MRC Ethics Committee (SCC 1085), and was conducted between November 2007 and May 2011. Willing mothers/guardians attending the Sukuta Health Centre to get their 2 month old infants vaccinated according to the EPI schedule were given verbal information about the study and given the subject information sheet (Appendix A). This sheet contained information on why the study was being conducted and what it entailed. The information sheet was in English, non-English speaking mothers/guardians had it translated in their native languages by the attending nurse/fieldworker. Exclusion criteria included being under normal weight-for-age, multiple births, having a congenital defect, vaccines not given according to the current EPI schedule, or likelihood of moving outside the study area. Once consent was obtained from the parent/guardian, the informed consent form (Appendix B) was signed with a parental signature or thumbprint.

2.3.2 Study Design

Randomization of the infants was conducted when the infants were 4 months of age. Selecting sequentially arranged envelopes (block randomized in groups of 12) determined which of the three study groups the infants were randomized into. Females and males were randomized separately for later analysis by sex. Three hundred (302) children were randomized (Figure 2.3). In Group 1 (MV group) children received the normal EPI schedule of DTwP3, HBV and OPV at 4 months; in Groups 2 (MV+DTwP group) and 3 (DTwP group), the children did not receive their DTwP3 dose but only received HBV and OPV. At the second study visit, i.e. at 9 months of age, Group 1 received a single intramuscular (im) dose of the
measles vaccine (MV) (Edmonston Zagreb, Serum Institute of India Ltd, Pune, India); Group 2 received im doses of MV and DTwP (provided by UNICEF); and Group 3 received a single im dose of DTwP alone (Figures. 2.4 and 2.5). Case report forms (CRFs) were used to record details about the child’s health, vaccines given and collection of samples. For the regulatory T cell phenotyping, data was available for only some of the participants, the numbers of participants in each group is outlined in Table 2.1. All the information held about the participants was doubled entered and verified by the unit data entry staff and stored in a secure Microsoft Access database created by the MRC Data Management team.

Figure 2.3: Study flow chart detailing the number of subjects in the study groups throughout the study
Table 2. 1; Number of participants with Treg frequency data by study group and sex at the different time points

<table>
<thead>
<tr>
<th></th>
<th>9 months</th>
<th>10 months</th>
<th>19 months</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MV All</strong></td>
<td>48</td>
<td>42</td>
<td>48</td>
</tr>
<tr>
<td>Males</td>
<td>26</td>
<td>24</td>
<td>31</td>
</tr>
<tr>
<td>Females</td>
<td>22</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td><strong>MVDTP All</strong></td>
<td>56</td>
<td>43</td>
<td>51</td>
</tr>
<tr>
<td>Males</td>
<td>24</td>
<td>19</td>
<td>27</td>
</tr>
<tr>
<td>Females</td>
<td>31</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td><strong>DTP All</strong></td>
<td>32</td>
<td>34</td>
<td>42</td>
</tr>
<tr>
<td>Males</td>
<td>13</td>
<td>15</td>
<td>23</td>
</tr>
<tr>
<td>Females</td>
<td>19</td>
<td>19</td>
<td>19</td>
</tr>
</tbody>
</table>

* MV received a single intramuscular (im) dose of the measles vaccine (MV) (Edmonston Zagreb) at 9 months; MVDTP received im doses of MV and DTwP at 9 months; and DTP received a single im dose of DTwP alone at 9 months. Participants with data available for all the three time points; MV (n=41), MVDTP (n=40) and DTP (n=32).

2.3.3 Sample Processing

Venous blood totalling 4.5 mLs was collected into heparinised tubes (containing 7.5 units of heparin per mL of blood) at 9 and 10 months of age (Figure 2.4). Samples were transported to the MRC laboratories in Fajara within 2 hours for processing. Upon arrival at the Infant Immunology laboratory, 600 µL of whole blood was used per participant for overnight
stimulation assays (100μL per antigen); the ex vivo flow cytometry assays used 100μL of whole blood per staining panel; and any remaining whole blood was spun and plasma collected and stored at -20°C. Peripheral blood mononuclear cells (PBMCs) were obtained with any remaining blood sample by density gradient centrifugation on Ficoll, and stored in liquid nitrogen for future use (outlined in Chapter 3, Figure 3.1).

**Figure 2.4: Bleeding Timepoints for the MV/DTP study (Part 1)**

All the infants were bled at 9 months (baseline) before vaccine administration and again at 10 months, 4 weeks after vaccination. All outstanding vaccines were administered at 11 months of age (yellow fever and OPV for the MV and MV+DTP groups; and MV, OPV and YF for the DTP group)
2.4 STUDY 2: INVESTIGATING THE FUNCTIONAL ROLE OF TREGS IN THE IMMUNE RESPONSE TO MEASLES VACCINE

2.4.1 Ethical Approval and Informed Consent Procedure

The study was approved by the Medical Research Council (MRC) Scientific Coordinating Committee (SCC) and the Joint Gambia Government / MRC Ethics Committee (Project no. SCC 1230) and the LSHTM Ethics Committee. Willing mothers/guardians attending the Sukuta Health Centre with their infants for their 9-month vaccines according to the EPI schedule (MV, YF and OPV) were provided information about the study and given the subject information sheet (Appendix C). The information sheet was in English, and non-literate mothers/guardians had it translated into their native languages by the attending nurse/fieldworker. Inclusion and exclusion criteria used in the recruitment of the study participants are shown in Figure 2.7. The parent/guardian signed the informed consent form (Appendix D) with their signature or thumbprint.

2.4.2 Study Design and Cohort

Infants aged 9 months (+/-2 weeks) were included in the study. Blood samples (5mL) were collected in vacutainers containing ethylenediamine tetra-acetic acid (EDTA) at baseline - Visit 1, before receiving a single intramuscular (im) dose of the Edmonston Zagreb measles vaccine (Serum Institute of India Ltd, Pune, India) and 2 weeks later - Visit 2 (Figure. 2.5). The ‘catch-up’ vaccines, yellow fever and oral polio vaccine (OPV) were given after the second blood sample was collected at Visit 2.
Study infants were bled at 9 months of age (median age of 40 weeks), MV administered, and infants returned for a follow-up visit 2 weeks later (Visit 2) when they were bled a second time. Yellow fever (YF) and Oral Polio Vaccine (OPV) were administered to the infants once they were bled.

A total of 50 infants were recruited in the study, with an equal number of males and females so as to allow for later analysis by sex (Fig. 2.6). Data on the medical history of the infant, the vaccines administered, and the date samples were taken, were entered in the Case Report Forms (CRFs). Information about the participant was confidential and stored in the secure Microsoft Access database created by the MRC Data Management team. A dropout form was completed if parents/guardians removed their child from the study.
Mothers/Guardians were approached and provided information about the study. Willing mothers were consented and infants recruited into the study. 3 children dropped out before the follow-up visit 2 weeks later.

### 2.2.3 Sample processing

The 5 mL blood sample was processed in the laboratory in Fajara within 2 hours of collection, according to the scheme in Figure 2.7. Plasma was collected and frozen, and cultures set up for flow cytometry. The remaining blood was used for PBMC separation and cryopreservation. PBMC were later shipped to the Vaccine and Infectious Diseases Lab, Department of Immunology and Pathology, Monash University, Melbourne for further detailed analysis.
<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
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<tbody>
<tr>
<td>• Healthy infants aged 9 months</td>
</tr>
<tr>
<td>• Weight for age z-scores within 2 standard deviations of normal</td>
</tr>
<tr>
<td>• Has received all EPI immunizations</td>
</tr>
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<table>
<thead>
<tr>
<th>Exclusion Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Acute disease at the time of vaccination</td>
</tr>
<tr>
<td>• Axillary temperature of more than 37 at the current visit</td>
</tr>
<tr>
<td>• History of allergic disease/reaction likely to be exacerbated by any component of the vaccine e.g. egg products, previous allergic vaccine reaction</td>
</tr>
<tr>
<td>• Presence of any underlying disease that might compromise evaluation of response to the vaccine, or on-going chronic illness requiring hospital specialist supervision</td>
</tr>
<tr>
<td>• Research Physician's assessment of lack of willingness by parents to participate</td>
</tr>
<tr>
<td>• Any history of anaphylaxis in reaction to vaccination</td>
</tr>
<tr>
<td>• Likelihood of travel away from the study area</td>
</tr>
</tbody>
</table>

**Figure 2. 7: Inclusion and exclusion criteria used to recruit study participants**

The research clinician assessed all of these criteria thoroughly before enrolling any participant into the study.
Figure 2. 8: Sample Processing Flowchart

Summarises how each of the study samples were processed on the two study visits.
2.5 PLASMA PREPARATION

Blood was inverted twice to mix the anticoagulant and prevent clotting. The blood was then spun in the vacutainers for 5 min at 1,800 rpm in order to separate the plasma. Using a sterile Pasteur pipette, plasma was gently removed and transferred into a pre-labelleed sterile 1.8 mL cryovial tube and stored at -20°C.

2.6 PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) PREPARATION

For the better recovery of PBMCs, RPMI medium (Sigma Aldrich) was added to the upper edge of the vacutainer label and mixed by inverting the tube gently. Using a sterile Pasteur pipette, the diluted blood was layered gently over 10mL Lymphoprep (Stem Cell Technologies) taking care not to disturb the interface. With the brake of the centrifuge turned ‘off’, the tube was spun for 30 minutes at 1,500 rpm at room temperature (18°C to 26°C). Once the spin was done, the tubes were gently removed so as not to disturb the layers (Figure 2.9). The PBMC layer was transferred to a 15 mL Falcon tube and labelled with the sample number. 5mL RPMI (Sigma Aldrich, UK) was added to the PBMC and tube was inverted to mix to homogeneity. With the brake on, the PBMC were spun in the centrifuge for 10 minutes at 1,500 rpm at RT. The supernatant was poured off after the spin and the wash was repeated once. After the second wash, the cells were re-suspended in 2 mL of R10F (RPMI containing 10% FCS, Sigma) after which a cell viability and cell count determination was performed using a haemocytometer.
Blood samples collected from the infants were diluted using sterile RPMI and then layered gently on 10mL of Lymphoprep. Following a 30-minute spin at 1,500 rpm (brake off) distinct layers were visible. RBC = Red blood cell.

2.7 PBMC COUNTING METHOD

The PBMC cell suspension was mixed gently using a vortex. From this suspension, 10µl was added to 90µl of Trypan Blue in a 96 well tissue culture plate (i.e. 1:10 dilution of the cell suspension). A cover slip was placed over the grid on the Neubauer haemocytometer and 10µl carefully dispensed at the edge of the coverslip and the cells allowed to settle down. Using a microscope with a 10X objective, viable cells (clear and shiny appearance) in a single quadrant (sixteen squares) were counted.

The following formula was used to determine the cell count:

\[ n \text{ (cells counted)} \times 10 \text{ (dilution factor)} \times 10^4 \text{ (correction factor)} \times 2 \text{ (resuspension volume)} \]
A PBMC processing worksheet was used to record the cell counts and viability results. A viability count was done by staining the cells with filtered 0.4% Trypan Blue (Thermo Scientific) viability dye. The dead cells appear blue whilst the live cells remained clear. PBMC viability greater than 95% was used as a standard as the cells were freshly isolated. The PBMCs were then prepared for storage, using 10 x 10^6 cells per cryovial.

### 2.8 PBMC CRYOPRESERVATION

The cryopreservation solution (CPS) was always freshly prepared on the day. The total amount prepared depended on total number of viable cells. In total, 9 parts of fetal calf serum (FCS) was added to 1 part Dimethyl sulfoxide (DMSO, Sigma Aldrich, UK) (10% DMSO, 100μL per cryovial). The tube containing the solution and the pre-labelled cryovials were placed on ice for 15 minutes before use. Whilst the CPS was being cooled, the PBMC were prepared for cryopreservation. The tube containing the PBMCs were washed and spun at 1,500 rpm for 10 minutes at room temperature with the brake on. After the wash, the supernatant was poured off and the cells were resuspended in the residual volume of RPMI (approximately 200μL).

The chilled CPS solution was added to the PBMCs drop wise, and the tube was swirled after every drop to mix the suspension, as DMSO is toxic to the cells. We used 1mL of CPS for 10x10^6 cells and aliquoted 1mL of the suspension into each of the prepared cryovials. This step was done on ice as the toxicity of DMSO increases with increased temperature. The cryovials were then transferred to a Mr Frosty pre-chilled to 2-8°C and placed in a -70/-80°C freezer overnight. The cryovials were then transferred to liquid nitrogen (in the vapour phase) after 24 hours for long-term storage.
2.9 PBMC THAWING

The PBMCs were rapidly thawed by placing in a 37°C water bath and transferred into 10mL tubes. First 1mL of thawing medium R10F (warm RPMI medium containing 10% FCS (Sigma Aldrich, UK) + 50 units/ml Benzonase [2µL of Benzonase stock per 10 mL of medium, Sigma Aldrich]) was added drop wise to the cells. The thawed cells were transferred to a 10mL FACs tube and R10F was added drop wise to fill the tube. The cells were then spun at 1,400 rpm for 10 minutes. Following the wash, the supernatant was poured off, AIM V medium added and cells were washed two more times. After the last wash, cells were resuspended in 1mL of the AIM V medium, and a cell count conducted.

2.10 COMPENSATION OF THE FLOW CYTOMETER

The compensation beads (E-Biosciences) were prepared. A drop was added from each bottle (A and B,) to the tubes. The single stains were added (at the required volume) to create single stain compensation tubes (except for unstained), vortexed, covered with foil and incubated at 4°C for 30 minutes. The tubes were then topped up and washed with 2mLs FACS buffer. Following the wash, the supernatants were removed and the beads were re-suspended in 200µL FACs buffer. Acquisition of the beads and cells was done immediately on a CyanADP (Beckman Coulter, USA) flow cytometer using Summit v4 software. At least 100,000 events were acquired per tube. Analysis was done using FlowJo v10.0.2 (Treestar, USA).
2.11 WHOLE BLOOD CULTURE AND INTRACELLULAR STAINING (ICS)

This technique was used in the studies presented in Chapters 3 and 5. To set up the overnight culture for the intracellular cytokine staining, whole blood was aliquoted in a 96 well round-bottom plate. Antigens were added to the designated wells as described in the chapter-specific methods. In general, we used a positive control, a negative control (RPMI medium with 10% fetal calf serum added (R10F) (Sigma Aldrich, UK), PPD and measles peptide/virus depending on the study.

The cells were incubated with the antigens at 37°C for 2 hours. After the incubation, 20μL of 100μg/ml Brefeldin A (BFA) was added and the plate was incubated overnight (37°C for 16 hours).

After the overnight stimulation, the plate was spun at 1,800 rpm for 5 minutes at room temperature and supernatants removed using a pipette tip. Using 200μL of lysis buffer, the blood was diluted and transferred to Cyan tubes. Once transferred, the buffer was topped up to 1mL by adding 800 μl lysis buffer (1:10). The blood was incubated in the buffer for 10 minutes at 4°C. After the lysing of the red blood cells, they were washed twice (1,800 rpm for 5 minutes) in 2mLs FACs buffer. Live-Dead Aqua (Invitrogen, USA) diluted 1:40 with distilled water was added to each tube and incubated at 4°C for 10 minutes covered in foil (in the dark).
The amount of antibody used was established using the titration method. As a gating control, we used a fluorescence minus one (FMO) control to determine where the positive and negative populations are (Figure 2.10).

![FMO- APC Cy7](image1)

![CD25 APC-Cy7](image2)

**Figure 2.10: Example of FMO gating control**

*Fluorescence Minus One (FMO) was used as a gating control in our multicolour panels. (A) A representative sample with all the antibodies added except for the CD25 APC-Cy7 antibody. (B) shows staining with CD25 APC Cy7 antibody added*

The cells were then stained with surface marker cocktail depending on the specifications of the panel, then incubated for 30 minutes at 4°C and washed with 1mL of FACS buffer (PBS/1% FCS/0.2% Sodium Azide). The supernatants were then removed by aspiration and the cells were washed in 1mL fix/perm solution (E-biosciences, UK). The cells were washed in 1mL perm/wash solution (E-biosciences, UK), 2μL of rat serum (E-Biosciences) was added as a blocking step to prevent non-specific binding, and the cells were incubated for 15 minutes at 4°C in the dark (covered in foil). FOXP3 and the antibodies from the intracellular cytokine
panels were added to the cells, mixed by vortexing gently, covered in foil and incubated for 30 min at 4°C.

2.12. **EX VIVO STAINING OF PBMC FOR FLOW CYTOMETRY**

A 100μL aliquot of whole blood was used for Treg phenotyping having optimised the assay to be performed in 96-well tissue culture plates. The cells were lysed by adding 200μL RBC lysing buffer diluted 10x with distilled water (BD Biosciences) at room temperature for 10 minutes and spinning for 10 minutes at 2,000 rpm. Cells were then washed and spun at 2,000 rpm for 5 minutes in 200μL FACS buffer (fluorescence activated cell sorter buffer, containing 0.5% Bovine Serum Albumin, 0.1% ethylenediamine tetra-acetic acid (EDTA) 0.1% sodium azide (NaN₃) in phosphate buffered saline (BSA), BD Biosciences) and incubated for half an hour at 4°C in the dark with the surface antibody cocktail. After incubation, plates were spun for 5 minutes at 2,000 rpm, the supernatant flicked off and cells mixed gently using the vortex. Cells were then permeabilized using 200μL of Cytofix/Cytoperm buffer (BD Biosciences, USA) and incubated at 4°C for 20 minutes covered in foil. The cells were then washed with 200μl of the FOXP3 wash (E-Biosciences), 2μL of rat serum (E-Biosciences) was added as a blocking step to prevent non-specific binding and incubated at 4°C for 15 minutes.

After the incubation, FOXP3 and the intracellular antibodies were added and incubated for 30 minutes at 4°C, then 200μL of the perm/wash buffer (BD Biosciences, USA) was added, the plate spun for 5 minutes at 2,000 rpm and the supernatant flicked off. This wash step was repeated a second time following which 150μL of FACS fix was added (containing 1% Paraformaldehyde [PFA]) to the cells. In preparation for acquisition, cells were transferred to
5 mL u-bottom falcon tubes (BD Falcon, Cedex, France), and acquired using the flow cytometer. The precise antibody panels used are described in the individual results chapters. Compensations were done automatically using the software. Upon compensation, we gated on FSC/SSC plots, and then 100,000 lymphocyte events were collected as a minimum per sample. I then gated on the single cells, following which I gated on live cells (Figure 2.11). Data were analysed using FlowJo software (Treestar, California, USA). The gating strategy for Tregs and other panels are shown in the respective chapters.
Figure 2. 11: The Gating Strategy Used for all panels

Cells were gated on the total lymphocytes (A), and then on Singlets (B), Live cells (C) and CD4+ and CD8+ T cells. Depending on the panels, gating was then done on the markers of interest.
2.13 SAMPLE SIZE CONSIDERATIONS AND STATISTICAL ANALYSIS

The original sample size calculation for the main MV/DTP study was made in consultation with one of the unit statisticians Dr Crozier; and was powered based on data from previous studies conducted at Sukuta Health Centre. One of our main hypotheses was that vaccination would alter Treg frequencies. Assuming a FOXP3+ Treg frequency of 2.5% (SD 0.5%) of CD4+ T cells (as observed in cord blood samples in a recent study (Flanagan, Halliday et al. 2010) then to detect a 0.5% difference between groups would require 23 in each group (n = 69 in total). Previous local data from several studies showed that the measles antibody titre post measles vaccination would be an average log2 6.9 (SD 1.8) (Njie-Jobe, Nyamweya et al. 2012) following vaccination at 9 months. To detect a 3-fold geometric mean difference in Ab level between the groups at the 1% significance level with 90% power would require 29 in each group. Preliminary results from our collaborators on the microarray aspect of the project suggested that 50 subjects per vaccine group should be sufficient to identify 2-fold differential expression with 90% power for at least 90% of the probes on the array with a significance level of alpha of 0.001 (correlated for multiple testing by Bonferroni). The 1% significance level was used for all calculations to allow for the multiple comparisons that were planned. Therefore the aim of ~100 per vaccine group (half males and half females) was thought to be a good compromise to obtain robust immunological data and detect significant differences post-MV and between vaccine groups and allow for study dropouts.

From the results obtained, we used the confidence interval to determine the statistical power of this part of the study. The observed differences between the medians of the Treg frequencies in the peripheral blood of Gambian infants at 9 months and 10 months of age in
all the study participants in the MV/DTP study is 0.72. The 95% confidence interval (CI) for the difference is estimated to be between -1.81 to 1.37.

Flow cytometry data was analysed using Flowjo (Version 10). All of the antigen stimulated cytokine responses had background (negative control) subtracted. The differences in the Treg frequencies; and measles antibody levels before and after vaccination were calculated using a 2-sided Mann-Whitney U test. Paired analyses were used where data was available for the same donor at both time points using the Wilcoxon matched-pairs test.

Treg frequencies were correlated with cellular and antibody responses and analysed using Spearman’s non-parametric correlation coefficients. Analyses were performed using STATA (SE Version 12 for Windows), GraphPad Prism (Version 7 for MAC, (GraphPad Software Inc, San Diego, USA) and Genstat Version 18. A p-value of less than 0.05 was considered significant. An r value of 0.4 was considered as a strong correlation (Cohen, 1988). The Bonferroni method was used to correct for multiple comparisons at a 5% significance level. Due to the number of parameters assessed in chapter 6, the Benjamini-Hochberg method was used at a False Discovery Rate (FDR) of 0.05 to correct for the multiple comparisons. The data values in tables and figures are shown as medians with the 25th and 75th quartiles shown in brackets.
CHAPTER 3: INVESTIGATING THE ROLE CIRCULATING TREGS PLAY IN THE CONTROL OF HUMORAL AND CELLULAR RESPONSES FOLLOWING MEASLES AND DTP VACCINATION

3.1 INTRODUCTION

Very little is known about the role Tregs play in controlling vaccine immunogenicity in animals or humans. There is a need to determine whether circulating Tregs at the time of vaccination interfere with favourable vaccine-induced responses. Since Tregs are induced by natural infections to regulate the inflammatory response, it is tempting to suggest that vaccination will also induce Tregs as part of the immune response, although this is generally not known (Ndure and Flanagan 2014). Though instrumental in preventing an over-exuberant immune response and immune pathology, there is evidence that Tregs can allow for persistent infection by preventing sterilizing immunity (Belkaid, Piccirillo et al. 2002). Whether Tregs may dampen the vaccine-induced response is unclear as data on how they influence the T cell response following vaccination are lacking. Studies on most vaccines have predominantly looked at the pro-inflammatory responses following vaccination, completely overlooking the regulatory response, which is also vital to better understand vaccine immunogenicity.

Recently, the adaptation of the immune system in the young child as compared to adults has been highlighted (reviewed in (Dowling and Levy 2014). Similar to other immune cells, several differences in CD4+FOXP3+ Tregs in early life as compared to that in adults have been described. Infants Tregs have been shown to be highly suppressive (Wing, Ekmark et al. 2002), and present in higher frequencies than in adults (Flanagan, Halliday et al. 2010). They have also been shown to be more naïve and less differentiated (Wing, Ekmark et al. 2002,
Fujimaki, Takahashi et al. 2008, Flanagan, Halliday et al. 2010. Whether Tregs have a role to play in the suboptimal responses to vaccination observed in infancy is not known, but could potentially offer a strategy to improve vaccine immunogenicity in early life. In this chapter we will explore the potential immunoregulatory role of Tregs in the context of measles vaccination and diphtheria-tetanus-whole cell pertussis (DTwP) vaccination.

Despite the availability of a highly effective live-attenuated vaccine, children continue to die daily from measles infection. Protection from infection relies upon humoral and cellular immunity. Measles gamma immunoglobulin (IgG) has effectively prevented infection and subsequent disease, including maternal measles IgG, which has also been shown to protect neonates and young infants against infection (reviewed by Plotkin 2010). However, patients with agammaglobulinaemia have the ability to clear infection with measles virus, underscoring the importance of the cell-mediated immune response in protecting against measles infection (Naniche 2009). Measles infection causes immunosuppression, resulting to increased susceptibility to secondary infections, which is the cause of most of the measles-related deaths (Griffin, Ward et al. 1994). The skewing of the immune response to a Th2-type and regulatory T cell response has been proposed as indirect mechanisms for measles-virus induced immunosuppression (Griffin and Ward 1993, Griffin 2010).

The humoral immune response to the live attenuated measles vaccine is generally considered to be the correlate of protection. Neutralising antibody titres of ≥120 mIU/ml has been shown to be protective against disease, and cases of vaccine failure have been attributed to the loss of measles IgG antibody (Mathias, Meekison et al. 1989). Variations in the immune response to Measles Vaccination, Sub-optimal antibody responses to the MV
have been observed in young children, especially in the developing world, and attributed to various host factors such as the mother’s immune status, the age of the child at vaccination, concurrent infections and nutritional status of the child (Kumar, Johnson et al. 1998, Bautista-Lopez, Vaisberg et al. 2001, Gans, Yasukawa et al. 2004).

Some studies have highlighted the importance of the cell-mediated immune response as an additional measure of vaccine efficacy (Ward, Boulianne et al. 1995). Measles vaccination activates both CD4+ and CD8+ memory T cells which produce essential pro-inflammatory cytokines to clear the virus, such as IFN-γ. In particular, the CD8 T cells have been shown to be important in measles virus clearance (reviewed by (Griffin, Lin et al. 2012); with the depletion of CD8 T cells in monkeys, rather than B cells, being associated with persistent MV infection (Permar, Klumpp et al. 2003, Permar, Klumpp et al. 2004). The role of Tregs in MV immunogenicity has not been explored, and it is not known if MV induces Tregs.

The aluminium adjuvanted inactivated DTwP vaccine plays a vital role in combating diphtheria, tetanus and pertussis infections in young children throughout the world. Unlike measles vaccination, which confers long-term protection, protection from the DTP vaccine is more short lived, hence three doses of this vaccine are given in the first 4 months of life in order to provide adequate protection. Classically, studies have analysed levels of antibodies to diphtheria toxoid (Dtx), tetanus toxoid (Ttx) and pertussis toxoid (Ptx) when investigating the immune response to DTP vaccination. The correlate of protection for the diphtheria and tetanus vaccines has been clearly documented. Antitoxins levels of 0.01 μg/mL after the administration of both vaccines has been shown to provide some protection, with levels reaching 0.1 providing complete protection against disease (Livorsi, Eaton et al. 2010).
Although rare, cases of infection have been reported despite the presence of high levels of antitoxin antibodies following vaccination. These infections have been mild, and are attributed to the poor diffusion of antitoxins to the sites of infection (de Moraes-Pinto, Oruamabo et al. 1995). A standard level of antitoxin, which provides protection against pertussis vaccination, is yet to be established.

The pertussis vaccine is usually given as part of a combination vaccine in either the acellular or whole-cell formulation. In The Gambia and much of the developing world the whole cell form is used in combination with tetanus and pertussis vaccines as DTwP. More recently, the pentavalent vaccine was introduced in The Gambia, in which the DTwP is combined with the HepB and Hib vaccines (Table 3.1).

<table>
<thead>
<tr>
<th>Vaccinations</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCG/OPV/HepB</td>
<td>Birth</td>
</tr>
<tr>
<td>DTwP-Hib-HepB/OPV</td>
<td>2 months</td>
</tr>
<tr>
<td>DTwP-Hib-HepB/OPV</td>
<td>3 months</td>
</tr>
<tr>
<td>DTwP-Hib-Hep/OPV</td>
<td>4 months</td>
</tr>
<tr>
<td>Measles/Yellow Fever/OPV</td>
<td>9 months</td>
</tr>
</tbody>
</table>

Table 3.1: The EPI schedule in the Gambia at the time the study was undertaken


Although the humoral response pays a vital role in protecting against diphtheria, tetanus and pertussis, as with many other bacterial infections, the role of the cell-mediated immune response also remains important. Unlike antibody levels which have also been shown to
decline following DTP vaccination, the cell-mediated immune response was shown to last longer (Cassone, Ausiello et al. 1997). The cellular immune response to whole cell pertussis has been shown to be predominantly a Th1 response, whereas acellular pertussis induces both Th1 and Th2 cytokines (Ausiello, Urbani et al. 1997). The role that Tregs play in regulating DTwP induced immunity in early life has not been investigated, but might provide useful insights into how the immunogenicity of this vaccine might be improved.

Sex differences in immunity (Klein and Flanagan 2016), and more specifically vaccine responses (Klein, Jedlicka et al. 2010) have been well described in the literature, but are often not considered in pre-clinical studies. We therefore predicted that Treg frequencies might be different in males and females, and further might influence vaccine responses in a sex-differential manner.

In this chapter we explore the potential role that CD4+FOXP3+ Tregs play in controlling the immune responses to MV and DTwP. We first analysed the Treg frequencies before and after vaccination, and then sought to investigate for the first time whether pre-existing circulating Tregs have an effect on the antibody and cellular responses to these vaccines.

**3.2 HYPOTHESES**

I hypothesised that:

- The CD4+FOXP3+CD127lo Tregs will be induced by vaccination
- Tregs have a role to play in immune activation and effector T cell function; and that circulating CD4+FOXP3+ Tregs at the time of MV and DTP vaccination will inversely correlate with markers of immune activation, T cell proliferation and perforin production.
• Circulating CD4+FOXP3+ Tregs at the time of MV and DTP vaccination will inversely correlate with measles and DTP antibody levels respectively
• Circulating Tregs will inversely correlate with vaccine-specific Th1/Th2 cytokine responses following vaccination and positively correlate with IL-10 responses
• The association between the Tregs and vaccine responses will be different in males and females

3.3 AIMS

• To determine ex-vivo Treg frequencies prior to vaccination at 9 months of age and 4 weeks post-vaccination in all vaccine groups
• To examine the correlation between the Tregs and the vaccine antibody responses
• To examine the correlation between the Tregs and Th1/Th2 cytokine responses
• To examine the correlation between the Tregs and T cell perforin production, Ki67 expression and activation marker expression (CD38, HLA-DR, B2M levels)
• To determine whether the above factors are different in males and females

3.4 METHODS

This study was nested within a larger study investigating the immunological effects of measles vaccination, DTP vaccination, or a combination of the two in 9 month old Gambian infants (Noho-Konteh, Adetifa et al. 2016). The overall study design and subject numbers are described in Materials and Methods 2.

The assays in this chapter were performed as part of a larger group. These individuals have been acknowledged in the acknowledgement section of the thesis.
3.4.1 Sample Processing

Venous blood totalling 4.5 mLs was collected into heparinised tubes (containing 7.5 units of heparin per mL of blood) at 9 and 10 months of age (Table 3.2). Samples were transported to the MRC laboratories in Fajara within 2 hours for processing. Upon arrival at the Infant Immunology laboratory, 600 µL of whole blood was used per participant for overnight stimulation assays (100µL per antigen); the ex-vivo flow cytometry assays used 100µL of whole blood per staining panel; and any remaining whole blood was spun and plasma collected and stored at -20°C. Peripheral blood mononuclear cells (PBMCs) were obtained with any remaining blood sample by density gradient centrifugation on Ficoll, and stored in liquid nitrogen for future use (Figure 3.1).

<table>
<thead>
<tr>
<th>Study Group</th>
<th>4 months</th>
<th>9 months</th>
<th>10 months</th>
<th>11 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>DTP</td>
<td>Bleed then MV</td>
<td>Bleed</td>
<td>YF/OPV (catch-up)</td>
</tr>
<tr>
<td></td>
<td>HBV, Hib</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>HBV, Hib</td>
<td>Bleed then MV+DTP</td>
<td>Bleed</td>
<td>YF/OPV (catch-up)</td>
</tr>
<tr>
<td>Group 3</td>
<td>HBV, Hib</td>
<td>Bleed then DTP</td>
<td>Bleed</td>
<td>MV/YF/OPV (catch-up)</td>
</tr>
</tbody>
</table>

Table 3. 2: Bleeding Time points for the MV/DTP study

*All the infants were bled at 9 months (baseline) before vaccine administration and again at 10 months, 4 weeks after vaccination. All outstanding vaccines were administered at 11 months of age (yellow fever and OPV for the MV and MV+DTP groups; and MV, OPV and YF for the DTP group).*
Figure 3.1: Flow chart showing how each sample was handled for the Treg part of the MV/DTP study.
3.4.2. Regulatory T cell phenotyping by flow cytometry

The flow cytometric staining was done by a group of individuals as it was nested within a larger study. Due to an error in the staining procedure by one of the technicians, not all the participants were stained for all the antibodies in the Treg panel. The number of participants with Treg data available is shown on Table 2.1 of the Methods section.

A 100μL aliquot of whole blood was used for Treg phenotyping. Cells were lysed by adding 200μL RBC lysing buffer diluted 10x with distilled water (BD Biosciences) at room temperature for 10 minutes and spinning for 10 minutes at 2,000 rpm. Table 3.3 shows the amount of antibodies used for the Treg panel. Cells were washed and stained with surface antibodies as described in Section 2.12. Cells were then permeabilized, incubated 4°C for 15 minutes with 2μL of rat serum (E-Biosciences) was added as a blocking step to prevent non-specific binding, and FOXP3 APC antibody (E-biosciences) was added and handled as described in Section 2.12. We gated on FSC/SSC plots, and then 100,000 lymphocyte events were collected as a minimum per sample. The range of FOXP3 events acquired was between 900 to 4,600 events. Data were analysed using FlowJo software (Treestar, California, USA). The gating strategy is shown in Figure 3.2.

3.4.3 Phenotyping of activated, proliferating and perforin producing T cells by flow cytometry

100μL of whole blood was used for this T cell functional panel; cells were lysed and stained with a pool of surface antibodies containing CD4 APC-Cy7, CD8 PB, HLADR PerCP and CD38
PE-Cy7 in the functional panel (Table 3.3) according to the staining procedure described in Section 2.12. Cells were then washed, fixed and permeabilized and then stained for intracellular cytokines Ki67 and Perforin (Table 3.3). This was done according to staining procedure in Section 2.12. After the incubation, the cells were resuspended in 150μl fix buffer, acquired on the Cyan Flow cytometer (CyanADP) and analysed using the Flowjo software (Treestar, California).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Laser (excitation wavelength)</th>
<th>Fluorochrome Channel</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treg Panel</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4 PerCP</td>
<td>Blue (488nm)</td>
<td>FL4</td>
<td>1/5</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD8 PB</td>
<td>Violet (407nm)</td>
<td>FL6</td>
<td>1/8</td>
<td>E-Biosciences</td>
</tr>
<tr>
<td>CD25 PE-Cy7</td>
<td>Blue (488nm)</td>
<td>FL5</td>
<td>1/5</td>
<td>E-Biosciences</td>
</tr>
<tr>
<td>CD127 PE</td>
<td>Blue (488nm)</td>
<td>FL2</td>
<td>¼</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>FOXP3 APC</td>
<td>Red (633 nm)</td>
<td>FL8</td>
<td>1/5</td>
<td>E-Biosciences</td>
</tr>
<tr>
<td><strong>Functional Panel</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4 APC-Cy7</td>
<td>Red (642nm)</td>
<td>FL9</td>
<td>1/30</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD8 PB</td>
<td>Violet (407nm)</td>
<td>FL6</td>
<td>1/30</td>
<td>E-Biosciences</td>
</tr>
<tr>
<td>HLADR PerCP</td>
<td>Blue (488nm)</td>
<td>FL4</td>
<td>1/15</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD38 PE-Cy7</td>
<td>Blue (488nm)</td>
<td>FL5</td>
<td>1/6</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Ki67 FITC</td>
<td>Blue (488nm)</td>
<td>FL1</td>
<td>1/6</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Perforin PE</td>
<td>Blue (488nm)</td>
<td>FL2</td>
<td>1/6</td>
<td>BD Biosciences</td>
</tr>
</tbody>
</table>

**Table 3.3:** The antibodies used for the Treg and functional panels in the MV/DTP study

*The dilution used for the staining, the fluorochrome channels, lasers and the source of the antibodies are also shown.*
To gate on the Tregs, gating was firstly done on the total lymphocytes, and then on the CD4+ subset within the total lymphocytes. Within this CD4+ subset, the cells characterized as FOXP3+ and CD127- were gated on and defined as the Tregs, and frequencies expressed as a percentage of CD4+ T cells (Figure 3.2).

![Figure 3.2: The Gating Strategy](image)

**Figure 3.2: The Gating Strategy**

The gating strategy for the (A) lymphocytes, (B) CD4+ and CD8+ T cells, (C) FOXP3+CD127lo Tregs, (D) HLADR+CD38+ activated, (E) Ki67+ proliferating and (F) Perforin-producing CD4+ and CD8+ T cells. *Cells were gated on the total lymphocytes, and then on the CD4+ and CD8+ subsets within the total lymphocytes. They were then gated on the markers of interest. A minimum of 100,000 events were collected within the lymphocyte gate.*
3.4.4 Beta-2 microglobulin assay

The plasma beta-2 microglobulin levels were assessed by an automated Microparticle Enzyme Immunoassay (MEIA) using an AxSYM automated machine (Abbott Laboratories, Wiesbaden, Germany) according to the manufacturer’s instructions. The plasma samples were collected from study participants at 9 months (baseline) and at 10 months (1 month post-vaccination) were thawed and placed in sample cups, and delivered by the sampling probe with the sample diluent and phosphate buffer to the wells of the reaction vessel. The plasma sample was diluted by the machine, and an aliquot of the diluted sample, with anti-β2m coated microparticles and AxSYM Solution 4 was dispensed into a well of the reaction vessel. An aliquot of the reaction mixture was then transferred to the matrix cell whereby the microparticles bind irreversibly to the glass fibre matrix and then the matrix cell is washed to remove unbound materials. In this reaction mixture, the β2m in the specimen binds to the anti-β2m coated microparticles forming an antigen-antibody complex. An Alkaline Phosphatase (ALP) conjugate is then dispensed onto the matrix cell and binds to the antigen-antibody complex to form the antibody-analyte-conjugate ‘sandwich’. The ‘sandwich’ is washed again and the 4-methylumbelliferyl phosphate (MUP) substrate added. The ALP conjugate then hydrolyses MUP to 4-methylumbelliferone (MU). The rate at which MU is generated from MUP is measured by the MEIA optical assembly, and is proportional to the concentration of β2m in the plasma sample. The readout is given in mg/mL.

3.4.5 Cell culture assay for cytokine measurement

Whole blood samples were cultured for 16hrs with 10µL of measles haemagglutinin peptide pool (consisting of 20-mer overlapping peptides of >95% purity spanning the haemagglutinin antigen of measles, final concentration of 1 µg/ml; Sigma-Genosys), tetanus toxoid (TT) (final
concentration 10µg/mL, Sanofi Pasteur, France) and purified protein derivative of *M. tuberculosis* (PPD) (final concentration 10µg/mL, Statens Serum Institute, Denmark). Anti-CD3 (αCD3) (5µg/mL, Becton-Dickinson (BD), USA) and anti-CD28 (αCD28) (5µg/mL, eBiosciences, UK) were used to stimulate T cells non-specifically, with RPMI (Sigma Aldrich) alone used as a negative control. After 16hr culture (overnight), plates were centrifuged at 2,000 rpm for 5 minutes and 50µl supernatant collected from each well into pre-labelled tubes. The collected supernatants were frozen at -70°C and later analysed by Luminex assays for cytokine concentrations.

### 3.4.6 Cytokine Luminex assay

The Bio-Plex 200 Suspension Array system (Bio-Rad Laboratories, France) was used for analysis of cytokines in plasma and culture supernatants. Plasma and supernatants that were collected from the overnight cultures were thawed and centrifuged at 1,200 rpm for 2 minutes to remove any debris. 25µl aliquots were plated out in 96 well plates and diluted 1:2 with medium containing serum (RPMI medium containing 10% BSA, Sigma) media.

For the initial identification of key cytokine/chemokines to investigate further, a 27-plex assay was used on 10-month (post-vaccination) culture supernatant samples from randomly selected donors. This contained pre-mixed beads specific for: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, Eotaxin, FGF basic, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1b, PDGFbb, RANTES, TNF-α and VEGF. The cytokines for further study were narrowed down to 10 cytokines / chemokines based on which showed the greatest differences between vaccine groups in the initial analysis, and all remaining 9 and 10 month samples were tested. Of these we selected to analyse for correlates between Tregs
and the 5 key cytokines which best differentiated vaccine groups in the final modelling analysis, namely TNF, IFN-γ, IL-1β, IL-4 and IL-10.

The Bio-plex assay was conducted according to the manufacturer’s instructions (Biorad). The standard came lyophilized in the kit. It was reconstituted in 500μl RPMI medium containing 10% bovine serum albumin (BSA) (VWR, UK), mixed by being vortexed and then incubated on ice for 30 minutes. The standard was serially diluted 1:4 using the RPMI medium to produce 8 standards. The standard ranges were set for the analytes using the standard curve.

The vacuum filter was first pre-wet using 100μl of assay buffer (Bio-Rad Laboratories, France), and buffer then removed by vacuum filtration using the Aurum vacuum manifold (BioRad, Belgium) with 1-2 mmHg pressure. The plate was washed twice with 100μl wash buffer. To remove excess fluid, the bottom of the plate was blotted using a lint free paper towel. The conjugated microsphere beads were vortexed for 20-30 seconds and then diluted with 1mL assay buffer. From the diluted mixture, 50μL was added to each well of the plate. The negative control (RPMI medium, Sigma) and the standards (50μL) were added in duplicates (S1-S8), and the test samples were added in single wells. The plate was then sealed and covered in foil, and the beads incubated with the samples and standards at room temperature on a shaker (30 seconds at 1,100 rpm, then for 30 minutes at 300 rpm). At the end of the incubation, the plate was washed three times with the vacuum manifold using 200μL per well of wash buffer. Following blotting, the biotinylated detection antibody (diluted according to the manufacturer’s instructions) was vortexed gently and 25μL added to each well. The plate was sealed with sealing tape, covered in foil and incubated for 30 minutes at room temperature (RT). The streptavidin-PE detection antibody was diluted 1:10, and 25μL
was added to each well of the assay plate and incubated for 10 minutes at RT on a gentle shaker. The plate was washed thrice with wash buffer and each well was resuspended in 125μL of assay buffer. The plate was then placed on a shaker for 30 seconds at 1,100 rpm before being read.

The plates were read using the Bio-Plex 200 Suspension Array system (Bio-Rad) and Bio-Plex Manager software version 4.1.1 (Bio-Rad Laboratories, Hercules, CA). The reader was calibrated before every run using CAL1 and CAL2 calibration kits (Bio-Rad, UK). All values less than the lowest value of the standard within the standard curve were given the value of half the lowest standard and all values higher than the highest standard were given the value of twice the highest standard. Background (unstimulated) results were subtracted from antigen-stimulated results to determine antigen-specific levels of cytokine production.

3.4.7. Diphtheria, tetanus and pertussis antibody measurements by multiplex immunoassay (MIA)

*These assays were performed in The Netherlands in collaboration with Fiona van der Klis at the National Institute for Public Health and the Environment RIVM. This technology has since been transferred to MRC The Gambia Unit as an extension of this collaboration.*

Antibody titres were measured in plasma samples to the diphtheria toxin (Dtx); tetanus toxin (Ttx); and pertussis toxin (Ptx), filamentous haemaglutinin (FHA), pertactin (Prn) and fimbriae type 2 (Fim-2) using multiplex technology. The assay allows for the simultaneous detection and quantification of the various vaccine antibodies in 1 small volume sample and was
conducted using specific in-house protocols as previously described (van Gageldonk, van Schaijk et al. 2008). In brief, the technology relies on a microsphere-based fluorescent system, quantifying total IgG antibodies in the sample against the vaccine antigens of interest. The plasma was diluted and added to the in-house standards containing beads coated in the antigen proteins. Antibodies specific for the diphtheria, tetanus and pertussis antigens bind to these beads and are subsequently detected upon addition of an R-phycoerythrin labelled goat anti-human conjugate. The immunoassay plate was read using the Bio-Plex 200 system.

3.4.8 HAI measles neutralisation assay

These assays were conducted by Jainaba Njie-Jobe and Lady Chilel Sanyang at the MRC laboratories in The Gambia

Plasma samples were analysed for measles Ab levels by haemagglutination inhibition assay (HAI) which is a functional assay using monkey red blood cells as described previously (Whittle, Campbell et al. 1990). The assay strongly correlates with neutralising antibodies (Samb, Aaby et al. 1995).

Serial 2-fold dilutions of the plasma samples (decomplemented at 56°C for 30 minutes in a water bath) were allowed to react with a fixed concentration of haemagglutinin antigen (HA). The residual HA is detected by addition of monkey red blood cells (Cercopithecus RBCs, 0.5% in HAI buffer solution) obtained from the Barbados Primate Research Centre. These cells have measles haemagglutinin receptors on their surface. The plasma sample was incubated
with the RBCs in a humidified incubator at 37°C, 5% CO₂ for 3 hours. Following this incubation, the plates were left to incubate at room temperature overnight.

Following the overnight incubation, there were two independent reads of each plate. A lack of agglutination occurs when the haemagglutinin is neutralized by the antibody present in the sample. Results are expressed as log₂ units, the minimum detection level being 31.2mIU, and a protective level defined as ≥125mIU antibody (log₂ titre ≥3) (Samb, Aaby et al. 1995).

### 3.4.9: Sample Size Considerations and Statistical Analysis

The original sample size calculation for the main MV/DTP study was made in consultation with one of the unit statisticians Dr Crozier; and was powered for the whole human transcriptome analysis which requires a sample size of approx. 50 per group. This is detailed in Section 2.13 of the Methods section.

The antigen stimulated cytokine responses were analysed with the background (negative control) value subtracted to determine net cytokine production above background. Negative values were transformed to 0 as these were below the detection limit of the assay.

Differences between the bleeding time points were analysed using 2-sided Mann-Whitney tests, paired analyses was used where data was available for the same donor at both time points using the Wilcoxon matched pairs signed rank test. Tregs were correlated with cytokine and antibody responses, and functional T cell markers. We calculated r values using the Spearman’s non-parametric correlation coefficients. Analyses were performed using
GraphPad Prism (Version 6 for MAC). A p-value of ≤0.05 was considered as significant. The Bonferroni method was used to correct for multiple testing according to the number of parameters assessed, at a significance level of 5%.

3.5 RESULTS

3.5.1 Treg frequencies almost halved by 19 months of age

To get an overview of the change in Treg frequencies overtime, we followed up the infants at 10 and 19 months of age and measured their Tregs again by ex-vivo flow cytometry. There was a highly significant decline in the Treg frequencies at 19 months compared to previous time points regardless of study group (p<0.001, Figure 3.3). This remained significant after correcting for multiple testing.

![Figure 3.3: Tregs at 19 months compared to at 10 months of age](image)

**CD4+FOXP3+CD127- Treg frequencies at 10 and 19 months in the 3 vaccine groups for all donors. Each dot represents a single individual with paired data shown on the right (B)**
at age 10 months (10mo) and 19 months (19mo) for all the study groups. Paired data were analysed using a two-tailed Wilcoxon matched-pairs signed rank test * = p ≤0.05, **= p ≤ 0.01, *** = p <0.001. Bonferroni correction was used to correct for multiple testing; α ≤0.008 was considered significant after correcting for 6 parameters. Data are shown for n = 136 infants, MV group n = 43, MV+DTP group n = 40, DTP n = 32

### 3.5.2 Sex differential changes in Treg frequencies following MV and DTP vaccination

We first analysed whether circulating Treg frequencies were altered following vaccination with MV, DTP, or both vaccines combined. When all donors were analysed together the Tregs declined in the DTP vaccinated group (p = 0.039), but did not change in the other two groups (Figure 3.4A,B). When males and females were analysed separately, we found an increase in circulating Tregs in the measles vaccinated females (p = 0.04) (Figure. 3.4C,D) and a decrease in the DTP vaccinated males (p = 0.029) (Figure 3.4E,F). When corrected for multiple testing, there was no change in Treg frequency in those vaccinated with MV and DTP together for all donors or by sex.
Figure 3. 4: CD4+FOXP3+CD127= Treg Frequencies before and after vaccination

CD4+FOXP3+CD127- Treg frequencies before and after vaccination in the 3 vaccine groups for all donors (A), and females (B) and males (C) separately. Each dot represents a single individual with paired data shown on the right panel (B, D and F) at baseline (9mo) and 4 weeks following vaccination (10) for all the study groups. Paired data were analysed using a
two-tailed Wilcoxon matched-pairs signed rank test * = p ≤0.05, ** = p ≤ 0.01, *** = p < 0.001. Bonferroni correction was used to correct for multiple testing; \( \alpha \leq 0.008 \) was considered significant after correcting for 6 parameters; \( \alpha \leq 0.004 \) was considered significant after correcting for 12 parameters for the sex-differential analyses. Data are shown for 9 month visit, MV group \( n = 48 \), MV+DTP group \( n = 55 \), DTP \( n = 32 \); 10 month visit. MV group \( n = 43 \), MV+DTP group \( n = 40 \), DTP \( n = 34 \). For the paired analysis, data shown for MV group \( n = 19 \), MV+DTP group \( n = 20 \), DTP \( n = 13 \).

3.5.3 Sex-differential increased immune activation and proliferation following simultaneous MV and DTP vaccination, but diminished after DTP vaccination alone

Early studies show that there are elevated levels of beta-2 microglobulin (\( \beta 2m \)), a marker of immune activation, in measles infection (Griffin and Ward 1993). As we were interested in the effect of vaccination on the immune activation status of the infants, we next evaluated the plasma levels of \( \beta 2m \) before and vaccination, and found that it increased after MV+DTP vaccination when all infants were analysed together (\( p = 0.051 \)), but not when analysed by sex (Figure 3.5A-F). When corrected for multiple testing, we did not observe any significant change in the \( \beta 2m \) levels following vaccination.

The co-expression of HLADR and CD38 indicate a classical marker of T cell activation widely used in HIV studies where it is associated with disease progression (Giorgi, Hausner et al. 1999). CD38+HLADR+ activated CD4 T cell frequencies were comparable for the MV alone and DTP alone groups, before and after vaccination. However, it increased significantly following vaccination in the MV+DTP group (\( p = 0.004 \)), and remained significant after
correcting for multiple testing (Figure. 3.6A). The MV+DTP vaccinated females also had increased activated CD4 T cells after vaccination (p = 0.049), this effect was however not observed when the p-value was corrected for multiple comparisons (Figure. 3.6C). No changes were observed among males for any vaccine group (Figure. 3.6E). By contrast we did not observe any change in the CD38+HLADR+ CD8 T cells for the vaccine groups with males and females combined (Figure 3.6B), or when females (Figure. 3.6D) and males (Figure. 3.6F) were analysed separately.
Figure 3.5: Beta-2 microglobulin before and after vaccination

Plasma β2m levels were analysed before and after vaccination for (A and B) all infants combined and (C and D) females and (D and E) males analysed separately. Each dot represents a single individual with paired data shown (right panel B, D and F) at baseline (9mo) and 4 weeks following vaccination (10) for all the study groups. Paired data were
analysed using a two-tailed Wilcoxon matched-pairs signed rank test * = p ≤0.05, **= p ≤ 0.01, *** = p <0.001. Bonferroni correction was used to correct for multiple testing; α ≤0.008 was considered significant after correcting for 6 parameters. Data are shown for 9 month visit, MV group n = 70, MV+DTP group n = 90, DTP n = 58; 10 month visit. MV group n = 88, MV+DTP group n = 96, DTP n = 67. For the paired analysis, data shown for MV group n = 70, MV+DTP group n = 90, DTP n = 56.
The co-expression of CD38 and HLADR was used as a marker of activated T cells. Activated (A) CD4+ and (B) CD8+ T cells are shown for all infants combined. When separated by sex, (C and D) females, and (E and F) males are also shown. Each dot represents a single individual with paired data shown (right panel (B, D and F) at baseline (9mo) and 4 weeks following vaccination (10) for all the study groups. Paired data were analysed using a two-tailed Wilcoxon matched-pairs signed rank test * = p ≤0.05, ** = p ≤ 0.01, *** = p <0.001. Bonferroni
correction was used to correct for multiple testing; \( \alpha \leq 0.008 \) was considered significant after correcting for 6 parameters; \( \alpha \leq 0.004 \) was considered significant after correcting for 12 parameters for the sex-differential analyses. Data are shown for paired samples, MV group \( n = 60 \), MV+DTP group \( n = 62 \), DTP \( n = 33 \).

CD4 T cell proliferation determined by intracellular Ki67 expression was also increased in the MV+DTP group (all infants \( p < 0.001 \); females \( p = 0.027 \)); and males experienced no change in CD4 T cell proliferation post-vaccination (Figure. 3.7A, C, E). There was no change in CD8 T cell proliferation in females, or in males or all infants analysed together (Figure. 3.7B, D, F). After correcting for multiple testing the MV+DTP females had increased activated and proliferating CD4+ T cells post-vaccination.

3.5.4 Sex differential altered perforin production by CD8 T cells post-vaccination

The other T cell function that was analysed was perforin production by CD8 T cells. CD8+ T cell perforin production increased in the MV+DTP group (all infants \( p = 0.002 \); females \( p = 0.01 \); males \( p = 0.03 \)), but declined in the DTP vaccinated groups (all infants \( p = 0.03 \)) (Figure. 3.8A-C). After correcting for multiple testing all infants combined had an increase in perforin+ CD8 T cells after MV+DTP and a decline after DTP alone, but the effect in males was no longer significant despite showing the same pattern.
Figure 3. 7: Frequencies of proliferating T cells before and after vaccination

Intracellular Ki67 was used as a marker of proliferating T cells. CD4 T cell proliferation was measured in all infants combined (A), females (C) and males (E) separately. CD8 T cell proliferation was similarly assessed in all infants (B), females (D) and males (F). Each dot represents a single individual with paired data shown at baseline (9mo) and 4 weeks following vaccination (10mo) for all the study groups. Paired data were analysed using a two-tailed Wilcoxon matched-pairs signed rank test * = p ≤0.05, **= p ≤ 0.01, *** = p <0.001. Bonferroni correction was used to correct for multiple testing; α ≤0.008 was considered
significant after correcting for 6 parameters; \( \alpha \leq 0.004 \) was considered significant after correcting for 12 parameters for the sex-differential analyses. Data are shown for paired samples; MV group \( n = 46 \), MV+DTP group \( n = 42 \), DTP \( n = 27 \).

![Graph](image)

**Figure 3.8: Perforin-producing Cd8 T cells before and after vaccination**

Intracellular perforin levels were analysed in CD8 T cells by flow cytometry in all infants combined (A), females (B) and males (C) separately. Each dot represents a single individual with paired data shown at baseline (9mo) and 4 weeks following vaccination (10mo) for all the study groups. Paired data were analysed using a two-tailed Wilcoxon matched-pairs signed rank test \( * = p \leq 0.05 \), \( ** = p \leq 0.01 \), \( *** = p < 0.001 \). Bonferroni correction was used to correct for multiple testing; \( \alpha \leq 0.008 \) was considered significant after correcting for 6 parameters; \( \alpha \leq 0.004 \) was considered significant after correcting for 12 parameters for the
sex-differential analyses. Data are shown for paired samples; MV group n = 42, MV+DTP group n = 39, DTP n = 27.

3.5.5 No evidence that Tregs play a role in altered immune activation, function, T cell proliferation or perforin production after vaccination

There was no evidence for a negative correlation between the circulating Tregs on the day of vaccination and circulating frequencies of activated (HLADR+CD38+), proliferating (Ki67+) or perforin producing (perforin+) T cells at the same time point (Figure 3.9). There was similarly no negative correlation between circulating Tregs four weeks after vaccination and the same parameters at this time point (Figure 3.10), suggesting that CD4+FOXP3+CD127- Tregs have no effect on CD4 or CD8 T cell activation and proliferation or CD8 T cell perforin production either before or after vaccination.

3.5.6 Baseline circulating Tregs on the day of vaccination negatively correlate with antibody responses to MV but not DTP

We next compared the antibody levels/titres at baseline and following MV and/or DTP vaccination (Figure 3.11). As expected, we saw a significant increase in HAI measles neutralisation titres (Figure 3.11A; p<0.001); and the DTx, Ttx, Ptx antibody levels (Figure 3.11B-C; p<0.001) following vaccination in the MV and DTP alone groups. This increase remained significant following correction for multiple testing.
Figure 3. 9: Correlations between Tregs and effector T cell function at the time of vaccination

Correlations between circulating Tregs and CD4 /CD8 T cell activation and proliferation or CD8 T cell perforin production at the time of vaccination. The frequency of Treg as a percentage of all lymphocytes at 9 months of age on the day of vaccination is shown on the x-axis. Frequencies of Ki67+, HLADR+CD38+ and Perforin+ cells, within the CD4 and/or CD8+ T cells, at the same time point are shown on the y-axis. Data are shown for all the 3 vaccine groups. The line indicates the best-fit correlation using Spearman’s rank correlation coefficient. MV group n= 48, MVDTP group n= 52 , DTP group n=29 . We did not observe any significant correlations.
Figure 3. 10: Correlations between circulating Tregs and effector T cell function a month after vaccination

Correlations between circulating Tregs and CD4 /CD8 T cell activation and proliferation or CD8 T cell perforin production four weeks after vaccination. The frequency of Treg as a percentage of all lymphocytes at 10 months of age (a month after vaccination) is shown on the x-axis. Frequencies of Ki67+, HLADR+CD38+ and Perforin+ cells, within the CD4 and/or CD8+ T cells, at the same time point are shown on the y-axis. Data are shown for all the 3 vaccine groups. The line indicates the best-fit correlation using Spearman’s rank correlation coefficient. MV group n=30, MVDTP group n=31, DTP group n=22. We did not observe any significant correlations.
Figure 3. 11: Vaccine Antibody plasma titres/levels before and after vaccination

(A) Measles HAI titres in measles vaccinated infants were measured using the HAI neutralisation assay. The levels of antibody to the (B) Diphtheria toxoid (DTx), Tetanus toxoid (TTx) and (C) Pertussis toxoid antigens were analysed in plasma collected from DTP vaccinated infants using the multiplex assay. Each dot represents a single individual with paired data shown at baseline (9mo) and 4 weeks following vaccination (10mo). Paired data were analysed using a two-tailed Wilcoxon matched-pairs signed rank test * = p ≤0.05, ** = p ≤ 0.01, *** = p <0.001. Bonferroni correction was used to correct for multiple testing; $\alpha$ $\leq$0.008 was considered significant after correcting for 6 parameters; $\alpha$ $\leq$0.004 was considered significant after correcting for 12 parameters for the sex-differential analyses. Data are shown for paired samples; MV group n = 77, DTP group n = 57.
We previously reported that measles antibody titres 4 weeks after vaccination were not affected by simultaneous administration of DTP; nor were tetanus toxoid (Ttx), diphtheria toxoid (Dtx) and pertussis toxoid (Ptx) titres post DTP vaccination altered by giving MV at the same time (Noho-Konteh, Adetifa et al. 2016). To increase statistical power, we therefore combined the MV and MV+DTP group to analyse for measles antibody correlations between baseline circulating Tregs and vaccine antibody levels 4 weeks later, and the DTP and MV+DTP groups to analyse for DTP antibody correlations. This showed a weak yet significant negative correlation between baseline Tregs and subsequent measles HAI neutralisation titres in infants who received MV (MV and MV+DTP groups combined) ($r = -0.208$, $p = 0.048$) (Figure. 3.12A); whereas there was no correlation with any of the DTP antibody readouts for DTP vaccinated infants (DTP or DTP+MV groups combined) (Figures. 3.12B-D). There were no significant correlations between Tregs and vaccine antibodies when corrected for multiple testing, and when males and females were analysed separately.
Figure 3. 12: Correlations between baseline circulating Tregs at the time of vaccination and vaccine antibodies 4 weeks later

The frequency of Treg as a percentage of all lymphocytes at 9 months of age on the day of vaccination is shown on the x-axis, and the IgG antibody titres at 10 months are shown on the y-axis. (A) Measles HAI titres are shown for measles vaccinated infants (MV and MV+DTP groups combined); and (B) Dtx titres, (C) Ttx titres and (D) Ptx titres are shown for DTP vaccine recipients (DTP and MV+DTP groups). The line indicates the best-fit correlation using Spearman’s rank correlation coefficient, and the correlation coefficient (r value) and p value for the correlation are shown. Bonferroni correction was used to correct for multiple testing; α
≤ 0.0125 was considered significant after correcting for 4 parameters. Data for 94 measles vaccinated (MV n=46, MV/DTP n=48) and 79 DTP vaccinated (MV/DTP n=41, DTP N=38) infants are shown.

**Figure 3.13**: Correlations between baseline circulating Tregs at the time of vaccination and the change in the antibody levels following vaccination

The frequency of Treg as a percentage of all lymphocytes at 9 months of age on the day of vaccination is shown on the x-axis, and the change in the antibody levels (calculated by subtracting the levels at 9 months from the levels at 10 months) is shown on the y-axis. Change in the (A) Measles HAI titres, (B) Dtx (C), Ttx and (D) Ptx antibody titres are shown. The line indicates the best-fit correlation using Spearman’s rank correlation coefficient.
Bonferroni correction was used to correct for multiple testing; \( \alpha \leq 0.0125 \) was considered significant after correcting for 4 parameters. Data shown for \( n=26 \) infants.

3.5.7 No correlations between circulating Tregs at vaccination and post-vaccination cellular responses to vaccine antigens

We could not perform functional Treg assays in this study for logistic reasons including the multiple assays being conducted, small blood volumes collected and lack of flow cytometry cell sorting facilities in The Gambia. We thus chose to analyse for correlations between CD4+FOXP3+CD127- Tregs and readouts of vaccine-specific cellular immunity. Measles-specific responses were assessed in supernatants from whole blood cultured with a measles haemagglutinin antigen peptide pool, and tetanus specific responses by measuring for cytokines in TT cultures. In the measles vaccinated infants, we observed a significant increase in all the levels of all four cytokines (IL-4, IL-10, IFN-\( \gamma \) and TNF) in measles peptide pool culture supernatants (Figure 3.14). In the DTP group, there was a significant increase in the IL-4, IFN-\( \gamma \) and TNF levels in TT culture supernatants, but not the IL-10 levels (Figure 3.14).

We questioned whether baseline Tregs on the day of vaccination correlated negatively with subsequent vaccine-specific pro-inflammatory (TNF), Th1 (TNF, IFN-\( \gamma \)) or Th2 (IL-4) cellular responses 2 weeks after vaccination, or positively or negatively with the immunosuppressive cytokine IL-10, which can also be produced by activated Th1 and Th2 cells. There was no evidence for any significant correlation between baseline Tregs and subsequent vaccine-specific cellular responses to suggest an immunoregulatory role for circulating Tregs at the time of vaccination (Table 3.4).
We questioned whether baseline Tregs on the day of vaccination correlated negatively with subsequent vaccine-specific pro-inflammatory (TNF), Th1 (TNF, IFN-γ) or Th2 (IL-4) cellular responses 2 weeks after vaccination, or positively or negatively with the immunosuppressive cytokine IL-10, which can also be produced by activated Th1 and Th2 cells. There was no evidence for any significant correlation between baseline Tregs and subsequent vaccine-specific cellular responses to suggest an immunoregulatory role for circulating Tregs at the time of vaccination (Figure 3.15).

Figure 3. 14: Antigen-specific cytokine levels before and after vaccination

The multiplex assay was used to measure the levels of the cytokines from supernatants collected following overnight stimulation with the measles ‘H’ pool (MV group), and TTx (DTP group). The levels from the medium stimulated wells were subtracted from the antigen-stimulated wells. Following background subtraction, we compared the cytokine levels at (A)
baseline (9mo) and (B) 4 weeks later (10mo). The cytokine levels are shown on the y-axis and are in pg/mL. Negative values were transformed to zero. The red line indicates the median values. The data were analysed using a two-tailed Mann Whitney U test. * = p ≤0.05, **= p ≤ 0.01, *** = p < 0.001. Bonferroni correction was used to correct for multiple testing; α ≤ 0.006 was considered significant after correcting for 8 parameters. Data shown for 9 month visit: MV group n =116, DTP group n = 109; 10 month visit: MV group n = 109, DTP n = 166.
Table 3. 4: Correlations between vaccine-specific cytokine response and Tregs at the time of vaccination and 4 weeks later

The multiplex assay was used to measure the levels of the cytokines from supernatants collected following overnight stimulation with the measles ‘H’ pool, TTx and PPD. The levels from the medium stimulated wells were subtracted from the antigen-stimulated wells. Following background subtraction, the post-vaccination (10mo) cytokine levels were correlated with the Treg frequencies as a percentage of all lymphocytes at baseline (9mo) and a month after vaccination (10mo). The r values are displayed, and were calculated using Spearman’s rank correlation coefficient. There were no significant correlations observed. Data shown for Tregs at 9mo n=24; Tregs at 10mo n=26.
3.5.7 Post-vaccination Tregs and correlations with vaccine-specific and control antigen cytokine responses at the same time

IL-10 is an immunosuppressive cytokine that plays a crucial role in regulating the human immune response. It strongly inhibits the production of pro-inflammatory cytokines such as IFN-γ, thereby counterbalancing the effects of the pro-inflammatory response. Therefore, we next investigated the association between pro- and anti-inflammatory cytokine ratios and Tregs before and after vaccination. There was a negative correlation between post-vaccination Tregs and the IFN-γ:IL-10 ratio in TT cultures in the DTP group only (p = 0.0202, r = -0.471) (Figure. 3.15A); and similarly the DTP group had a negative correlation between Tregs and measles-specific IFN-γ:IL-10 responses (p = 0.0475, r = -0.4001) (Figure. 3.15B). None of these correlations remained significant following correction for multiple testing. There was no correlation between post-vaccination Tregs and cytokine responses to the unrelated antigen PPD and positive control aCD3/CD28 for any vaccine group to support an immunoregulatory role.

3.5.8 Baseline Circulating Tregs and correlations with the change in the vaccine-specific antigen cytokine responses

We next questioned whether the Tregs at the time of vaccination (9mo) correlate with the increase/change in the vaccine-specific antigen cytokine responses in the measles and DTP vaccinated infants. There was no evidence for any significant correlation to suggest that the circulating Tregs at the time of vaccination suppress the cellular responses in either measles (Figure 3.16) or DTP vaccinated infants (Figure 3.17).
Figure 3.15: Correlations between post-vaccination circulating Tregs and vaccine-specific cellular responses

The frequency of post-vaccination Treg as a percentage of all lymphocytes is shown on the x-axis, and the post-vaccination cytokine response to the indicated antigen are shown on the y-axis. The line indicates the best-fit correlation using Spearman’s rank correlation coefficient, and the correlation coefficient (r value) and p value for the correlation are shown following overnight stimulation with (A) TTx and (B) measles ‘H’ pool antigens. Bonferroni correction was used to correct for multiple testing; α ≤ 0.025 was considered significant after correcting for 2 parameters. Data shown for n= 25 DTP vaccinated infants.
Figure 3. 16: Correlations between baseline circulating Tregs and the change in the measles-specific cytokine responses

The frequency of Tregs as a percentage of all lymphocytes at 9 months of age on the day of vaccination is shown on the x-axis. The difference in the cytokine levels (calculated by subtracting the levels at 9 months from the levels at 10 months) is shown on the y-axis. The multiplex assay was used to measure the levels of the cytokines from supernatants collected following overnight stimulation with the measles ‘H’ pool. Change in the levels of (A) TNF, (B)
IFN-γ, (C) IL-4 and (D) IL-10 are shown for the infants vaccinated with MV alone. The line indicates the best fit correlation using Spearman’s rank correlation coefficient. Data shown for \( n=28 \) infants.

Figure 3. 17: Correlations between baseline circulating Tregs and the change in the TTx-specific cytokine responses

The frequency of Tregs as a percentage of all lymphocytes at 9 months of age on the day of vaccination is shown on the x-axis. The difference in the cytokine levels (calculated by subtracting the levels at 9 months from the levels at 10 months) is shown on the y-axis. The multiplex assay was used to measure the levels of the cytokines from supernatants collected following overnight stimulation with the TTx antigen. Change in the levels of (A) TNF, (B) IFN-γ, (C) IL-4 and (D) IL-10 are shown for the infants vaccinated with DTP alone. The line
indicates the best fit correlation using Spearman’s rank correlation coefficient. Data shown for n=22 infants.

3.6 DISCUSSION

This study was nested into a study investigating the non-targeted immunological effects of measles vaccination (MV) and the combined diphtheria, tetanus and pertussis (DTP) vaccine in nine-month old Gambian infants, the results of which have been published (Noho-Konteh, Adetifa et al. 2016). The published data show that the different vaccine groups had different immunological profiles post-vaccination, which varied according to sex. The DTP group had lower plasma pro-inflammatory responses following vaccination; MV+DTP group females, but not males, had enhanced pro-inflammatory cytokines; while MV males, but not females, similarly had enhanced pro-inflammatory cytokines. Thus the cytokine milieu may vary depending on what vaccine is administered, and our first question was whether other ex-vivo parameters including Tregs, were affected by vaccination.

I hypothesised that vaccination would likely induce Tregs. The decline in Tregs following DTP vaccination, particularly in males, is interesting. This could potentially allow for a greater inflammatory response in DTP vaccinated males compared to females, which is in keeping with the significantly greater Th1 cytokine response to T cell stimulation among males compared to females in our published study from this cohort (Noho-Konteh, Adetifa et al. 2016). A plausible explanation may be that there is preferential activation and expansion of conventional T cells following vaccination with DTP. A flow cytometry panel containing more phenotypic markers including different homing receptors would have provided information
about which subset of T cells are expanded and where they home to. Indeed aluminium adjuvanted vaccines have been shown to preferentially expand Tregs in draining lymph nodes (Plebanski et al., unpublished), which could lead to a paradoxical decline in peripheral blood.

The decline in Tregs was more pronounced in DTP-vaccinated males than females. In contrast, there was an increase in Tregs post-MV in females but not male infants. Sex differences in immunity have been well described for antibody responses to many vaccines (Klein 2012), (Klein and Flanagan, 2016; Klein and Flanagan, 2017) with hints that cellular immunity to vaccination might differ between the sexes (Flanagan 2015). However this is the first description of sex-differential changes in Treg frequencies following infant vaccination.

Wild-type measles infection is known to cause a prolonged immunosuppression, and while increased IL-10 production has been implicated (Griffin and Ward 1993, Moss, Ryon et al. 2002), FOXP3+ Treg induction may be another mediator of this suppressive effect. Whether the live attenuated vaccine strain similarly causes immunosuppression by inducing Tregs has not been previously investigated.

Analysis for changes in markers of activation, proliferation, and T cell function following vaccination provided results consistent with our previous published study. The MV+DTP females had increased activated and proliferating CD4+ T cells consistent with the enhanced immunity previously shown in this group (Noho-Konteh, Adetifa et al. 2016). Plasma β2m also increased in the MV+DTP vaccinated infants, as did CD8 T cell perforin production. In contrast, there was a decline in the CD8 T cell perforin production in the DTP vaccinated
infants. These data further support the theory that immunity may be enhanced when the two vaccines are given at the same time.

We found that Tregs were significantly higher at 9 and 10 months compared to 19 months of age, indicating a significant decline of Treg frequencies from infancy to the second year of life. Such high Treg numbers during infancy, which have been shown to be highly suppressive in previous studies (Wing, Ekmark et al. 2002), may contribute to the suboptimal responses to infections and vaccinations observed in infants. Only a few previous studies support a functional role for Tregs in vaccine-induced immunity, mainly in animal tumour models where Tregs have been associated with for survival as they block anti-tumour immunity (Ndure and Flanagan 2014). Expression of the chemokine CCL2 by tumours attract Tregs by binding to CCR4, which is expressed in high numbers by functional Tregs (Curiel, Coukos et al. 2004). A recent study using humanized mice shows that blocking Tregs using anti-CCR4 monoclonal antibodies enhances anti-tumour immunity therefore suppressing the growth of the tumours (Chang, Peterson et al. 2016). The administration of anti-CD25 Abs to deplete Tregs enhanced the immunogenicity of BCG and hepatitis B vaccines (Moore, Gallimore et al. 2005), and pre-existing Tregs correlated with subsequent immunity to BCG in mice (Ho, Wei et al. 2010). A human study showed marked increases in CD4+CD25hiFOXP3+ Tregs following vaccination with a dendritic cell-based HIV vaccine, and enhanced T cell immunity in vitro following Treg depletion (Macatangay, Szajnik et al. 2010). Infant BCG vaccination has been shown to induce CD4+CD25+FOXP3+ Tregs (Burl, Adetifa et al. 2010). The induced Tregs failed to negatively correlate with IFN-γ reactivity to PPD in this latter study, suggesting that they do not regulate the BCG vaccine response, although in vitro functional assays were not performed to confirm this.
One of the key objectives of this study was to investigate whether CD4+FOXP3+CD127- Tregs correlate with vaccine-specific antibodies and cellular immunity. The data suggest that circulating Tregs at the time of vaccination may suppress the primary antibody response to measles vaccination, but had no effect on secondary DTP vaccine antibody responses. This novel finding could be due to suppression of helper T cells required for Ab responses, although other mechanisms are feasible. If confirmed in future human studies, Tregs could provide a functional target for enhancing vaccine antibody responses in infancy, the target age group for many vaccines, and in whom responses are notoriously low compared to adults (Levy 2007).

Our results show no evidence that circulating Tregs at the time of vaccination have any effect on subsequent vaccine-specific cellular immunity. This is encouraging since infants have high numbers of circulating CD4+FOXP3+CD127- circulating Tregs, and it would be problematic if they suppressed the induction of vaccine specific cellular immunity.

The inverse correlation between the circulating CD4+FOXP3+CD127- Tregs post-DTP vaccination and the IFN-γ:IL-10 ratio in TT and measles pool cultures at the same time point supports an immunoregulatory role. No such correlations were observed in the MV or MV+DTP groups to support the presence functional Tregs, suggesting that some vaccines, but not others, may induce suppressive Tregs. Indeed, Plebanski et al have found that mice immunized with aluminium adjuvant have a preferential expansion of the highly suppressive CD4+CD25+FOXP3+TNFR2+ Tregs in draining lymph nodes compared to the TNFR2- subset, providing a potential mechanism whereby Tregs may be more functionally suppressive.
following vaccination with the aluminium adjuvanted DTP vaccine (M Plebanski, personal communication). However, the Tregs also declined in the DTP group compared to pre-vaccination, but only in males when the sexes were analysed separately, suggesting that DTP vaccination may increase function but decrease numbers of circulating Tregs. There were no significant inverse correlations when analysing by sex, but this may be due to smaller numbers of infants in each group. The significance of these findings would need to be evaluated further in functional studies.

Nevertheless, there are a number of mechanisms whereby immune responses may differ in males and females (Klein and Flanagan 2016). These include the immunological effects of sex hormones, and differing expression levels of sex-linked immune response genes (Fish 2008) and microRNAs (Pinheiro, Dejager et al. 2011). Multiple immune cells express sex-hormone receptors; and oestrogens and androgens have broadly opposing immunological effects - oestrogens being pro-inflammatory and androgens being immunosuppressive. Even in early life, sex hormone levels are different in males and females, with males experiencing an early testosterone surge (a mini-puberty) in the first 6 months of life, and females having higher oestrogen levels throughout the first year of life (Flanagan 2015). FOXP3 itself is encoded on the X-chromosome (Fish 2008) so it may not be surprising that the sexes differ in the intracellular expression of FOXP3.

We recognise certain limitations in this study. Most importantly, associations do not necessarily equate with cause and effect, and functional assays would need to confirm an immunoregulatory role. Confounding factors such as nutritional status, concomitant infection, genetic and environmental factors were not adjusted for in our analysis. However,
all children were afebrile and clinically well at the time of bleeding, and the majority were of Mandinka ethnicity. HIV infection rates are extremely low in this community, and helminth infection rates are also low. Another limitation is that of identifying true functional Tregs by phenotyping. A number of different phenotypic markers have been used in human studies, with the CD4+FOXP3+CD127- subset being one that is commonly used. We only had one post-vaccination time point and this does not tell us what the dynamics of the Treg response is in males and females. It is possible that the dynamics rather than the quality of the Treg response differ in the sexes.

In summary, the novel findings in this chapter suggest that Treg frequencies can be affected by vaccination, and that the sexes may differ in their susceptibility to this effect. They also hint at immunoregulatory effects of pre-existing CD4+FOXP3+CD127lo Tregs on measles antibody responses to vaccination. To investigate whether other Treg phenotypes correlate with vaccine effector read-outs, the recruitment of a selected cohort of MV vaccinated infants was opted for going forward. This would better allow for the characterization and phenotyping of the Tregs alongside the effector subsets; and to elucidate the mechanisms used by the Treg in influencing the MV vaccine response in 9 month-old Gambian infants.
CHAPTER 4: OPTIMISATION OF REGULATORY T CELL SUPPRESSION ASSAYS IN INFANTS

4.1. INTRODUCTION

Regulatory T cells ensure the maintenance of immune homeostasis by preventing autoimmunity and immune pathology. This state of immune homeostasis is achieved when there is a balance between the regulatory and effector response (Belkaid 2007). Different mechanisms of Treg suppression have been described. A vital mechanism of ensuring peripheral tolerance used by Tregs is the suppression of effector T cell expansion and proliferation (Shevach 2009, Sakaguchi, Miyara et al. 2010). Additionally, the suppression of cytokine responses in humans is another mode of action of Tregs in humans (Allan, Song-Zhao et al. 2008). The role of Tregs can be easily studied in vivo in murine models; whereas in human studies, Treg function has to be tested in-vitro, with co-culture suppression assays often being used as a surrogate measure of their regulatory potential in vivo (Takahashi, Kuniyasu et al. 1998, Thornton and Shevach 1998). These early studies showed that the murine CD4+CD25+ population was anergic in vitro and suppressed the production of IL-2 by CD4+CD25- effector cells. By using transwells to prevent cell-cell contact the authors were able to demonstrate that the suppression was cell-to-cell contact dependent. The assay is employed by many as it is simple and easy to set up, however as described then, it largely led to the conclusion that the mechanism of action used by the Tregs to confer suppression was solely through the production of inhibitory cytokines, although several mechanisms of action of Tregs have been proposed (Vignali, Collison et al. 2008). More recently, Collison and Vignali have published alternative protocols of the in-vitro co-culture assay, and suggest that the protocols should be used based on the experimental question and applicability of the assay. Instead of using the Thymidine incorporation assay as described in the original
protocol, more recent studies have used the 3-(4,5-dimethylthiazol-2-yl)-2,5-
diphenyltetrazolium bromide (MTT) assay and the Carboxyfluorescein succinimidyl ester
(CFSE) proliferative assay as a readout of Treg suppression. With the latter, the number of
cell divisions with or without the addition of Tregs can also be determined by flow cytometry
(Collison and Vignali 2011). The ease and simplicity of the co-culture suppression assay
coupled with development in the techniques employed, makes it ideal to employ when
studying Treg function in the context of many diseases. This is important, as Tregs remain
potential targets for therapeutic interventions in humans given their important role in the
maintenance of peripheral tolerance and autoimmunity.

In murine studies, Tregs can be purified from the spleen in order to provide sufficient
numbers for in-vitro co-culture assays. In humans, tissue is rarely available; therefore
peripheral Tregs are usually isolated from blood. Although earlier studies have shown that
10-15% of murine CD4+ T cells are CD25+ and thus potentially Tregs (Thornton and Shevach
1998); Baecher Allan et al show that in human peripheral blood, only 1-3% of CD4+ T cells
with the highest expression of the CD25 surface marker (CD25hi) exhibit some regulatory
capacity (Baecher-Allan, Brown et al. 2001). Another advantage of conducting Treg studies in
murine models is that the Tregs can be studied extensively in vivo. In humans, such in vivo
studies cannot be conducted hence Treg function is tested by using in vitro co-culture
suppression assays as a measure of their regulatory potential in vivo (Takahashi, Kuniyasu et
al. 1998, Thornton and Shevach 1998). To overcome some of these problems, human Tregs
may be pre-activated or expanded as this will increase Treg numbers available and these
have been shown to be more suppressive as compared to freshly isolated naïve Tregs (Liu,
Sun et al. 2006).
The characteristic marker for human and murine Tregs is the transcription factor Forkhead Box P3 (FOXP3) (Fontenot, Gavin et al. 2003), as CD25 is expressed not only by Tregs but also by activated effector T cells. As FOXP3 is in the nucleus, it is not possible to isolated/depleted Tregs for use in functional assays using this marker, as permeabilisation of the cells causes cell death. There is also evidence that some activated effector T cells express FOXP3 (Wang, Ioan-Facsinay et al. 2007, Kmieciak, Gowda et al. 2009). CD127 plays an important role in the proliferation and differentiation of most mature T cells and is therefore expressed on their surfaces. Conversely, on Tregs, there is low expression of CD127, and its expression inversely correlates with FOXP3 expression (Liu, Putnam et al. 2006). This makes CD127lo/- an additional marker for Tregs, and can be used to further distinguish between activated effector T cells and Tregs in humans.

This chapter describes the optimization experiments for Treg isolation and suppression assays. We describe performed these assays using blood samples from 9 month-old measles vaccinated infants by two methods of Treg isolation:

(i) Isolating CD4+CD25+CD127lo/- Tregs using magnetic beads, setting up a co-culture suppression assay, using the IFN-γ ELISpot assay as a read-out of Treg suppression of cytokine production.

(ii) Depleting the CD25hi Tregs by flow cytometry and evaluating the effect of this depletion on effector T cell proliferation and cytokine responses by flow cytometry.
4.1.3 HYPOTHESES

I hypothesised that addition of isolated Tregs to conventional T cells in culture will suppress the proliferation and pro-inflammatory cytokine production of these cells.

4.1.4 AIMS

- To determine whether adding MACS-isolated CD4+FOXP3+CD127lo Tregs to the PBMCs will suppress IFN-\(\gamma\) production in-vitro using the ELISpot co-culture suppression assay; and use this as a readout of Treg suppression in measles vaccinated infants.

- To determine whether the depletion of the CD25hi subset of CD4+ T cells by FACS will cause an increase in the expression of the proliferative marker Ki67 and production of pro-inflammatory cytokines IFN-\(\gamma\) and IL-2 by the CD4+ T cells.

4.2 METHODS

4.2.1 Cell Isolation and Co-culture Suppression Assay

4.2.1.1 Infant subjects and sample size

To optimise the co-culture suppression assay, we recruited ten 9 month-old healthy Gambian donors who were yet to receive the measles vaccine according to the Gambia EPI schedule. They were recruited using the inclusion and exclusion criteria as shown in Section 2.2.2. In total, 20 blood samples were collected 10 before vaccination and another 10 paired samples
at a second visit, 2 weeks following vaccination. No formal sample size calculation as performed for this section since the aim was to see if I could demonstrate consistent Treg mediated suppression in vitro. The flow chart in Figure 4.1 describes how the samples were handled after each bleed.

### 4.2.1.2 Treg Isolation Using Magnetic Beads

The isolation of the Treg population was done according to the Manufacturer’s instructions using the Miltenyi Biotec CD127-/lo isolation kit (Miltenyi Biotec, Germany), which uses the immunomagnetic separation. Using this kit, the first step included depleting the non-CD4+ (CD8, CD19, CD123) and CD127high cells, thus negatively selecting the CD4+ and CD127lo/- populations. In the next isolation step, the CD4+CD127lo/- cells well stained with CD25 microbeads to positively select for the CD4+CD25+CD127lo/- population. This was our isolated Treg population used for the co-culture ELISpot suppression assay. The steps are described in detail below.

All solutions/buffers used were pre-cooled to minimize cell death; filtered and de-gassed using vacuum filtration. PBMCs were washed with 5mL pre-chilled RPMI containing 10% FCS (Sigma Aldrich, UK) and spun at 1,500 rpm for 10 minutes. The supernatant was poured off and the cell pellet resuspended in 40µL of isolation buffer (per 10⁷ cells). The cells were then incubated in 10µL of the T cell biotin-antibody cocktail II (provided in the kit- cocktail CD8, CD19, CD123 and CD127 antibodies) for 10 minutes at 4°C. Following the incubation, 30µL of the isolation buffer and 20µL of the anti-biotin microbeads were added to the cells, vortexed, and incubated further at 4°C for 15 minutes. The cells were then washed using 2mL R10F,
spun for 10 minutes at 1,500 rpm. The supernatant was poured off, and cells re-suspended in 500µL of the isolation buffer.

The LD columns (MACS columns, Miltenyi Biotec, Germany) were placed in the MACS separator and pre-wet using 2mL of buffer. The cell suspension was poured into the column using a Pasteur pipette. The effluent unlabelled cells (pre-enriched CD4+ fraction) passed through the column and was collected in a pre-labelled FACS tube. The column was washed twice with 2mL of the buffer to collect residual cells in the column.

The effluent (CD4+ fraction) in the buffer was spun at 1,500 rpm for 10 minutes. The supernatant was poured off and the pellet re-suspended in 90µL of buffer. To the re-suspended pellet, 10µL of the CD25 MicroBeads II (anti-CD25 monoclonal antibody) was then added, vortexed and left to incubate at 4°C for 15 minutes. Following the incubation, cells were washed by adding 2 mL of the buffer and spun at 1,500 rpm for 10mins. The supernatant was poured off and pellet re-suspended in 500µL of the buffer.

To positively select for the CD4+CD25+CD127- Tregs, the MS column was used. The columns were prepared by placing in the MACS separator and rinsing with 500µL of buffer. The cell suspension was put into the column using a Pasteur pipette. The cells flowing through contained the unlabelled cells. The column was washed twice with 500µL of buffer. The column was then removed from the separator, 1mL of the buffer was added to the column, and the magnetically labelled cells were flushed through into a FACS tube containing the isolated CD4+CD25+CD127lo/- population.
Figure 4. 1: Flow diagram of how samples were handled for the optimisation of the cell isolation and co-culture suppression assays.
4.2.1.3 Analysis of cell subset purity by flow cytometry

The purity of the isolated Tregs was analysed by flow cytometry using the following fluorochromes: CD127-PE, CD4-PerCP, CD8-PB, CD25 PE-Cy7 and FOXP3 APC. PBMCs were first washed in 2mL FACS buffer at 1800 rpm for 5 minutes. The supernatant was poured off and pellet re-suspended in residual buffer. The staining was done as described in Section 2.12. The surface antibody cocktail (25µL) containing CD127-PE, CD4-PerCP, CD8-PB, CD25 PE-Cy7 was added to the cells, vortexed and incubated at 4°C for 30 minutes. Following the incubation, the cells were permeabilized, and stained with FOXP3 APC as described in Section 2.12. Following which, the cells were washed once in 2 mL FACS buffer, spun and resuspended in 150 µL of FACS fix for acquisition on the CyAn.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Laser (excitation wavelength)</th>
<th>Fluorochrome Channel</th>
<th>Volume added (µL)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Blue (488nm)</td>
<td>FL4</td>
<td>5</td>
<td>BD Biosciences</td>
</tr>
<tr>
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<td>Violet (407nm)</td>
<td>FL6</td>
<td>3</td>
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<td>FL5</td>
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<td>E-Biosciences</td>
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<td>Blue (488nm)</td>
<td>FL2</td>
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<td>BD Biosciences</td>
</tr>
<tr>
<td>FOXP3 APC</td>
<td>Red (633 nm)</td>
<td>FL8</td>
<td>5</td>
<td>E-Biosciences</td>
</tr>
</tbody>
</table>

Table 4. 1: Treg purity flow cytometry panel

The fluorochromes used, the volume added to each sample and the source of the antibodies.
4.2.2 Co-culture *in vitro* suppression ELISPOT assay

The IFN-γ ELISPOT assay was used as a readout of Treg suppression for the co-culture suppression assay. The assay was set up depending on the cell numbers isolated. In samples where the cell numbers were low, single wells were used rather than duplicate wells for all the conditions. All steps were conducted in aseptic conditions in a safety cabinet.

96 well ELISPOT plates (MAIPS4510, Merck Millipore, Germany) were coated with 100µL/well of 10µg/mL anti-human IFN-γ monoclonal antibody diluted in sterile PBS (coating antibody). The coating antibody was added to the plate using a multi-channel pipette (100µL/well) ensuring that membrane was fully covered. The plate was then left to incubate at 4°C overnight (16 hours), after which it was flicked off and blotted on a paper towel. It was then washed 4 times with 200µL / well of sterile PBS using a multi-channel pipette with sterile pipette tips. After the last wash, the PBS was flicked off, the plate was blotted on a paper towel and 200µl/well of sterile RPMI (Sigma) with 10% FCS (Sigma) (R10F) was added. After adding R10F, the plate was then placed in the incubator (37°C 5% CO₂) for at least 1 hour or overnight at 4°C.

4.2.2.1 Adding cells to the ELISPOT plate/ Setting up the Co-culture Assay

After the blocking step as described above, the R10F was flicked off, and the plate was set up as shown in Figure 4.2. A Treg: PBMC ratio of 1:2 was used. We used 50,000 cells from the CD4+CD25+CD127lo/- fraction and 100,000 PBMCs. Each condition was done in duplicate in donors with enough cell numbers. The total volume of each well was made up to 200 µl with RPMI medium containing 10% foetal calf serum, FCS (RPMI medium and FCS from SIGMA
Aldrich, UK). One row on the plate contained PBMC only with the respective test antigens, allowing us to determine whether suppression occurred upon the addition of the CD4+CD25+CD127lo fraction in the row below.

For each donor, the first row on the plate contained PBMCs (100,000 cells in 25µL of R10F) which was topped up to 100µL volume by adding 25µL R10F medium and 50µL of diluted antigen in R10F. The second row contained PBMCs (100,000 cells in 25µL) along with the isolated Treg cells (500,000 cells in 25µL R10F) and respective antigen diluted in 50µL R10F. The antigens were added using the concentrations 10µg/mL for Purified Protein Derivative (PPD) and Staphylococcus Enterotoxin B (SEB) at 1ng/mL. SEB was used as a positive control, and PPD was used to determine whether the response was vaccine-specific or not. Four wells were used for the measles virus incubation; two wells for the replicates of live measles virus which had been grown on a culture of Vero cells for 3 days in R10F medium, and two wells for the ‘Vero Mock’ which are uninfected Vero cells grown in R10F medium for 3 days and is a control well for virus infected well. The ELISpot plate set-up is shown in Figure 4.2. I infected 100,000 cells with the live virus/vero mock at a Multiplicity of Infection (MOI) of 0.1.

4.2.3 Overnight Stimulation

PBMCs were stimulated with PPD, SEB, live measles virus or the Vero mock as described above. A control well in which only R10F medium was added in place of an antigen was used as a negative control for the PPD and SEB stimulated wells. Concentrations used are shown in Table 4.2. PBMCs were incubated with the respective antigens for 18hrs (overnight) at 37°C and 5% CO₂. Cells were then re-suspended in 2mL R10F, centrifuged at 2000 rpm for 5 minutes.
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Dilution</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live MV</td>
<td>1:20</td>
<td>MOI 0.1</td>
</tr>
<tr>
<td>Medium (RPMI)</td>
<td>NA (20 µL added)</td>
<td>NA</td>
</tr>
<tr>
<td>SEB (50µg/mL)</td>
<td>1:10</td>
<td>5µg/mL</td>
</tr>
<tr>
<td>PPD (100µg/mL)</td>
<td>1:10</td>
<td>10 µg/mL</td>
</tr>
</tbody>
</table>

Table 4. 2: The dilution of the antigens used in the overnight stimulation assays

SEB (Staphylococcus enterotoxin B), PPD (purified protein derivative), MOI (multiplicity of infection).

![Figure 4. 2: Example of the plate layout for the IFN-γ ELISpot Suppression Assay](image)

For each donor, the first row on the plate contained the replicates of PBMCs and the respective antigens only (Wells A1-A6 for Donor A; and Wells C1-C6 for Donor B). The second row contained PBMCs along with the isolated Treg cells (ratio of 2:1) and the respective antigens (Wells B1-B6 for Donor A; and Wells D1-D6 for Donor B). Wells A8-D9 contained cells infected with the Live MV with the isolated Treg cells being added to the infected PBMCs.
in replicates (*B8*-9 for Donor A, and *D8*-9 for Donor B). Wells A10-D11 contained PBMCs infected with the control (*Vero Mock*).

Following the spin, the supernatant was discarded, and cells re-suspended in 1mL R10F. A 100µL of the cell suspension was added per well (i.e. estimated to contain 100,000 cells). Cells were added to the plate and antigen added as per the plate map. The cells were thoroughly mixed with the antigen by pipetting up and down 10 times. The plate was then placed in the incubator overnight (18 hours).

Immediately before the end of the incubation, the biotinylated mouse-anti human IFN-γ secondary antibody solution was diluted to 1µg/mL (1:1000 dilution of 1mg/mL stock MabTech clone 7-B6-1) in assay diluent (0.5% FCS in PBS). The cells were flicked off the ELISpot plates into a Virkon trough, and the wells washed six times with 200µL/well of PBS wash buffer using a multichannel pipette. The plates were blotted on a blue paper towel following which 100µl of biotinylated antibody from the reagent reservoir was added to each well of the ELISpot plate. The plate was then covered in foil and left to incubate on the bench at RT for 2 hours. The streptavidin-alkaline phosphatase conjugate was prepared in a 50mL falcon by diluting to 1µg/mL (1:1000 dilution of 1mg/mL stock, MabTech) in assay diluent (0.5% FCS in sterile PBS). At the end of the 2-hour incubation with the biotinylated secondary antibody, the plate was washed 6 times with 200µL/well of PBS using a multi-channel pipette. The Streptavidin-ALP solution was then added using 100µL per well and incubated for 1 hour in the dark (covered in foil) at room temperature.
The BCIP/NBT substrate was left to reach room temperature and filtered using a 0.45µm filter. After the incubation, the streptavidin-ALP solution was flicked off and the plate washed 6 times with 200µL/well of PBS. The BCIP/NBT substrate was then added (100µl/well) and left to incubate for 5 minutes at room temperature in a drawer (in the dark). After the 5 minutes of incubation, the plate was placed under running tap water to stop the reaction. The under-drain from the plate was removed, excess water blotted off the plate using paper towels, and it was left to dry in the drawer (in the dark) overnight. The spots in the wells of the developed ELISpot plates were read using the ELISPOT reader (Autoimmune Diagnostika software version 5.0, Strassburg, Germany).

Responses were scored as being suppressed if the wells in which the isolated Tregs were added to the PBMCs contained an average number of SFU count less than 2 standard deviations from the average SFU count in the wells in which only PBMC was added. This equates to a significant difference between the test wells (isolated Tregs + PBMCs) and the control wells (PBMCs alone) at the 5% level. We also report the percentage inhibition of the IFN-γ response (SFU count) when the isolated Tregs were added to the PBMCs compared to the wells with only PBMCs added.

4.2.4 CD25 depletion by Fluorescence activated cell sorting (FACS)

These assays were performed in the Vaccine and Infectious Diseases Laboratory at Monash University in Melbourne, Australia using cryopreserved PBMC samples. For this experiment, paired samples were assayed. In brief, eight infant PBMC samples were thawed (i.e. 4 paired pre-vaccination and post-vaccination samples). We started with a minimum of 10 million
PBMCs per donor. Additionally, PBMCs from a healthy adult donor (C1) was also thawed as a control. The PBMCs were stained with CD4 APC-Cy7, CD25 PE-Cy7, Live-Dead PO, and CD8 Q605 antibodies. Cells were gated first based on forward and side scatter to exclude cell debris, and a lymphocyte gate was created. Cells in the lymphocyte gate were then further gated to remove duplets and dead cells using the Live-Dead marker, and then gated on CD4 expression. Within the CD4 population, the cells were sorted into three populations based on CD25 expression; CD25lo (C), CD25int (B) and CD25hi (A) (Figure 4.3E). An FMO for CD25 was used to set the gate and ensure there was consistency between the samples assayed. Samples were sorted with CD25 being depleted (C only) or CD25hi fraction being depleted (contained B and C). A tube also contained all of the CD25 fractions (A, B and C). Following the sorting, the samples were stimulated with aCD3/CD28 (concentrations of 2.5μg/mL and 1.25 μg/mL respectively) for 8 hours, acquired on the BD LSR II (Beckton Dickinson, USA) and assessed for Ki67 (proliferative marker) expression using Flowjo (Treestar California). This is described in more detail below.

The PBMCs were rapidly thawed by placing in a 37°C water bath and transferred into 10mL tubes. First 1mL of thawing medium (warm AIM V medium containing 10% human AB serum (Life Technologies, UK) + 50 units/ml Benzonase [2μL of Benzonase stock per 10 mL of medium, Sigma Aldrich]) was added drop wise to the cells. The thawed cells were transferred to a 10mL FACs tube and AIM V medium (Life Technologies, UK) was added drop wise to fill the tube. The cells were then spun at 1,400 rpm for 10 minutes. Following the wash, the supernatant was poured off, AIM V medium added and cells were washed two more times. After the last wash, cells were resuspended in 1mL of the AIM V medium, and a cell count conducted. The cells were then washed in 200μL MACS buffer (PBS containing 0.5% BSA and
2mM EDTA) at 1,400rpm for 5 minutes. The supernatant was discarded and the cells were stained using CD4, CD8, CD25 and Live/Dead Aqua Antibodies (Table 4.3).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Laser (Excitation Wavelength)</th>
<th>Fluorochrome Channel</th>
<th>Dilution</th>
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<td>Live/Dead PO</td>
<td>Violet (405nm)</td>
<td>FL8</td>
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<td>Life Technologies</td>
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</table>

Table 4. 3: Staining Panel for the flow cytometry sorting of Tregs

The antibodies were diluted in MACS buffer. Starting with 10 million cells, 150μL of the Ab mastermix was added to the cells, and they were left to stain in the dark (covered in foil) at RT for 20 minutes. Following the incubation, the cells were washed in 200μL of MACS buffer twice. Following the wash, the supernatant was poured off, and the cells resuspended in 1mL AIM V medium containing 20% human serum (Thermo Scientific). The cells were then filtered into a sterile FACS tube.

Cells were gated on the lymphocyte population, duplets excluded, then gated on the live cells, followed by the CD4+ population. Within the CD4+ population, cells were sorted based on CD25 expression - CD25hi (A), CD25int (B) and CD25lo (C) populations (Figure 4.3). We collected the sorted populations A, B and C till there was no sample remaining.
The PBMCs were rapidly thawed as described in Section 2.9. After the last wash, cells were resuspended in 1mL of the AIM V medium, and a cell count conducted. The cells were then washed in 200μL MACS buffer (PBS containing 0.5% BSA and 2mM EDTA) at 1,400rpm for 5 minutes. The supernatant was discarded and the cells were stained using CD4, CD8, CD25 and Live-Dead antibodies (Table 4.3).

A 96 well plate was pre-coated with 50μL of 2.5μg/mL of purified anti-CD3 (Invitrogen), left to incubate for 1hr at 37°C and 5% CO₂. After the incubation, the plates were flicked off to remove any unbound anti-CD3. Sorted cells were separated into 3 tubes, Tube 1 contained fractions B+C (CD25hi negative); Tube 2 contained fraction C only (CD25 negative); and Tube 3 contained all the fractions (All). The cells in each tube were counted and re-suspended in AIM V, 5% HS, anti-CD28 at 1.25 μg/mL. Cells were then transferred into the appropriate well of the 96 well plate pre-coated with anti-CD3 and the incubated in a humidified incubator at 37°C and 5% CO₂ for 72 hours. Following the TCR stimulation, cells were stained with Ki67 V450 (BD Horizon) and CD3 Q655 (Invitrogen) antibodies as per the cell staining method (Section 2.11), and cells acquired using the BD LSR II (Beckton Dickinson, USA) and analysed using Flowjo Vx (Flowjo, Treestar, California) to determine whether removal of the CD25hi and CD25+ fractions results in increased expression of the proliferative marker Ki67.
Figure 4.3: Gating strategy for the depletion assay

First, the duplets and dead cells were excluded by gating on the single and viable cells. The cells were then gated on the CD4 cells and then sorted based on CD25 expression. (A) illustrates the lymphocyte gate, (B) singlets, (C) live cells, (D) CD4+ and CD8+ lymphocytes, E) illustrates the sorted fractions based on CD25 expression: The A gate was set on the CD25\text{hi} cells; B was set on CD25\text{int} cells and C on the CD25\text{lo/-} cells.
4.3 RESULTS

4.3.1. Low purity of Tregs isolated using the MACS separation technique

Following the isolation, we performed a purity analysis using flow cytometry. Although samples were shown to be enriched for CD4+CD25+CD127lo cells, there still remained some FOXP3- and CD127+ cells (Figure 4.4). For all the participants, there was a median purity of 79.9% (min 53%, max 94%).

4.3.2. Optimisation of live measles virus stimulation IFN-γ ELISpot assay

Co-culture suppression assays were used to determine the suppressive capacity of the MACS-isolated Tregs when added to the PBMC population for each donor. In order to assess whether measles-specific Tregs were induced by measles vaccination, we initially used a measles peptide pool spanning the length of the ‘H’ haemagglutinin antigen, as used in Chapter 3. However, the cells gave minimal IFN-γ ELISpot responses even after vaccination, hence after 2 donors (4 samples, Visits 1 and 2), we decided to use the live measles virus which had previously only been used to infect cells for a maximum period of 2 hours at an MOI of 1, after which high levels of cell death occur. We therefore used an MOI of 0.1 and 0.05 to infect cells for 18 hours (overnight) at 37°C and 5% CO₂. Following the overnight stimulation, and development of the ELISpot plate, the spot count was 87 in 100,000 cells for a post-vaccination sample (shown in Figure 4.5). This can be converted to 870 Spot forming units (SFU) per million PBMCs, which is a robust response. For the mock-stimulated cells (background) from the same donor, we observed a spot count of 90 SFU per million PBMCs.
Figure 4.4: FACS plots showing the phenotype of samples before and after isolation

Samples from the study participants were enriched for Tregs using the CD4+CD25+CD127lo/- Miltenyi Biotec Isolation kit. Frequencies of cells which were CD4+CD25+FOXP3+CD127lo cells before (top panel) and after (bottom panel) isolation using the kit. 
Figure 4.5: Purple spots in the wells of the ELISpot plate following stimulation with the live measles virus

The plate was developed and read using the AID reader. The live virus were added to the cells at an MOI (multiplicity of infection) of 0.1 (B11) and 0.05 (B12) and incubated overnight (18 hours). Well B9 (mock) contained the negative control for the assay-Vero mock cells.

4.3.3 IFN-γ responses before and after measles vaccination following overnight stimulation

Following overnight stimulation, I evaluated the IFN-γ response before and after measles vaccination (Figure 4.6) and compared the response in SFU per million PBMCs with and without the addition of the MACS-isolated Tregs (Figure 4.6). In both PBMC alone and PBMC+Treg wells, I observed a significant increase in the IFN-γ response post-vaccination following stimulation with the live measles virus (p = 0.002), Figure 4.6). I also observed a decrease in the response to SEB stimulation at the post-vaccination visit (Visit 2) compared to baseline (Visit 1), which was only significant for PBMC+Tregs (p=0.049). There was no significant change following measles vaccination in the PPD-stimulated wells. We did not observe any significant difference when we compared the IFN-γ response (in SFU per million

Mock

<table>
<thead>
<tr>
<th>MOI</th>
<th>Well</th>
<th>SEB SFU</th>
<th>IFN-γ SFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>22</td>
<td></td>
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</tr>
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</table>
PBMCs) with and without the addition of the MACS-isolated Tregs (Figures 4.6 and 4.7) in any of the stimulated cultures.

**Figure 4. 6: Co-culture suppression IFN-γ ELISpot responses**

IFN-γ ELISpot responses when cells are stimulated overnight and with (PBMC+Treg; P+T) and without (PBMC; P) the addition of the MACS-isolated Tregs. Cells were incubated overnight with either PPD, live measles virus or SEB. Comparisons were made at baseline (Visit 1) and post-vaccination (Visit 2). Each dot represents a response from an individual participant and shows the IFN-γ response following background (response from the medium-stimulated wells) subtraction. The median is shown and the whiskers show the interquartile ranges (IQR). The
y-axis shows the response in SFU per million PBMCs. Paired data for n = 10 infants are shown. Data were analysed using a two-tailed Wilcoxon matched-pairs signed rank test. Bonferroni correction was used to correct for multiple testing; \( \alpha \leq 0.004 \) was considered significant after correcting for 12 parameters.

Figure 4. 7: IFN-\( \gamma \) ELISpot responses when PBMCs are co-cultured with MACS-isolated Tregs

IFN-\( \gamma \) ELISpot responses when cells are stimulated overnight with and without the addition of the isolated Tregs. Cells were incubated overnight with either PPD, live measles virus or SEB. Comparisons were made between wells containing PBMCs alone and wells containing PBMCs with the Tregs added from the same individual. Each dot represents data from an individual and shows the IFN-\( \gamma \) response following background (response from the medium-stimulated wells) subtraction. This is shown for (A) baseline (Visit 1) and (B) postvaccination (Visit 2). The y-axis shows the IFN-\( \gamma \) response in SFU per million PBMCs. Paired data for n = 10 infants are shown. Data were analysed using a two-tailed Wilcoxon matched-pairs signed rank test.
I also analysed the percentage of inhibition of the IFN-γ response (in SFU per million PBMCs) when MACS-isolated Tregs are added to the PBMC fraction compared to PBMCs alone, to compare the response before (Visit 1) and after vaccination (Visit 2). We saw a trend in increase in inhibition in the SEB-stimulated cells post-vaccination at Visit 2, this was however not significant (Figure 4.8).

![Graph](image)

**Figure 4.8**: Inhibition of IFN-γ ELISpot responses when PBMCs are co-cultured with MACS-isolated Tregs

*Percentage inhibition of IFN-γ ELISpot responses in SFU per million PBMCs when Treg cells are added to PBMCs compared to PBMCs alone. Cells were incubated overnight with either PPD, live measles virus or SEB. Each dot represents an individual and shows the % inhibition of the IFN-γ response to that antigen. The line shows the median for the different stimulations at both visits. The y-axis shows the % inhibition of IFN-γ response. Paired data for n = 10 infants are shown.*

*Data were analysed using a two-tailed Wilcoxon matched-pairs signed rank test.*
4.3.4. Variability in the suppressive capacity of the Tregs in the co-culture suppression assay

I went on to explore the proportion of infants that had evidence of suppressive Tregs according to our ELISpot co-culture suppression assays. Based on responses from the mock control, we scored responses as being suppressed if the wells in which the isolated Tregs were added to the PBMCs contained an average number of SFU count less than 2 standard deviations from the average SFU count in the wells in which only PBMC was added. This is assuming a significant difference between the Test wells (isolated Tregs + PBMCs) and the control wells (PBMCs alone) at the 5% level.

In the 20 samples assayed, suppression of the IFN-γ response was observed in 6 samples stimulated with SEB (30% of samples; 3 at visit 1 and 3 at visit 2); 7 samples stimulated with PPD (35% of samples; 3 at visit 1 and 4 at visit 2) and only 2 out of the 16 samples stimulated with the live measles virus (12.5% of the samples, all at visit 2). Figure 4.9 shows the proportion of samples where suppression occurred following stimulation with the respective antigens at Visits 1 and 2. In the SEB stimulated wells, the proportion of samples where suppression occurred was comparable at both visits. In the samples where we observed suppression in one visit, this effect was not sustained in the next visit therefore making the sensitivity of this assay questionable. In addition, in some wells where the isolated Tregs were added to the PBMCs, we observed higher IFN-γ responses (Figure 4.2, Sample A).
Figure 4. 9: Proportion of infants exhibiting Treg-mediated suppression of the IFN-γ response

*Proportions of samples in which suppression occurred (red pie) and where no suppression occurred (blue pie) following stimulation with (A) SEB, (B) PPD and (C) live measles virus in 10 donors, before (V1) and after (V2) vaccination.*
Using percentage inhibition as the read-out, we compared the individual responses to evaluate whether inhibition was sustained at both study visits (Figure 4.10). We observed that this varied, as there was inhibition of the IFN-γ response for some infants at both visits, whilst for some inhibition was not sustained (Figure 4.10).

**Figure 4. 10: Inhibition of IFN-γ ELISpot responses before and after measles vaccination**

*Percentage inhibition of IFN-γ ELISpot responses in SFU per million PBMCS when Treg cells are added to PBMCs compared to PBMCs alone, at both study visits. Cells were incubated overnight with PPD, live measles virus or SEB. The paired individual data is shown. Each dot represents an individual and shows the % inhibition of the IFN-γ response to that antigen. The y-axis shows the % inhibition of IFN-γ response. Paired data for n = 10 infants are shown. Data were analysed using a two-tailed Wilcoxon matched-pairs signed rank test.*
To investigate whether the Treg purity influenced the variability in the suppression observed, we next compared the purities of the isolated Tregs in the samples in which suppression was observed with at least one of the stimulations and the samples in which no suppression was observed following all 3 stimulations. We found that the samples in which we observed suppression of IFN-γ response upon the addition of the isolated Tregs, these had a higher median purity of the isolated fraction than those in which no suppression was observed (Figure 4.11). However, this difference was not statistically significant.

Figure 4.11: Median CD4+ Treg purities in the suppression assays

Comparing the assays where suppression was observed to the assays that did not suppress. The y-axis shows the percentage purity comparing to CD4+CD25+FOXP3+CD127lo phenotype. NO (blue dots) = samples in which suppression was not observed; YES (red squares) = samples in which suppression was observed; ALL (green triangles) = all the samples combined. The median values are shown by the horizontal bar. P values were calculated using a two-tailed
Kruskal-Wallis test but there was no significant difference between suppressed and non-suppressed assays.

4.3.5 Depletion of CD25hi cells by FACS

Due to the marked variability and lack of consistency in suppression observed using MACS bead isolated Tregs, we decided to try other approaches to assess Treg function. This was done while working in the lab in Melbourne which has a cell sorter facility which was not available in The Gambia. First we decided to deplete the CD25hi cells and investigate the effect on T cell proliferation by Ki67 expression, hypothesizing that depletion of Tregs would lead to enhanced proliferation.

Using the gating strategy in Figure 4.12, we analysed by flow cytometry for Ki67 expression by T cells when the CD25hi population was depleted, or the whole CD25+ population was depleted or without any depletion (Figure 4.13). We did not observe any obvious change in proliferation when the CD25+ or CD25hi fractions were removed (Figure 4.14).
Figure 4. 12: The Gating Strategy used to gate on the CD3+CD4+ cells for the depletion assay

Cells were first gated on forward and side scatter to exclude dead cells and cell debris, and a (A) lymphocyte gate was created. Cells were then gated on (B) singlets and (C) live cell. Gating was then done on (D) CD3+CD4+ cells.
Figure 4.13: Flow plots showing Ki67+ expression following the depletion assay

Within the CD3+CD4+ population, we gated on Ki67 expression and gated on the CD25+Ki67+ cells. We compared the proliferating cells in the 3 different samples (A) CD25hi depleted, (B) CD25 depleted (C) all fractions (not depleted).
Figure 4.14: Expression of the proliferation marker Ki67 by the CD4+ T cells

(A) Expression of Ki67 in samples with all the fractions, i.e. undepleted PBMC (All = control), CD25 depleted samples (-CD25) and CD25hi depleted samples (-CD25hi) at baseline (Visit 1) and 2 weeks after (Visit 2) administration of the measles vaccine. (B) Paired data for n = 4 infants are shown. The y-axis shows the percentage of CD4+Ki67+ T cells. Each dot represents an individual participant. Paired data were analysed using a two-tailed Wilcoxon matched-pairs signed rank test.

We further assessed for the functionality of the responses by evaluating the proportions of T cells producing IL-2, IFN-γ, or both cytokines with and without the depletion of the CD25hi cells using the ICS assay as described in Section 2.11. We saw slightly higher IFN-γ responses in the CD25hi-depleted cells at both time points (Figure 4.16), however the proportion of cells producing multiple cytokines did not differ when we compared the CD25hi-depleted samples to the control (not depleted) (Figures 4.15).
Figure 4.15: Proportions of CD4+ cytokine producing cells before and after measles vaccination

Proportions of CD4+ cytokine producing cells in all participants (A) before and (B) after MV in non-depleted (All-control), CD25 depleted (-CD25) and CD25hi depleted (-CD25hi) samples. The red slice shows the proportion of cells producing IL-2 alone, green slice shows the proportion of cells producing both IL-2 and IFN-γ, the blue slice is the proportion of cells producing IFN-γ alone. Data represents 4 study participants at both visits.
Figure 4. 16: Absolute frequencies of CD4+ cytokine producing cells before and after measles vaccination

The graph shows the absolute frequencies of cytokine producing CD4+ cells in all participants (A) before and (B) after MV in non-depleted (All-control), CD25 depleted (-CD25) and CD25hi depleted (-CD25hi) samples. The lines show the median values. Data represents 4 study participants at both visits.
4.3.6 Insufficient cell numbers to conduct co-culture suppression assay using CD25hi Tregs

To investigate the suppression of proliferation of the CD25- effector population, we aimed to sort CD25hi Tregs by flow cytometry and add back into the effector cell cultures and measure effects of added Tregs on effector T cell lymphoproliferation by thymidine incorporation. We added the CD25hi cells (Treg population) to the CD25- (effector population) at a reasonably high ratio of 1:2 in order to observe robust suppression. However, sorting on CD25hi cells provided very few Tregs with which to conduct suppression assays, both for the adult sample, but more so for the infants. After the sorting, compared to thawed PBMCs from the healthy adult control, the infant donors had a very small number of CD25hi cells. For the adult donor, in 10,000 events, there were 46 cells in the CD25hi gate, as compared to only 7 cells in the CD25hi gate after acquiring the same number of events (Figure 4.17). As a result, we were unable to conduct the \textit{in-vitro} co-culture suppression assay using the FACS sorted CD25hi cells and investigate suppression of proliferation by using the Thymidine incorporation assay as initially planned. We therefore opted for an alternative approach to assess for Treg functionality, which will be discussed in the next chapter.
A. Infant Sample

![Cytometer](image)

B. Adult Control

![Cytometer](image)

**Figure 4. 17: Recovery of CD25hi cells following the FACS sorting**

*Comparison between (A) an infant sample and (B) the healthy adult control. We got a greater proportion of CD25hi cells in the adult control as compared to the infant sample.*
4.4 DISCUSSION

Treg suppression assays were first developed in the murine model where large cell numbers from tissue can be obtained and samples from multiple mice combined. The application of these assays to human peripheral blood samples remains challenging; particularly in infants where the blood volumes available are limited and Treg yields are very low. The assay was originally thought to work on the concept that the Tregs being added to the culture are anergic and would not contribute to proliferation or cytokine production, with early studies finding them to be hypo-responsive \textit{in-vitro} (Taams, Smith et al. 2001). However, in one study 22% of peripheral blood CD4+CD25+CD127loFOXP3+ Tregs were found to be Ki67+ and therefore proliferating (Vukmanovic-Stejic, Agius et al. 2008), and it is no longer thought that they are anergic. Indeed, they have been shown to expand \textit{in vivo} in the presence of IL-2 produced by surrounding effector T cells (Walker, Chodos et al. 2003) and respond to cytokines in the environment (reviewed in (Walker 2004). Some Tregs have even been shown to produce IFN-\gamma under Th1 polarising conditions (Wei, Wei et al. 2009, Dominguez-Villar, Baecher-Allan et al. 2011). This may partially explain why in some of our assays, we observed higher cytokine responses when the Treg fraction was added rather than suppression, although lack of a pure Treg population would have compounded this problem.

Another issue with this approach is that human Treg studies are generally limited to peripheral blood samples, but the Treg population in tissues may have different suppressive capacity. The circulating Tregs may not provide a surrogate marker of Treg activity during a disease process, and may decline in peripheral blood because they have localized to a site of inflammation. For example functional Treg activity markers such as IDO, TGF-\beta and CD80 in untreated HIV patients were shown to be higher in the tonsillar tissues than in the periphery.
(Andersson, Boasso et al. 2005). This suggests that there is not an equal distribution of Tregs in different compartments. Recent data also suggests that Tregs in the secondary lymphoid organs in humans may have even greater proliferative capacity than that observed in peripheral blood Tregs (Peters, Koenen et al. 2013). It is because of these issues that simply measuring circulating Tregs without any assessment of function could provide misleading results. It is for this reason that we wanted to assess Treg function.

Using the MACS sorting technique, I sorted the CD4+CD25+CD127lo/- cells and co-cultured these with PBMCs. The purity of the sorted fraction was, however questionable as it was below 90% and possibly contaminated with other cell types. Additionally, we were unable to use the CD25- fraction as the effector population as it had antibodies bound. Due to the few cell numbers, we had insufficient blood to obtain APCs and without APCs, effectors would likely not respond following stimulation. We therefore used whole peripheral blood mononuclear cells (PBMCs) as our effector population. The PBMC fraction is composed of several cell types such as monocytes, dendritic cells and lymphocyte populations. The lymphocytes can be further divided into T and B cells and NK cells. Although the T helper cells are major cytokine producers, NK cells have been shown to be the most prolific cytokine producers, and gamma-delta (γδ) T cells are also known to produce cytokines such as IFN-γ when activated (Wu, Wu et al. 2014). It is possible that these cells also contributed to cytokine production further underestimating the suppressive capacity of the Tregs. To overcome this problem with lack of T cell specificity, peptides of varying lengths can be appropriately selected to optimally stimulate antigen-specific CD4 and CD8 T cells in an ELISpot assay. In general, peptide lengths of between 8-9 amino acids induce MHC class I restricted T cell responses whilst for MHC class II stimulation peptide lengths of between 12-
15 amino acids are required. Other studies show that shorter peptides can be used to stimulate CD4 T cells but at much higher concentrations (Hammer, 2000). Interestingly, a supra-abundant peptide, MV-C.084.09 peptide (KLWESPQEI), which spans amino acids 84–92 from the non-structural C protein, binds to HLA-A*02:01 has been shown to induce strong CD8 T cell responses in measles infected individuals, and results in the clearance of infected host cells in HLA-A2 positive Gambian children with (Jaye et al., 2003). Although not used in our study, this is a promising approach to stimulate optimal CD8+ T cell responses.

Another limitation of our assay was that the isolation process was time-consuming and as the infant cells are not very robust, there is a possibility that some of the cells may have died during the process. Using the ELISpot assay as a read-out, cell-death may be interpreted as suppression as it was not possible to exclude dead cells. The MACS technique also introduced an additional limitation of the presence of microbeads on the surface of the cells. This has been shown to influence the activation/suppression of the cells (reviewed in (McMurchy and Levings 2012)).

Despite all these limitations we were able to demonstrate some functional suppression by infant Tregs in some of the donors tested. Importantly, while there was no suppression of measles-specific responses pre-vaccination, 25% of infants had suppression post measles vaccination demonstrating for the first time the presence of Tregs in humans capable of suppressing a measles-specific response following vaccination. However, the antigen specificity of the response would require more detailed investigation.
In these add-back experiments, the Tregs were used at a ratio of 1:2 Treg:PBMC, which is artificially high compared to the in-vivo situation where <5% of the lymphocytes are Tregs (reviewed in (McMurchy and Levings 2012). However, as mentioned above, Tregs become enriched at sites of inflammation so may reach much higher ratios in inflamed tissue than in peripheral blood. Vignali et al nicely illustrated some of the modes of actions of Tregs, both the techniques employed in the suppression assays do not take into consideration other modes of Treg action which have been described such as mopping up the available IL-2 leading to IL-2 deprivation and eventual effector T cell death, and the production of immunosuppressive cytokines such as IL-10, TGF-β and IL-35 (reviewed in(Vignali 2008). However, we only assessed for suppression of IFN-γ responses and effector T cell proliferation; but the Tregs may also suppress other modalities that we did not assess for.

We went on to use flow-cytometry to sort the Tregs using the cell sorting facility in Melbourne in an attempt to obtain more pure Tregs for the co-culture suppression assays. This method allowed for the evaluation of live effector T cell proliferation, greater purity, and to determine the change in proliferation (using Ki67) and effector cytokine response (by ICS) simultaneously. There was a trend of an increase in proliferation when the CD25hi and CD25+ cells were depleted which was promising, however, with the small cell numbers, we could not perform the co-culture suppression assay from the depleted fractions as planned. A good marker for human Tregs is the master regulator of Treg development and function FOXP3 (Fontenot, Gavin et al. 2003, Hori, Nomura et al. 2003). However, Tregs cannot be isolated/depleted using this marker as it is found in the nucleus and not on the surface of the cells, and cells have to be permeabilized and thus killed to stain for it. Moreover, some activated effector T cells also express FOXP3. We therefore chose to sort on CD25hi T cells
which characterise human Tregs and constitute 1-3% of all CD4+ T cells (Baecher-Allan, Brown et al. 2001). Unfortunately the yield of Tregs was prohibitively low and this approach had to be abandoned. For effective suppression, Tregs would need to be added to the effector T cells at high ratios, and with the cell numbers obtained, this was not feasible. Although high Treg:effector ratios work in-vitro; it does not mimic what occurs in vivo as this ratio is unlikely to occur naturally in the host (reviewed in (McMurchy and Levings 2012).

Taking into consideration all the technical and practical caveats described above to assess Treg function in humans, more so in young children, we opted to further evaluate Treg function by analysing for associations between Tregs and effector responses as will be described in the next chapter.
CHAPTER 5: INVERSE CORRELATION BETWEEN PRE-EXISTING CD4+FOXP3+CD25HI TREGS AND MEASLES-SPECIFIC EFFECTOR RESPONSES POST-VACCINATION

5.1. INTRODUCTION

Considering their major role in human immunity, and their potential as therapeutic targets, identifying functional Treg phenotypes with regards to vaccination would be of considerable value. In the previous chapter, the technical and practical caveats of using in vitro suppression assays to evaluate the functionality of Tregs in humans, especially in young children, was highlighted. We therefore opted to evaluate Treg function by using the association between the Tregs and effector responses as a surrogate indicator of suppressive function.

A number of cell surface markers have been used to define Tregs including the IL-2 receptor alpha chain (CD25), CD39 (Borsellino, Kleinevietfeld et al. 2007), cytotoxic T lymphocyte antigen 4 (CTLA4) (Read, Malmstrom et al. 2000), and glucocorticoid inducing tumour necrosis factor receptor (GITR) (McHugh, Whitters et al. 2002). However, none of these markers uniquely identify Tregs but are also expressed on conventional T cells. CD127 expression has been shown to inversely correlate not only with expression of FOXP3, but also with the functional capacity of Tregs (Liu, Putnam et al. 2006). Other studies of human Tregs have identified the CD25hi Tregs as the more functionally suppressive subset (Baecher-Allan, Brown et al. 2001), including studies using cord blood samples (Mayer, Bannert et al. 2012), and to date these remain the most widely studied Treg phenotype in humans. Studies have shown that in addition to cytokine receptors such as the receptor for IL-2, CD25, serving as a
phenotypic marker of Tregs, these receptors have been shown to have a role in the function of Tregs. Similar to CD25, TNFR2 has been shown to be another important cytokine receptor that is preferentially expressed by CD4+FOXP3+ Tregs (Chen and Oppenheim 2010). Chen and colleagues made a seminal discovery in mice that the CD4+FOXP3+TNFR2+ Tregs were maximally suppressive (Chen, Baumel et al. 2007). They show that TNFR2 plays a vital role in not only the activation but also the expansion and proliferation of the CD4+FOXP3+ functional Tregs. More recent human studies have also shown that the CD4+CD25hiFOXP3+ Tregs expressing (Chen and Oppenheim 2011) tumour necrosis factor receptor 2 (TNFR2) are more functionally suppressive than the TNFR2- fraction (Minigo, Woodberry et al. 2009), yet these have never been studied in human infants. Furthermore, the pro-inflammatory cytokine TNF has been shown to induce Tregs via its receptor TNFR2, and this is vital in maintaining FOXP3 expression and the functionality of Tregs (Chen, Baumel et al. 2007). Whether TNFR2 defines functional Tregs in the context of infant vaccination remains unknown.

Vaccines or vaccine adjuvants which induce TNF have also been shown to induce TNFR2+ Tregs (Wilson, Xiang et al. 2015); hence we were particularly interested to see if functional TNFR2+ Tregs are induced by infant vaccination. In this chapter we therefore evaluate the correlation between circulating CD4+CD25hiFOXP3+ Tregs at the time of infant measles vaccination with measles-specific CD4+ and CD8+ T cell effector responses and measles IgG levels following measles vaccination. The aim was to determine whether the numbers of Tregs changed following vaccination, and whether they inversely correlated with measles-
specific effector responses. We further investigated whether TNFR2 expression by these Tregs is enhanced by vaccination, and whether the TNFR2+ Treg population in particular inversely correlates with the effector readouts.

5.2 HYPOTHESES

I hypothesised that:

- Circulating Tregs at the time of measles vaccination would inversely correlate with post-vaccination measles-specific cellular and humoral responses.
- Measles vaccination would cause a sex-differential upregulation of circulating Tregs, including TNFR2+ Tregs.
- Post-vaccination Tregs would inversely correlate with measles-specific pro-inflammatory cytokine responses.

5.3 AIMS

- To determine the Treg frequencies using classic Treg markers before and after measles vaccination
- To measure measles-specific antibody and cytokine responses before and after measles vaccination
- To investigate the correlation between pre-existing and post-vaccination Tregs and the measles antibody response.
- To investigate the correlation between pre-existing and post-vaccination Tregs and the measles-specific CD4 and CD8 T cell cytokine responses
5.4 METHODS

5.4.1 Study design

Infants visiting the Sukuta Health Centre (SHC) for their routine 9-month vaccinations were recruited into the study. After obtaining signed or thumb printed informed consent, a baseline 5 mL blood sample was collected into a heparin tube immediately before the administration of a standard intramuscular dose of the Edmonston-Zagreb (EZ) strain of the measles vaccine (Serum Institute of India, Pune) into the left deltoid. A second 5mL blood sample was collected 2 weeks later (Visit 2). The follow-up vaccines yellow fever (YF) and oral polio vaccine (OPV) were also administered at the follow-up visit after the blood was taken. The full details on the study design, how infants were recruited and mothers consented are described in Section 2.4.2.

5.4.2 Blood culture conditions

200μL of whole blood was cultured overnight with either the vaccine-specific live measles virus (1:20 dilution of available stock), the unrelated antigen purified protein derivative (PPD, 10g/mL); staphylococcus enterotoxin B (SEB, 5g/mL) as a positive control, and RPMI medium alone as the negative control as described in Section 2.11. After a 2-hour incubation at 37°C, brefeldin A (BFA) was added, and the plate was incubated overnight (37°C for 16 hours). Cells were then washed and prepared for flow cytometric analysis.

5.4.3 Regulatory T cell phenotyping and intracellular cytokine staining by flow cytometry

The stimulated cells were stained according to the procedures in Section 2.12. The surface fluorochrome markers used were CD4 PerCP, CD8 PB, CD25 APC-Cy7, TNFR2 FITC and the viability marker live/dead aqua. The intracellular antibodies used were FOXP3 PE, IFN-γ PE-
CY7 and IL-2 APC. Following the final incubation, the cells were fixed and the samples analysed using the CyAn ADP flow cytometer.

The samples were analysed using the Flowjo Software (Treestar, California). To gate on the Tregs, gating was firstly done on the total lymphocytes, and then on the singlets and live cells. We then gated on the CD3+CD4+ and the CD3+CD8+ subsets within the viable singlets. Within these subsets, gating was done on the IFN-γ and/or IL-2 producing cells to determine the frequency of the CD4/8 cytokine-producing cells. Tregs were characterized as FOXP3+ and CD25hi, and then further differentiated into TNFR2 positive and negative. We also analysed for the median fluorescence intensity (MFI) of TNFR2 within the CD25hi subset to determine whether its expression is enhanced following vaccination (the gating strategy is illustrated in Figure. 5.1).
Acquired cells were first gated on (A) lymphocytes, then (B) singlets and then dead cells excluded using the (C) live-dead marker. Live lymphocytes were then gated on (D) CD4 and CD8 T cells. The CD4 regulatory T cell subsets analysed in this study were the (E) CD4+FOXP3+TNFR2+ and (F) CD4+CD25hiFOXP3+ Tregs, and the MFI of TNFR2 within this population. (F). IL-2 and IFN-γ cytokine production by (G) CD4 and (H) CD8 T cells was analysed by ICS using Boolean gating.
5.4.4 Measles IgG ELISA

The anti-measles virus human IgG *in vitro* ELISA kit (Abcam, UK, ab108750) was used to measure the measles IgG levels in the plasma samples. The reagents and plasma samples were left to equilibrate at room temperature. The plasma samples were diluted 1:100 using the IgG sample diluent provided in the kit. To dilute the sample, 10 μL was added to 1mL of the diluent in a tube and vortexed gently. The kit included a plate with the 96 well strips supplied. There were 3 standards (Measles virus IgG Positive, Measles virus IgG Negative and Measles virus IgG Cut-off), and a blank substrate control well was also included. The assays were done in replicates of two, 100μL of the standards were added onto the plates. For the samples, 100μL was added in single wells. The plate was covered in foil and incubated for 1 hour at 37°C in the water bath. Following the incubation, the contents of the wells were aspirated and each well of the plate was washed 5 times using 350μL of the 1x washing solution. This was done gently to avoid a spill over into neighbouring wells. The solution was decanted after the last wash and excess fluid was removed by blotting on a clean paper towel.

After blotting, 100μL of the measles virus anti-IgG horseradish peroxidase (HRP) conjugate was added into all the wells except for the blank substrate well. The plate was then covered with foil and left to stand on the bench for 30 minutes (room temperature). After this incubation, the plate was washed again five times using 350 μL of the 1x washing solution. The plate was blotted using a paper towel after which 100 μL of the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution was added into all wells. The plate was covered in foil and the substrate solution was left to incubate for 15 minutes at room temperature. After the incubation step, 100 μL of Stop Solution was added into all the wells.
When the Stop Solution was added, the blue colour seen in the well turned yellow, and highly positive samples appeared darker as they caused precipitates of the chromogen to be darker. The plate was read within 30 minutes using the ELISA reader (MultiSkan EX, Thermo Scientific) and Multiskan Ascent software (Ascent V2.6) set at a wavelength of 450 nm. The absorbance values of the controls were within the acceptable ranges (Substrate blank: Absorbance value < 0.100; Negative control: Absorbance value <0.200 and < cut-off; Cut-off control: Absorbance value 0.150 – 1.300; Positive control: Absorbance value > cut-off). The cut-off control value is the mean absorbance value of the cut-off control wells. The mean background subtracted absorbance was calculated for each sample and compared to mean cut-off control value. The absorbance value is the reading provided by the ELISA reader for each sample. The cut-off is a control standard provided in the kit which is assayed in duplicates. The average of absorbance value of the replicate wells containing the cut-off standard is calculated to obtain the mean absorbance value for the cut-off control,

5.4.5 Statistical Analysis

Antigen stimulated cytokine responses had background (negative control) subtracted to determine the net response. Differences between the two time points (V1 and V2) were calculated using a paired Wilcoxon signed rank test. Correlations were analysed using Spearman’s non-parametric correlation coefficients. Flowjo software (Treestar, California) was used for analysis of flow cytometric data. For the CD4 and CD8 cytokine responses, we used SPICE (Version 5, NIH) for the visual representation of the data showing the proportions of cells producing IL-2 and/or IFN-γ. Analysis was done using GraphPad Prism Version (GraphPad Software Inc, San Diego, USA). A p-value of less than 0.05 was considered as significant and an r-value of 0.4 was considered as a strong correlation (Cohen, 1988).
Bonferroni method was used at a 5% significance level to correct for multiple testing depending on the number of parameters assessed. The Genstat (18th Edition) statistical software was used to produce the correlograms, which provide a qualitative representation of the associations between the Tregs and the cytokine responses.

5.5 RESULTS

5.5.1 Participant Characteristics

Fifty participants were recruited, bled, and had measles vaccine (MV) administered, and 47 participants returned for bleeding for the follow-up visit 2 weeks later. Anthropometry data were collected for all the participants at both time points to get an overview of the clinical characteristics of the infants before and after vaccination.

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<td>14.72 (14-16)</td>
</tr>
</tbody>
</table>

Table 5.1: Characteristics and anthropometry results

Data are shown for both visits - before the vaccine was administered and 2 weeks later at the follow-up visit. Bpm- beats per minute, MUAC- mean upper arm circumference.
5.5.2 Measles vaccination did not induce TNFR2+ Tregs in 9 month-old infants

To investigate whether measles vaccination led to an upregulation of potentially functional Tregs as shown in females in the study in Chapter 3, we measured frequencies of the CD4+CD25hiFOXP3+ and CD4+FOXP3+TNFR2+ cells in the whole blood samples incubated in medium alone (Figure 5.2 A & B), and also evaluated whether there was a sex difference in Treg levels (Figure 5.2 C & D). Based on previous data from Chapter 3, I hypothesised that measles vaccination would result in an upregulation of Tregs in females 2 weeks after vaccination but were unable to confirm the same effect in this cohort (Figure 5.2 C & D). We saw a significant decrease in the CD4+CD25hiFOXP3+ cell frequencies at visit 2 compared to visit 1 in all participants (p = 0.01) and in males analysed by sex. When corrected for multiple testing, this decrease was not significant. The CD4+FOXP3+TNFR2+ cell frequencies did not change following MV. We also measured the overall median fluorescence intensity (MFI) of TNFR2 within the CD4+CD25hiFOXP3+ population to investigate whether measles vaccination increases the TNFR2+ subpopulation, but did not observe any difference to pre-vaccination levels in our cohort (5.2 E).
Figure 5. 2: Frequencies of Tregs before and 2 weeks after measles vaccination

(A) CD4+CD25hiFOXP3+ and (B) CD4+FOXP3+TNFR2+ Treg frequencies were measured after overnight incubation in medium. (C) CD4+CD25hiFOXP3+ and (D) CD4+FOXP3+TNFR2+ Treg frequencies in males and females measured after overnight incubation in medium. (E) The median fluorescence intensity (MFI) of TNFR2 within the CD4+CD25hiFOXP3+ population and
(F) The MFI of TNFR2 within the Tregs analysed by sex. Frequencies are expressed as a % of the total CD4+ T cell population. Each dot represents the frequency of the Tregs from a single individual with the lines connecting paired sample points. P values were calculated using a two-tailed Wilcoxon Test. A p value of ≤0.05 was considered significant. * represents a p value of ≤0.05, ** represents a p value of ≤0.01. V1 = Visit 1 (before vaccination); V2 = Visit 2 (2 weeks after vaccination). Bonferroni correction was used to correct for multiple testing; α ≤ 0.008 was considered significant after correcting for 6 comparisons.; α ≤ 0.004 was considered significant after correcting for multiple testing for the sex-differential analyses.

5.5.3 Decrease in the frequency of IFN-γ producing measles-stimulated CD4+ T cells in following MV

We observed a significant increase in the CD4+IFN-γ+ T cells from whole blood incubated in medium following measles vaccination (p<0.001) (Figure 5.3 A); whilst in the measles stimulated cultures, there was a significant decline (Figure 5.4 A). When corrected for multiple testing, the latter did not remain significant. There was no such change in SEB cultured IFN-γ or IL-2 production by CD4+ or CD8 +T cells (Figure 5.3 B). There was also no change in the ICS responses for CD4 or CD8 T cells in PPD cultures following vaccination (Figure 5.4 B).
Figure 5. 3: Intracellular cytokines in medium and SEB stimulated whole blood cultures

(A) Intracellular cytokine staining (ICS) responses for whole blood incubated with medium for CD4 and CD8 T cells. (B) ICS responses following overnight culture with SEB for CD4 and CD8 T cells. Data shown for n=50 at v1 and n=47 at v2. Each dot represents frequency of cytokine from a single individual with the lines connecting paired sample points. P values were calculated using a two-tailed Wilcoxon Test. A p value of ≤0.05 was considered significant. *** represents a p value of ≤0.001. Bonferroni correction was used to correct for multiple testing; α ≤ 0.0063 was considered significant after correcting for 8 comparisons.
Figure 5.4; ICS cytokines in live measles virus and PPD cultured cells

Plot shows CD4+ and CD8+ T cell IL-2 and IFN-γ responses following whole blood stimulation with (A) live measles virus and (B) PPD. Paired data are shown for 37 participants (measles virus cultured cells), and 36 participants (PPD cultured cells) before and after measles vaccination. Each dot represents frequency of cytokine from a single individual with the lines connecting paired sample points. P values were calculated using a two-tailed Wilcoxon matched pairs signed rank test. * = p <0.05. Bonferroni correction was used to correct for multiple testing; α ≤ 0.0063 was considered significant after correcting for 8 comparisons.

5.5.4 Increased proportion of IL-2 producing CD4 and CD8 T cells following measles vaccination

We used SPICE software to further analyse the quality of the ICS cytokine responses before and after vaccination, by investigating the proportions of single and double positive cytokine producing T cells. There was an increase in measles virus stimulated IL-2+ CD4 T cells with a concomitant decease in IFN-γ+ CD4 T cells following vaccination, consistent with the
reduction in CD4+IFN-γ+ T cells described earlier (Figure 5.5A). Conversely, in the PPD cultures, the proportion of CD4+ T cells producing both IL-2 and IFN-γ increased following vaccination, alongside a decrease in the proportion of IL-2 producing CD4 T cells (Figure 5.5B). The absolute frequencies of the ICS+ CD4 T cells did not change significantly post-MV compared to pre-MV in measles cultures (Figure 5.5C) or PPD cultures (Figure 5.5D).

With the CD8+ T cells response, there were no apparent changes in the proportions of the 3 populations following vaccination in the measles virus stimulated cultures (Figure 5.6A). In the PPD-stimulated cells, the CD8 cytokine response mirrored what was observed with the PPD-stimulated CD4+ T cells with an increase in double positive cells and a decline in the IL-2+ subset for measles or PPD stimulated cells (Figure 5.6B). There was no significant change after MV in the absolute frequencies of the ICS+ CD8 T cells in measles stimulated (Figure 5.6C) or PPD stimulated cells (Figure 5.6D).
Figure 5.5: Proportions of CD4 T cell cytokine producing cells before and after measles vaccination

Proportions of CD4+ cytokine-producing cells following overnight stimulation with the live (A) measles virus and (B) PPD before (V1) and 2 weeks after measles vaccination (V2). The proportion of cells producing both IL-2 and IFN-γ is shown in red, proportion of cells producing IL-2 alone in green, and IFN-γ alone is shown in blue. Absolute frequencies of cytokine producing cells following background subtraction in (C) measles virus and (D) PPD cultures. Data shown for n= 48 at Visit 1 and n=46 at Visit 2.
Figure 5.6: Proportions of CD8 T cell cytokine producing cells before and after measles vaccination

Proportions of CD8+ cytokine-producing cells following overnight stimulation with the (A) live measles virus and (B) PPD before (V1) and 2 weeks after measles vaccination (V2). The proportion of cells producing both IL-2 and IFN-γ is shown in red, proportion of cells producing IL-2 alone in green, and IFN-γ alone is shown in blue. Absolute frequencies of cytokine producing cells following background subtraction in (C) measles virus and (D) PPD cultures. Data shown for n=48 at Visit 1 and n=46 at Visit 2.
5.5.5 Inverse correlation between baseline Tregs and subsequent measles IgG Ab responses

Using the measles IgG ELISA kit, the measles-specific IgG levels were measured before and after vaccination. The levels increased significantly, as expected at the post vaccination visit (P<0.001, Figure 5.7A). I calculated the difference between the IgG levels at baseline and post-vaccination to determine the change in antibody level. We observed a borderline significant negative correlation between the change in antibody levels and the circulating Tregs at the time of vaccination (r= -0.305 p=0.052, Figure 5.7B). This was however not significant when corrected for multiple testing.

To investigate whether circulating Tregs at the time of vaccination inversely correlate with post-vaccination measles IgG antibody responses, we tested the association between the baseline CD4+CD25hiFOXP3+ Tregs from the medium control wells and the post-vaccination measles IgG levels. This showed a significant inverse correlation between the baseline Tregs and the post-vaccination IgG response (r= -0.4128, p= 0.017, Figure 5.7C). The correlation remained significant when corrected for multiple testing. We further analysed whether the post-vaccination CD4+CD25hiFOXP3+ Tregs correlated with the measles IgG antibody levels at the same time point to evaluate whether they become activated or expanded by vaccination and inhibit the antibody response. There was no evidence that this occurred (r=0.03, p=0.857, Figure 5.7D).
Figure 5. 7: Baseline Tregs correlate with measles antibody responses post-vaccination

(A) The measles IgG levels before and 2 weeks after vaccination. Scatter plots showing the relationship between the CD4+CD25hiFOXP3+ Treg frequencies before vaccination (Pre) and (B) change in measles IgG antibody responses; (C) measles IgG antibody response 2 weeks post-vaccination (Post). (D) shows the relationship between the circulating CD4+CD25hiFOXP3+ Treg frequencies and measles IgG antibody response 2 weeks after vaccination. Comparison was done using Spearman’s Rank Correlation Coefficient. Bonferroni correction was used to correct for multiple testing; \( \alpha \leq 0.025 \) was considered significant after correcting for 2 comparisons.
5.5.6 Associations between Tregs and effector T cell cytokine responses before vaccination

We employed a summary correlation diagram to give a graphic representation of the relationship between the measles-specific and non-specific (PPD-stimulated) cytokine responses and the three subsets of Tregs being evaluated. We measured the partial correlation controlling for sex and age in weeks. Before vaccination, we observed a moderate positive correlation between the CD4+CD25hiFOXP3+ Tregs in medium and the measles-specific CD4+IFN-γ+ response, and a strong positive correlation between this subset and the measles-specific CD4+IL-2+ response (Figure 5.8A). When we evaluated the measles-specific CD8 cytokine response, we observed a weak inverse correlation between this subset and the CD8+IFN-γ+ response, while the association with the CD8+IL-2+ response was a moderate positive correlation (Figure 5.8A).

When we looked at the association of the TNFR2-expressing cells within the CD4+CD25hiFOXP3+ subset with the measles-specific effector read-outs, the association was generally positive, with the correlation with the CD4+IFN-γ+, CD4+IL-2+ and CD8+IL-2+ being a moderate to strong positive correlation. The CD8+IFN-γ+ response however, inversely correlated with the MFI of TNFR2 within the CD4+CD25hiFOXP3+ subset. We saw the reverse with the CD4+FOXP3+TNFR2+ subset, with which the CD4+IFN-γ+, CD4+IL-2+ and CD8+IL-2+ responses had a moderate to strong negative correlation; and a strong positive association with the CD8+IFN-γ+ response (Figure 5.8A).

To evaluate the antigen specificity of this effect, we then examined the relationship between the PPD-specific cytokine responses and the three subsets of Tregs. Whilst the CD4+IFN-γ+...
response had a moderate negative association with the CD4+CD25hiFOXP3+ subset, the negative correlation was stronger with the TFR2-expressing cells within this subset (Figure 5.8B). A moderate negative correlation was also observed between the MFI of TNFR2 within the CD4+CD25hiFOXP3+ cells and the CD8+IL-2+ cells. We did not observe any correlations between the CD4+FOXP3+TNFR2+ subset and the PPD-specific CD4 and CD8 cytokine responses.
Figure 5. 8: Correlations between Treg frequencies and T cell cytokine responses before vaccination

Partial correlation graphs showing associations between Treg frequencies and T cell cytokine responses before vaccination following overnight stimulation with (A) live measles virus and (B) PPD. The grey scale is used to depict the strength of the association, the darker the shade, the stronger the correlation. Key: 1=CD4+CD25hiFOX3+ Tregs, 2=Median Fluorescence Intensity of TNFR2 within CD4+CD25hiFOX3+ Tregs, 3=CD4+FOX3+TNFR2+ Tregs, 4=CD4+IFN-γ+, 5=CD4+IL-2+, 6=CD8+IFN-γ+, 7=CD8+IL-2.
5.5.7 Associations between Tregs and effector T cell cytokine responses after measles vaccination

We next evaluated the post-vaccination response to investigate whether the Tregs become activated or functionally altered by vaccination and inhibit effector cytokine responses. To investigate this, we looked at the association between the Tregs in medium 2 weeks after vaccination and the measles-specific and non-specific (PPD-stimulated) cytokine responses at the same time point (Figure 5.9). We observed a moderate inverse correlation between the measles-specific CD4+IFN-γ+ response and the CD4+CD25hiFOXP3+ subset. However, we did not observe any other significant correlations between the Treg subsets and the measles-specific cytokine responses at this time point.

To evaluate the antigen specificity of the above inverse correlation post-vaccination, we also analysed for associations between Tregs and PPD-specific cytokine responses 2 weeks after MV. A moderate positive correlation was observed between the CD4+CD25hiFOXP3+ cells and the PPD-stimulated CD4+IFN-γ+ cells and CD8+IL-2+ cells. The MFI of TNFR2 within the CD4+CD25hiFOXP3+ subset and the CD8+IL-2 response were moderately positively correlated. We did not observe any correlations between the CD4+FOXP3+TNFR2+ subset and the PPD-specific CD4 and CD8 cytokine responses.
Figure 5.9: Correlations between Treg frequencies and T cell cytokine responses after vaccination

Partial correlation graphs showing associations between Treg frequencies and antigen-specific T cell cytokine responses after vaccination following overnight stimulation with A) live measles virus and B) PPD. The grey scale is used to depict the strength of the association, the darker the shade, the stronger the correlation. Key: 1=CD4+CD25hiFOX3+ Tregs, 2=Median Fluorescence Intensity of TNFR2 within CD4+CD25hiFOX3+, 3=CD4+FOX3+TNFR2+ Tregs, 4=CD4+IFN-γ+, 5=CD4+IL-2+, 6=CD8+IFN-γ+, 7=CD8+IL-2+. 
5.5.8 Inverse correlation between pre-existing CD4+CD25hiFOXP3+ Tregs and measles-stimulated CD4+IFN-γ+ T cell response 2 weeks post-measles vaccination

We also evaluated the association between the pre-existing Tregs in medium at the time of vaccination (V1) with the T cell ICS responses in measles virus and PPD cultures 2 weeks after vaccination (V2) to investigate the role of Tregs at the time of vaccination in regulating subsequent T cell effector responses. We analysed for an association between the pre-existing CD4+CD25hiFOXP3+, the MFI of TNFR2 within this subset or CD4+FOXP3+TNFR2+ Tregs and the post-vaccination cytokine responses in measles virus and PPD cultures (Figure 5.10A and B). We observed a strong inverse correlation between the CD4+CD25hiFOXP3+ Tregs and the measles-specific CD4+IFN-γ+ T cell response supporting an immunoregulatory role for this subset (Figure 5.10A). When we looked at the PPD-specific response, a moderate negative correlation between the CD4+FOXP3+TNFR2 subset and the CD8+IL-2+ T cell response was observed (Figure 5.10B).
Figure 5. 10: Correlations between Tregs at the time of vaccination and post-MV T cell cytokine responses

Partial correlation graphs showing associations between Tregs at baseline (v1) and post-vaccination antigen specific cytokine responses following overnight stimulation with (A) live measles virus and (B) PPD. The grey scale is used to depict the strength of the association, the darker the shade, the stronger the correlation. Key: 1=CD4+CD25hiFOX3+ Tregs, 2=Median Fluorescence Intensity of TNFR2 within CD4+CD25hiFOX3, 3=CD4+FOX3+TNFR2+ Tregs, 4=CD4+IFN-γ+, 5=CD4+IL-2+, 6=CD8+IFN-γ+, 7=CD8+IL-2+. 
5.6 DISCUSSION

In Chapter 3 we showed an increase in CD4+FOXP3+CD127lo Tregs in measles vaccinated females but not males. However, in this study we were unable to replicate these findings and did not see a significant increase in CD4+CD25hiFOXP3, TNFR2-expressing CD4+CD25hiFOXP3+ or CD4+FOXP3+TNFR2+ Treg populations following MV. In fact we unexpectedly found a significant decline in the CD4+CD25hiFOXP3+ cells following measles vaccination. There are several potential reasons for this discrepancy. One is that the increase in females in our first study was not significant after correcting for multiple testing and therefore may not have been a real effect. Another factor is that we measured responses at 2 weeks in this study compared to 4 weeks post-MV in the previous study, and the dynamics may be such that Tregs increase later than 2 weeks after MV in order to counteract an early inflammatory response following vaccination. Indeed, Jaye et al. showed that cellular responses following measles vaccination (specifically measles-specific CD8 T cell responses) were similar to those seen following natural infection. They found that the CTL responses were stronger in the earlier weeks after vaccination than later when the responses were found to wane (Jaye, Magnusen et al. 1998) which could possibly be due to an increase in Tregs. Another difference is the Treg phenotype used for the two studies since CD4+FOXP3+CD127lo Tregs were analysed in the earlier study, whereas in this study we chose to focus on the CD4+CD25hiFOXP3+ and TNFR2+ Treg populations to evaluate whether these suppressive subsets in humans have a regulatory role following measles vaccination in infants.

The inverse correlation between the CD4+CD25hiFOXP3+ Tregs at the time of vaccination and post-vaccination measles-specific IgG responses was also found in our previous study, and further supports our hypothesis that Tregs at the time of measles vaccination can
influence the induction of vaccine-induced antibodies. To our knowledge, the role of this subset in regulating or suppressing infant vaccine antibody responses has not been previously described. The mechanism is not known but there is evidence that Tregs can directly suppress B cell responses via cell-to-cell contact mechanisms, and that TGF-β and CTLA4 may play a role (Lim, Hillsamer et al. 2005). Earlier studies suggest that Treg induced B cell suppression may also occur via the suppression of T helper cell responses, which are vital for B cell expansion and activation (Lim, Hillsamer et al. 2004).

Measles vaccination was associated with a decline in IFN-γ production by medium-incubated CD4+ T cells but not CD8+ T cells, while measles-, SEB- and PPD-specific ICS responses remained quantitatively unchanged in both T cell subsets following MV. The lack of priming of measles-specific responses may be due to the fact that we stimulated with the live measles virus and not the vaccine antigen, but may also be due to the sensitivity of the assay since we were able to detect measles virus specific IFN-γ responses by ELISpot assay in preliminary assays in the previous chapter. Another explanation may be that the cells were cultured overnight, and a longer incubation period is required to detect measles-specific T cell responses by ICS. Since the responses did not change post-MV it is possible that the ICS responses detected to measles virus at baseline and 2 weeks after vaccination are to intrinsic innate pattern associated molecular patterns expressed by the virus, rather than adaptive responses. The cytokine profile of the CD4 T cells cultured with measles virus did alter after MV towards increased IL-2 production, and decreased IFN-γ; whereas the CD8 profile did not change significantly. This is consistent with the decline in CD4+IFN-γ+ T cell frequencies. The PPD responses also altered towards an IL-2/IFN-γ double positive CD4+ population, again with little change in the CD8 profile.
The strong inverse correlation between the baseline Treg frequencies and the post-vaccination measles-specific CD4+IFN-γ+ response is also consistent with our hypothesis that Tregs at the time of vaccination might suppress the induction of vaccine-specific cellular immunity. The significant decrease in CD4+IFN-γ+ responses in medium following MV lends further support to this hypothesis. Furthermore, the CD4+CD25hiFOXP3+ Tregs 2 weeks after vaccination inversely correlated with measles-specific CD4+IFN-γ+ responses at the same time, but this was not seen when the baseline Tregs were correlated with baseline CD4+IFN-γ+ cells in measles cultures. Thus, although measles vaccination did not cause an upregulation of Tregs, the circulating Tregs may have developed the functional capacity to suppress vaccine-specific cellular immunity 2 weeks after vaccination. Ideally, this should be confirmed in future functional studies, however cord blood studies have confirmed that this Treg subset is functionally suppressive in neonates (Nettenstrom, Alderson et al. 2013) and we have also shown it to be functional post-measles vaccination in certain infants in the previous chapter.

Baseline CD4+CD25hiFOXP3+ Tregs inversely correlated with baseline CD4+IFN-γ+ ICS responses to PPD suggesting a regulatory function, although this was not the case 2 weeks after MV. There was also evidence that the TNFR2+ Tregs have an immunoregulatory role. At baseline, the TNFR2 MFI in the CD4+CD25hiFOXP3+ Tregs inversely correlated with measles-specific CD8+IFN-γ+ ICS responses at the same time point, and the CD4+FOXP3+TNFR2+ Tregs inversely correlated with measles-specific CD4+IFN-γ+, CD4+IL-2+ and CD8+IL-2+ responses. The baseline MFI levels in the CD4+CD25hiFOXP3+ Tregs also inversely correlated with baseline CD4+IFN-γ+ and CD8+IL-2+ PPD responses. These observations are exploratory but do suggest that steady state
TNFR2+ Tregs may have a regulatory role, although don’t appear to influence the subsequent vaccine-specific cytokine responses.

In the cellular response induced by the measles vaccine, when stratified by sex, a previous study reported enhanced pro-inflammatory innate immunity in males but not females (Noho-Konteh, Adetifa et al. 2016). In our study MV males had increased CD4+IL-2+ cells in medium while MV females had lower CD4+IFN-γ+ T cells in medium, supporting the findings in the previous study and suggesting that MV may be pro-inflammatory in males and immunosuppressive in females. Proposed reasons for early life sex differences in cellular immunity include the influence of sex hormones which differ between males and females even at this pre-pubertal stage in development (Flanagan 2015). Multiple immune cells express sex-hormone receptors, and thus sex hormones have multiple immunological effects, with oestrogens generally considered pro-inflammatory and androgens generally immunosuppressive (Klein 2012). Clearly the pattern of higher inflammation in measles vaccinated males and lower in females does not fit with effects of sex steroids where the opposite pattern would be expected. Another possible mechanism for sex differences in immunity is the multiple immune response genes (Fish 2008) and microRNAs (Pinheiro, Dejager et al. 2011) expressed by the X-chromosome, including the innate viral receptors (toll-like receptor (TLR) 7 and 8), multiple interleukin receptors, transcriptional and translational regulators and immune response proteins. Males express only one X-chromosome, whereas one of the two in females undergo X-inactivation, a process that is incomplete leading to differences throughout the body in X-chromosome gene expression.
In summary, our data support a functional role for CD4+CD25hiFOXP3+ Tregs at the time of vaccination in controlling the subsequent humoral and CD4+IFN-γ+ response to MV. We also show evidence that the TNFR2+ subset has an immunoregulatory role, but not specifically related to vaccination. Further evaluation of the role of CD4+CD25hiFOXP3+ Tregs in measles vaccine immunogenicity is therefore warranted. Understanding the role they play and the mechanisms used by these Tregs to regulate vaccine responses would contribute to the knowledge base required to improve the efficacy of current childhood vaccines. To our knowledge, this is the first evidence that functional peripheral Tregs negatively regulate functional effector readouts to measles vaccination in infants.

We recognise a number of limitations. Associations do not necessarily mean cause and effect, as the association may be coincidental and other factors may be responsible. However, the fact that we found the same inverse correlation between Tregs and measles antibodies in our previous study further supports the possibility that this finding is real. The single time point for follow-up samples did not allow us to assess the dynamics of the Treg to T effector response which is likely to change over time. Potential confounding factors include differences in nutritional status, genetic, environmental factors and underlying infections; although all infants were healthy and well on the day of vaccination, were all resident in the same environment and HIV and helminth infection rates are low in this cohort (Noho-Konteh, Adetifa et al. 2016).
CHAPTER 6: DETAILED FUNCTIONAL PHENOTYPING OF TREGS TO INVESTIGATE POTENTIAL MECHANISMS OF ACTION

6.1 INTRODUCTION

We have shown in the previous chapter that there is a significant inverse association between pre-existing CD4+CD25hiFOXP3+ Tregs and measles-specific IgG response post-vaccination. This significant inverse association was also observed with the TNFR2-expressing CD4+CD25hiFOXP3+ Tregs and CD4+FOXP3+CD127- Tregs from the first part of this study. Our data from previous chapters therefore supports a functional role for Tregs in controlling the antibody response to infant measles vaccination. However, the full phenotype of functional Tregs in African infants is not clear, nor is the mode of action whereby circulating Tregs at the time of vaccination may alter vaccine responses. FOXP3+ Tregs employ a variety of mechanisms to mediate suppression, but are also flexible in that they can adapt the mechanism according to their local environment (reviewed by (Wing and Sakaguchi 2012), raising the possibility that they might change following vaccination, although this has not been previously investigated. A better understanding of the mechanism(s) of action used by Tregs to suppress vaccine responses could contribute further to therapeutic interventions aimed at improving vaccine responses in infants.

CD4+ Tregs can be further divided according to CD45RA expression, which can distinguish naïve Tregs (nTregs) or resting Tregs (rTregs) (FOXP3loCD45RA+) and memory activated Tregs (FOXP3hiCD45RA-) (Miyara, Yoshioka et al. 2009). The memory Treg subset can be further divided into central memory (TregCM) and effector memory (TregEM) according to the expression of chemokine receptor 7 (CCR7) with TregCM being CCR7+ and TregEM being CCR7- (Sallusto, Lenig et al. 1999, Tosello, Odunsi et al. 2008). Which of these subsets predominate in 9-month-old infants, and whether they are altered by vaccination, is not
known. Other phenotypic markers of FOXP3+ Tregs that have been described include the co-expression of the ectoenzymes CD39 and CD73 on the Tregs, which activate the A2A receptor on the effector T cells and production of adenosine, which causes effector cell suppression (Deaglio, Dwyer et al. 2007). The role of the CD39+CD73+ Treg subset in the context of vaccination is yet to be investigated.

The first stage of measles infection is initiated by the interaction of the cellular receptors to the ‘H’ protein on the measles virus. This interaction then triggers the ‘F’ protein to initiate fusion between the plasma membranes of the host cells and the virus (Lin and Richardson 2016). The two measles virus surface receptors that have been identified are CD46 and CD150/SLAM (signalling lymphocyte-activation molecule). CD150/SLAM is selectively expressed on B and T cells whilst CD46 is expressed on all nucleated cells. Wild-type measles virus strains use CD150/SLAM as a cellular receptor (Tatsuo, Ono et al. 2000), while vaccine strains such as the Edmonston strain can use both SLAM and CD46 as receptors (Naniche, Varior-Krishnan et al. 1993). More recently, a newly discovered epithelial receptor called Nectin-4 has been described. Unlike the first two, this is a secondary exit receptor (Lin 2016). To better understand the immunological effects of measles vaccination, we were interested in studying the expression of the receptors the virus uses to gain entry into host cells. Previous studies have shown that measles virus down-modulates the expression of the two entry receptors CD150 and CD46 on the host cells during infection, possibly suggesting a role of these receptors in the measles virus-induced immunosuppression observed during infection (Welstead, Hsu et al. 2004). Whether the same down-modulation of the receptors occurs following measles vaccination in children is not known. In our measles vaccinated cohort, the Edmonston
strain of the vaccine was used; hence we investigated the expression of both receptors by cell subsets before and after vaccination.

We also wanted to further characterize the effector T cell response following MV. The differentiation and commitment of T cells into the different lineages is facilitated by the expression of different transcription factors, and is directed by cytokines in the environment when the naïve T cell encounters an antigen. Investigating the expression of transcription factors is a useful tool for understanding the cellular mechanisms of immune regulation. For instance, differentiation of Th1 and Th2 cells are driven mainly by IL-12 and IL-4 respectively via the transcription factors STAT4 and STAT6 (Kaplan, Sun et al. 1996, Thierfelder, van Deursen et al. 1996). Being an important mediator of STAT6 function, GATA3 is expressed predominantly by Th2 cells and is vital in their differentiation (Zheng and Flavell 1997, Ouyang, Lohning et al. 2000). T-bet is the transcription factor critical in the differentiation of Th1 cells and influences IFN-γ production (Szabo, Kim et al. 2000). T-bet inhibits Th2 cytokine expression whilst GATA3 inhibits Th1 cytokine expression (Lee, Takemoto et al. 2000, Mullen, High et al. 2001). NFATc1 is also implicated as being an important transcription factor expressed by Th1 cells which influences the production of the Th1 cytokine IL-2.

The co-expression of HLADR and CD38 indicate a classical marker of T cell activation widely used in HIV studies where it is associated with disease progression (Giorgi, Hausner et al. 1999). The CD95 marker is used as an apoptotic marker (Peter and Krammer 2003) whilst CCR4 has been shown to aid regulatory T cell migration to inflamed tissues (Faustino, da Fonseca et al. 2013).
To explore the suppressive mechanisms of Tregs in measles vaccinated 9 month-old infants, we conducted detailed phenotyping of Tregs from infants before and 2 weeks after MV. The pro-inflammatory cytokine TNF has been shown to induce Tregs via TNFR2, allowing the maintenance of FOXP3 expression and Treg function. Hence, we further generated 3-day short term cell lines (STCL) for further detailed study; adding IL-2 into the cultures to enhance Treg function and proliferation. In addition, the pro-inflammatory cytokine TNF has counter intuitively been shown to induce Tregs via its receptor TNFR2, and this is vital in maintaining FOXP3 expression and the functionality of Tregs (Chen, Baumel et al. 2007), hence we also added TNF to selected cultures to investigate whether the characteristics of the Tregs are altered.

6.2 HYPOTHESES

I hypothesised that:

- Measles vaccination alters the phenotype of the Tregs.
- Measles vaccination will change the differentiation of the Treg subsets.
- Measles vaccination causes a downregulation of the measles virus receptors on the host cells and this may contribute to a Th1 to Th2 or immunosuppressive shift.
- Culturing cells in IL-2 and TNF will induce Tregs especially the TNFR2 subset.

6.3 AIMS

- To determine the detailed phenotype of the Tregs before and after measles vaccination
- To determine the functional characteristics of the Tregs before and after measles vaccination.
• To analyse for expression of the measles receptors CD46 and CD150 by the CD4+ and CD8+ T cells
• To investigate the role of key cytokines and transcription factors in the suppressive effects of Tregs.
• To determine whether the Tregs correlate with CD4+ and CD8+ T cell cytokine responses and transcription factors expression when samples are cultured in either IL-2 alone or IL-2 and TNF for 3 days.

6.4 METHODS

6.4.1 Study Samples

Frozen PBMC from the same infant cohort described in the previous chapter were shipped to the Vaccine and Infectious Diseases Lab, Monash University, Melbourne, Australia for further analysis. Paired samples pre- and post-MV from 10 participants were selected based on the PBMC numbers for this part of the study. Equal numbers of males and females were selected.

6.4.2 Multiparameter (11-colour) flow cytometric analysis

This part of the study was done in Melbourne because 11-colour flow cytometry is not available in The Gambia.

Ten pairs of samples (total of 20 samples; visit 1 and 2) were selected to do extensive phenotyping to determine which markers best defined the functional Tregs in this age group, and to identify transcription factors that might associate with Treg function. Selection was done based on the samples with the highest cell numbers. Out of the 20 samples, equal number of males and females were selected from both study visits.
The PBMCs were rapidly thawed and washed as described in Section 2.9. Following the final wash, the supernatant was discarded and the cells were resuspended in 1mL of Aim V medium and a cell count performed. Following the cell count, the suspension volume was topped up to 3 mL and cells were washed and centrifuged at 2,000 rpm for 3 minutes, supernatant discarded and cells resuspended at a final concentration of 5x10^6 cells/mL in Aim V media containing 5% human AB serum.

The thawed PBMCs were stained with the six multicolour flow cytometry panels described in Tables 6.1 and 6.2 using the procedure described in Section 2.12. Panels 1 and 4 were given priority if cell numbers were insufficient.

For Panel 1, 300 μL of the cell suspension was plated out into a 48 well plate. The cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) and ionomycin (1 μg/ml), and samples incubated for two hours at 37°C/5% CO₂. Brefeldin A (BFA; 10μg/mL) was then added and the cells were incubated again for 6 hours under the same conditions. Cells were stained using the antibodies in Panel 1 (Table 6.1). For the remaining panels (panels 2 to 6), 100 μl of the cells suspension was plated out for each sample in a V bottom plate, and staining was done using the antibodies in Tables 6.1 and 6.2 as described in Section 2.12.

Isotype controls were used to determine non-specific binding. Cells were resuspended to a concentration of 2x10^6 cells/mL. In each control well, 100 μL of cell suspension was plated in a V-bottom 96-well plate. Staining was done as in section 2.12, replacing respective antibodies with the isotype control. This was done for both the surface and intracellular staining.
6.4.3 Short-term 3 day cultures

In order to examine the biological activity of the Tregs, thawed PBMCs were used to set up short term 3 day cultures by adding recombinant IL-2 (20 units/mL, BD Pharmingen) to stimulate and expand the T cells and identify which transcription factors were involved in the effector cytokine response before and after measles vaccination; and whether this is regulated by pre-vaccination Tregs. We examined whether adding TNF (20 ng/mL, BD Pharmingen) enhanced the function of the TNFR2 expressing Tregs, and whether this influenced the cytokine response.

From the 20 samples selected for this part of the study, only 16 had enough cells remaining following the phenotypic analysis described above. At day 0, cells were stimulated with IL-2 (20 units per mL) and/or TNF (20ng/mL), or with AIM V medium containing 5% human serum (negative control). We started with 2 million cells per well, and used 6 wells per donor (i.e. visit 1 and 2: IL-2 stimulated, IL2+TNF, medium alone). The cells were cultured in a flask in a humidified incubator at 37°C, 5% CO₂ for 3 days.

On Day 3, the cells were washed twice by adding 2 mL AIM V media containing 5% human AB serum medium and spun at 2,000 rpm for 3 minutes. Following the final incubation for both the overnight stimulated (8-hour) and 3-day STCL, the cells were fixed and stained using panels 1 and 4, then acquired using the LSRII flow cytometer.
Table 6. 1: Flow cytometry staining panels (1-3) used for phenotypic and functional analysis of Tregs

<table>
<thead>
<tr>
<th>Panel 1</th>
<th>Panel 2</th>
<th>Panel 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibody</strong></td>
<td><strong>Dilution</strong></td>
<td><strong>Source</strong></td>
</tr>
<tr>
<td>CD3 G655</td>
<td>1/300</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>CD4 APC-Cy7</td>
<td>1/50</td>
<td>BD Pharm.</td>
</tr>
<tr>
<td>CD8 Q605</td>
<td>1/800</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>CD25 PE-Cy7</td>
<td>1/40</td>
<td>BD Pharm.</td>
</tr>
<tr>
<td>FOXP3 AF700</td>
<td>1/40</td>
<td>E-Bio</td>
</tr>
<tr>
<td>IFN- FITC</td>
<td>1/100</td>
<td>BD Pharm.</td>
</tr>
<tr>
<td>IL-2 APC</td>
<td>1/50</td>
<td>BD Pharm.</td>
</tr>
<tr>
<td>IL-10 PE</td>
<td>1/10</td>
<td>E-Bio</td>
</tr>
<tr>
<td>IL-4 PerCP</td>
<td>1/50</td>
<td>BD Pharm.</td>
</tr>
<tr>
<td>Ki67 V450</td>
<td>1/100</td>
<td>BD Horizon</td>
</tr>
<tr>
<td>DCS PO</td>
<td>1/500</td>
<td>Life Tech.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Antibody</strong></th>
<th><strong>Dilution</strong></th>
<th><strong>Source</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3 G655</td>
<td>1/300</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>CD4 APC-Cy7</td>
<td>1/50</td>
<td>BD Pharm.</td>
</tr>
<tr>
<td>CD8 Q605</td>
<td>1/800</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>CD25 PE</td>
<td>1/40</td>
<td>BD Pharm.</td>
</tr>
<tr>
<td>FOXP3 AF700</td>
<td>1/40</td>
<td>E-Bio</td>
</tr>
<tr>
<td>TNFR2 FITC</td>
<td>1/20</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>CCR7 APC</td>
<td>1/20</td>
<td>BD Pharm.</td>
</tr>
<tr>
<td>CD127 PerCP</td>
<td>1/10</td>
<td>BD Pharm.</td>
</tr>
<tr>
<td>CCR4 PE-Cy7</td>
<td>1/40</td>
<td>BD Pharm.</td>
</tr>
<tr>
<td>Ki67 V450</td>
<td>1/100</td>
<td>BD Horizon</td>
</tr>
<tr>
<td>DCS PO</td>
<td>1/500</td>
<td>Life Tech.</td>
</tr>
</tbody>
</table>

The dilutions used for the staining and the source of the antibodies is also shown.
Table 6. 2: Flow cytometry staining panels (4-6) used for the phenotypic and functional analysis of Tregs

The dilutions used for the staining and the source of the antibodies is also shown.
6.4.4 Gating of cell subsets of interest

Following acquisition of the cells, the data were analysed using Flowjo V10.1 for Mac (Treestar, California). The gating strategy for the various cell subsets and markers is shown in Figures 6.1 - 6.3. For all panels we first gated on the total lymphocytes; using a wider gate to include expanded cells, then on the singlets and live cells. We then gated on the CD4+ T cells or CD8+ T cells and from there, gated on the individual markers being analysed in each panel (Tables 6.1 and 6.2). During the acquisition, approximately 200,000 lymphocyte events were acquired, with a range of 1011-3953 events in the FOXP3+ gate within the CD4 T cells.

For markers within the CD4+CD25hiFOXP3+ population in which there were fewer cells, we used the contour plots for gating. Infant cells do not stain the same as adult cells, with certain markers less highly expressed in infants. We therefore included a healthy adult control donor for each experiment to ensure that the staining had worked well (Figure 6.4).
Figure 6. 1: Gating Strategy for Tregs, HLADR, CD95 and CCR7

We first gated on (A) lymphocytes, then (B&C) single cells, then the (D) live cells followed by (E) CD4+ and CD8+ T cells within the CD3+ T cells.

The Tregs were characterized as (F) CD4+CD25hiFOXP3+, and were further gated for (G)HLADR, (H) CD95 and (I) CCR7 expression.
Figure 6.2: The Gating strategy used to gate on further markers of interest within the CD4+ T cell population

Expression of (A) CD45RO, (B) CCR7, (C) CCR4, (D) HLADR, (E) CD95 by gated CD4+ T cells. The transcription factors (F) GATA3, (G) STAT4, (H) Tbet, and (I) NFATc1+ were also gated within the CD4+T cells.
Figure 6.3: The Gating strategy used to gate on the CD4+ and CD8+ cytokine producing cells

CD4 and CD8 T cells were gated as shown in Figure. 6.1. We then gated on cells producing the individual cytokines for (A-E) CD4+ and (F-J) CD8+ T cells.
Figure 6.4: Comparison of adult and infant samples for the CD4 and CD8 staining

(A) Healthy adult donor PBMC had more distinct CD4 and CD8 populations compared to (B) study infants. Thus a healthy adult sample was stained in each experiment as an additional control.

6.4.5 Statistical analysis

Data were mainly analysed by descriptive statistics, as this part of the study is exploratory in nature, and the sample sizes are generally too small to determine changes in subset frequencies post-MV. Nevertheless, differences in parameters between the two visits were analysed using a Wilcoxon matched-pairs signed rank test, and a p value of ≤0.05 was considered as significant. For the CD4 and CD8 T cell cytokine responses, we used SPICE (Version 5, NIH) for the visual representation of the data showing the proportions of cells producing IL-2 and/or IFN-γ. The Benjamini-Hochberg method was used at a false discovery rate (FDR) of 0.05 to correct for multiple testing. Analyses were performed using GraphPad Prism (Version
6 for MAC).

### 6.5 RESULTS

#### 6.5.1 Effect of measles vaccination on CD4+ T cell phenotype and function

We first investigated the expression of phenotypic and functional markers by the CD4+ and CD8+ T cells before and after measles vaccination. The expression of the measles receptor CD46 by CD4+ T cells was significantly increased ($p=0.031$), but CD150 receptor expression levels were unchanged. We also observed a significant increase in the proportion of CD4+ T cells producing IL-10 following MV ($p=0.04$), with only a negligible percentage co-expressing the Th-1 cytokine IFN-γ (<1%). However, when corrected for multiple comparisons, the change in the expression of CD46 and IL-10 by the CD4+ T cells was not significant.

We did not observe any significant change in both the dual and single expression of the activation markers HLADR and CD38 by CD4 T cells, or the CD39 and CD73 markers. The expression of the transcription factors STAT4, GATA3, T-bet and NFATc1 by CD4 T cells were also comparable before and after vaccination, although T-bet expression did almost double (from 2.44% to 4.48% of CD4 T cells). We also found no significant change in expression of Ki67, CCR4 or CD95/Fas following vaccination (Table 6.3).
<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Median % (25th-75th quartiles) at Visit 1</th>
<th>Median % (25th-75th quartiles) at Visit 2</th>
<th>P Value</th>
<th>Adjusted P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ (% of CD3+ lymphocytes)</td>
<td>10</td>
<td>66.45 (60.85-73.43)</td>
<td>64.45 (48.78-70.83)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Ki67+</td>
<td>10</td>
<td>5.84 (4.385-8.143)</td>
<td>5.53 (3.933-11.36)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CCR4+</td>
<td>8</td>
<td>11.5 (7.5-18.05)</td>
<td>13.8 (9.95-14.48)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CCR7+</td>
<td>8</td>
<td>21.4 (18.925-29.95)</td>
<td>21.2 (18.13-31.05)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD95+</td>
<td>8</td>
<td>32.85 (28.65-36.23)</td>
<td>36.05 (30.4-52.75)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD45RO+</td>
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<td>17.8 (13.8-24.98)</td>
<td>17 (15.85-27.5)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD45RA+</td>
<td>8</td>
<td>54.55 (41.45-55.48)</td>
<td>54 (51.35-57.35)</td>
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<td>NS</td>
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<td>HLA DR+</td>
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<td>GATA3+</td>
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<td>NS</td>
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<td>NFATc1+</td>
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<td>4.055 (2.693-5.795)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD38+</td>
<td>8</td>
<td>17.8 (13-20.4)</td>
<td>15.35 (13.65-19.4)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD46+</td>
<td>8</td>
<td>18.9 (12.9-24.4)</td>
<td>22.4 (13.3-36.5)</td>
<td>0.031</td>
<td>NS</td>
</tr>
<tr>
<td>CD150+</td>
<td>8</td>
<td>13.4 (8.69-13.9)</td>
<td>10.7 (9.013-12.5)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>STAT4</td>
<td>8</td>
<td>1.09 (0.425-2.06)</td>
<td>1.01 (0.405-3.068)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>T-bet</td>
<td>8</td>
<td>2.44 (1.97-5.885)</td>
<td>4.475 (3.27-6.723)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IL-4</td>
<td>9</td>
<td>3.470 (2.138-5.270)</td>
<td>5.240 (1.735-7.458)</td>
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<td>NS</td>
</tr>
<tr>
<td>IL-10</td>
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<td>0.960 (0.5875-2.013)</td>
<td>1.835 (0.7750-3.215)</td>
<td>0.04</td>
<td>NS</td>
</tr>
<tr>
<td>IFN- γ+</td>
<td>10</td>
<td>0.545 (0.3625-0.7925)</td>
<td>0.9750 (0.600-1.585)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IL-2</td>
<td>10</td>
<td>0.2450 (0.1445-0.8300)</td>
<td>0.2650 (0.1203-0.7925)</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 6.3: Phenotypic analysis of CD4+ T cells before and after measles vaccination

PBMCs were gated on CD4+ cells within the viable and single CD3+ T cell population.

The median percentage of the gated CD4+ T cells expressing each marker is shown at the top with the 25th-75th quartiles shown in the brackets below for both study visits.

N = no. of paired samples tested, NS = not significant. P values derived from a two-
tailed Wilcoxon matched-pairs signed rank tests. The Benjamini-Hochberg method was used at a false discovery rate (FDR) of 0.05 to correct for 19 comparisons.

6.5.2 Effect of MV on CD8+ T cell phenotype and function

Among the CD8 T cell population, there was a borderline increase in the frequency of Ki67+ proliferating CD8+ T cells following MV (p=0.07) (Table 6.4). In contrast to the CD4 T cells where we observed a significant increase in the IL-10 producing cells, this was not observed with the IL-10 producing CD8+ T cells post-MV (Table 6.4). We did not observe any significant increase in the expression of the other markers of interest in the CD8 population.
Table 6.4: Phenotypic analysis of the CD8+ T cells

PBMCs were gated on CD8+ T cells and the median percentage of CD8 T cells expressing each marker is shown with the 25th-75th quartiles shown in the brackets for both study visits. N = no. of paired samples tested, NS = not significant. P values derived from two-tailed Wilcoxon matched-pairs signed rank tests. The Benjamini-Hochberg method was used at a false discovery rate (FDR) of 0.05 to correct for 11 comparisons.

6.5.3 The phenotypic characteristics of the CD4+CD25hiFOX3+ Tregs are comparable before and after measles vaccination

To further define the CD4+CD25hiFoxp3+ Treg population before and after MV, we gated on this Treg subset and analysed for co-expression of phenotypic and
functional markers before and after measles vaccination. Following MV, we did not observe any significant change in any of the markers of interest. However, numbers were very small and there was an increase in Ki67 expression from 29.05% to 38.5% of the Tregs, which might support increased proliferation of this subset. NFATc1 expression decreased from 8.55% to 5.77%, which could support decreased expression of the transcription factor. The median fluorescence intensity (MFI) of TNFR2 within the CD4+CD25hiFOXP3+ Tregs did not alter significantly following MV, although did increase from 281 to 314 (Table 6.5).

6.5.4 No evidence that CD25hiFOXP3+ Tregs suppress proliferating CD4+ T cells

We next sought to find out whether Treg function is altered following measles vaccination. We investigated for a correlation between the circulating CD4+CD25hiFOXP3+ Tregs and the CD4+Ki67+ T cells at baseline or 2 weeks post-MV. There was no significant correlation between Tregs and the CD4+Ki67+ T cells at either time point and in fact the relationship was generally on a positive slope indicating increased CD4 proliferation with increased Tregs (Figure 6.5).
<table>
<thead>
<tr>
<th>Marker</th>
<th>N</th>
<th>Median % (25th-75th Quartile) at Visit 1</th>
<th>Median % (25th-75th Quartile) at Visit 2</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(CD4)CD25hiFOXP3+</td>
<td>8</td>
<td>0.855 (0.4725-1.485)</td>
<td>0.675 (0.3675-1.165)</td>
<td>NS</td>
</tr>
<tr>
<td>Ki67+</td>
<td>10</td>
<td>29.05 (21.93-39.78)</td>
<td>38.5 (31.43-42.7)</td>
<td>NS</td>
</tr>
<tr>
<td>CCR4+</td>
<td>8</td>
<td>14.5 (11.6-18.15)</td>
<td>17.9 (12.45-22.03)</td>
<td>NS</td>
</tr>
<tr>
<td>CCR7+</td>
<td>8</td>
<td>40.2 (26.6-62.23)</td>
<td>35.75 (28.28-52.8)</td>
<td>NS</td>
</tr>
<tr>
<td>CD95+</td>
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<td>55.15 (47.68-60.83)</td>
<td>58.7 (46.98-75.02)</td>
<td>NS</td>
</tr>
<tr>
<td>DC45RA+</td>
<td>8</td>
<td>51.3 (34.03-59.63)</td>
<td>52.15 (37.75-62.05)</td>
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</tr>
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<td>CD45RO+</td>
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<td>22.95 (13.8-30.95)</td>
<td>24 (18.53-29.55)</td>
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<td>HLADR+</td>
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<td>49.2 (33.85-66)</td>
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</tr>
<tr>
<td>GATA3+</td>
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<td>14.4 (12.05-19.1)</td>
<td>14.05 (7.04-17.9)</td>
<td>NS</td>
</tr>
<tr>
<td>NFATc1+</td>
<td>8</td>
<td>8.545 (4.838-12.9)</td>
<td>5.77 (3.138-8.495)</td>
<td>NS</td>
</tr>
<tr>
<td>CD38+</td>
<td>8</td>
<td>19.4 (17.8-22.5)</td>
<td>17.9 (12.45-31.95)</td>
<td>NS</td>
</tr>
<tr>
<td>CD46+</td>
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<td>29 (17.5-42.9)</td>
<td>32.05 (15.75-43.23)</td>
<td>NS</td>
</tr>
<tr>
<td>CD150+</td>
<td>8</td>
<td>19.7 (14.2-26.6)</td>
<td>19.5 (18.55-25.03)</td>
<td>NS</td>
</tr>
<tr>
<td>STAT4</td>
<td>8</td>
<td>4.31 (0.968-7.105)</td>
<td>2.72 (2.115-9.175)</td>
<td>NS</td>
</tr>
<tr>
<td>T-bet</td>
<td>8</td>
<td>2.045 (0.8775-2.81)</td>
<td>2.0935 (1.65-3.623)</td>
<td>NS</td>
</tr>
<tr>
<td>MFI TNFR2</td>
<td>8</td>
<td>281 (226.5-462.5)</td>
<td>314 (255-402.8)</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Table 6.5: Phenotypic analysis of CD4+CD25hiFOXP3+ Tregs**

The median percentage of the gated Treg cells expressing each marker is shown at the top with the 25th-75th quartiles shown in the brackets below for both study visits.

N = no. of paired samples tested, NS = not significant. P values derived from Wilcoxon matched-pairs signed rank tests.
Figure 6. 5: Association between circulating CD4+CD25hiFOXP3+ Treg frequencies and proliferating CD4 T cells at the same time-point

Correlation between CD4+CD25hiFOXP3+ Tregs and the Ki67-expressing CD4+ T cells (A) at baseline and (B) 2 weeks after measles vaccination. Data from n = 10 infants shown. Comparison was done using Spearman’s Rank Correlation Coefficient.

6.5.5 Evaluation of cells expressing the CCR7 lymph node homing marker

There was no significant change in the frequency of CD4 T cells expressing the lymph node homing marker CCR7 following vaccination, a marker of central memory T cells (Figure 6.6). However, although it was not significant, we did observe a decline in CCR7 expression among the CD4+CD25hiFOXP3+ Tregs suggesting a possible increase in effector phenotype of the Tregs 2 weeks after MV (Figure 6.6).
The $CD4^+$ and $CD4^+CD25^{hi}FOXP3^+$ T cells had comparable levels of CCR7 expression at baseline (V1) and 2 weeks after (V2) administration of the measles vaccine. The y-axis shows the percentage of CCR7+. Paired data for $n=8$ infants are shown. Each dot represents a single individual with paired data shown for the study visits; V1-Visit 1 V2- Visit 2. Paired data were analysed using a two-tailed Wilcoxon matched-pairs signed rank test.

6.5.6 Gambian infant Tregs are mostly of a CD45RA+CCR7- TEMRA phenotype

Staining for CD45RA and CCR7 demonstrated that the majority of CD4+ T cells were CD45RA+ and CCR7- indicative of TEMRA cells rather than true naïve ($p=0.008$). This effect remained significant when adjusted for multiple comparisons (adjusted $p=0.021$). Much fewer CD4+ T cells were CD45RA-, and of those that were, more were CCR7- (TEM) than CCR7+ (TCM). Similarly, the majority of the CD4+CD25hiFOXP3+ Tregs were of a CD45RA+ phenotype (Table 6.5, Figure. 6.7) both before and after vaccination, with no significant change in frequency from baseline to 2 weeks post-MV. The CD45RA+ Tregs were equally divided into CCR7- and CCR7+ subsets suggesting a mixture of TEMRA and true naïve populations.
(Figure 6.7). By contrast, the majority of the CD45RA- memory Tregs showed a CCR7+ central memory phenotype, with only a minority being of a CCR7- effector memory phenotype (p<0.001) (Figure 6.7). This observation remained significant following adjustment for multiple comparisons (adjusted p=0.0027).

Figure 6.7: CD45RA and CCR7 expression among CD4+ T cells and CD4+CD25hiFOXP3+ Tregs

(A) Gating strategy used to gate on subsets of CD45RA and CCR7 expressing (CD4)CD25hiFOXP3+ Tregs. (B) Frequencies of CD45RA and CCR7 expressing CD4+ T cells and (C) Frequencies of CD45RA and CCR7 expressing CD4+CD25hiFOXP3+ cells within the CD4+ T cells at baseline (V1) and 2 weeks after (V2) the administration of
MV using multi-parameter flow cytometric analysis. The box plots show the median values. The whiskers represent the 5th and 95th percentiles. Paired data shown for n = 8 infants. Differences between paired samples were analysed using a two-tailed Wilcoxon matched-pairs signed rank test. A p value of ≤0.05 was considered significant. *represents a p value of ≤0.05, ** represents a p value of ≤0.01, *** represents a p value of ≤0.001. The Benjamini-Hochberg method was used at a false discovery rate (FDR) of 0.05 to correct for 14 comparisons.

6.5.7 Analysis for resting and activated Tregs

Miyara et al. describe two functional Treg subsets depending on FOXP3 and CD45RA expression (Miyara et al., 2009). Regulatory T cells with the phenotype CD45RA+FOXP3hi were described as naïve activated Tregs, and the CD45RA-FOXP3hi were defined as memory activated Tregs; whereas the FOXP3lo Tregs were defined as resting Tregs. Within the CD4+CD25hiFOXP3+ subset of Tregs, we firstly gated on the FOXP3hi and FOXP3lo subset and then used co-expression of CCR7 and CD45RA, as done previously (Section 6.4.6) to define the functional Treg subsets. Generally, the FOXP3hi Tregs appeared to be more differentiated than the FOXP3lo subset, with a significantly higher number of CM Tregs in the FOXP3hi subset as compared to the FOXP3lo subset (Figure 6.8, p<0.001). This observation remained significant when adjusted for multiple comparisons (adjusted p=0.024). There was however, no significant change in the resting or activated Treg frequencies following MV.
Figure 6. 8: CD45RA and CCR7 expression among activated and resting CD4+CD25hiFOXP3+ Tregs

(A) Gating strategy used to gate on the FOXP3lo (resting) and FOXP3hi (activated) CD4+CD25hiFOXP3+ Treg subsets. (B) Co-expression of CD45RA and CCR7 used to separate the (C) FOXP3lo Tregs and (D) FOXP3hi Tregs into functional T cell subsets. Data shown for n=8 infants. The box plots indicate median frequencies and the
whiskers show the 5th and 95th percentiles. Differences between paired samples were analysed using a matched-pairs two-tailed Wilcoxon Test. A p value of ≤0.05 was considered significant. * represents a p value of ≤0.05, ** represents a p value of ≤0.01 and *** represents a p value of ≤0.001. The Benjamini-Hochberg method was used at a false discovery rate (FDR) of 0.05 to correct for 14 comparisons.

6.5.8 Investigating for the induction of IL-10 producing CD4 T cells

We next investigated the induction of CD4+IL-10+ T cells in more detail. Among the IL-10 producing CD4+ T cells, we analysed for FOXP3 and CD25hi expression. We found that only 6.875% and 5.605% of this subset also expressed FOXP3 at Visits 1 and 2 respectively (Table 6.6). The frequency of CD25hi cells within this subset was even less with 1.590% and 2.430% at Visit 1 and Visit 2 respectively (Table 6.6). Furthermore, the percentage of CD4+CD25hiFOXP3+ T cells producing IL-10 was negligible and did not change after vaccination (Table 6.6). T regulatory 1 (Tr1) cells are IL-10 producing Tregs that do not require the expression of CD25 or FOXP3. It is likely that the expanded CD4+IL-10+ T cells post-MV are Tr1 cells with an immunoregulatory role. To exclude the possibility that these may be Th1 or Th2 cells; we evaluated the expression of IFN-γ and IL-4 within the CD4+IL-10+ cells. We found that only 0.99 and 1.37% were IFN-γ + at visit 1 and visit 2 respectively. We found that only 6.87% and 6.91% of the IL-10 producing CD4 T cells were IL-4+ (Table 6.6). This further supports our suggestion that these may be Tr1 cells.
6.5.9 Investigating T cell pro-inflammatory cytokine responses before and after MV

We next investigated the dynamics of the IL-2 and IFN-γ cytokine responses before and after MV in order to further evaluate the effector T cell response. We analysed for the proportions of monofunctional IL-2+ and IFN-γ+ T cells and T cells producing both cytokines. We gated on CD3+ lymphocytes, then on either the CD4 or CD8 T cells. Then we looked at the co-expression of IL-2 and IFN-γ using Boolean gating. Overall, in all the study participants, we observed a greater proportion of CD4+ T cells producing both cytokines at Visit 2 compared to Visit 1 (Figure 6.9 A & B). The proportion of IFN-γ producing CD8+ cells was also greater at Visit 2 than was observed at Visit 1 (Figure 6.10 A & B).

<table>
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<tr>
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<td>0.0 (0-1.190)</td>
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</tr>
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<td>CD4+IL-10+ Cells</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>IFN-γ+</td>
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<td>1.37 (0.565-1.75)</td>
<td>NS</td>
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<tr>
<td>IL-4+</td>
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<td>6.91 (1.90-14.48)</td>
<td>NS</td>
</tr>
<tr>
<td>FOXP3+</td>
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<td>5.605 (3.728 -10.28)</td>
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</tr>
<tr>
<td>CD25hi</td>
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<td>1.590 (0.5100-3.773)</td>
<td>2.430 (1.858-4.938)</td>
<td>NS</td>
</tr>
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</table>

Table 6.6: IL-10 producing Cd4+ T cells and CD4+CD25hiFOXP3+ Tregs

The table shows the median percentage of CD4+IL-10+ cells expressing each marker with the 25th-75th quartiles shown in the brackets below for both study visits. The median frequency of the CD4+CD25hiFOXP3+ Tregs producing IL-10 at both visits is shown at the bottom of the table with the 25th -75th quartiles shown in the brackets.
for both study visits. N = no. of paired samples tested, NS = not significant. P values derived from Wilcoxon matched-pairs signed rank tests.

Figure 6.9: CD4+ cytokine producing T cells before and after measles vaccination

(A) Proportions of CD4+ cytokine producing cells following 8-hour stimulation with PMA/Ionomycin in all participants at baseline (Visit 1) and two weeks after MV (Visit 2). PBMCs were stained for intracellular cytokines IL-2 and IFN-γ; and analysed using Flowjo. Boolean gating was used to determine the frequencies of cytokine producing cells. The red slice shows the proportion of cells producing IL-2 alone, the green slice is the proportion of cells producing both IL-2 and IFN-γ, and the blue slice is the proportion of cells producing IFN-γ alone. (B) Absolute frequencies of cytokine producing CD4+ T cells. The red dots show the frequency of cells producing IL-2 alone; green dots the frequency of cells producing both IL-2 and IFN-γ; blue dots the frequency of cells producing IFN-γ alone. Data represents 10 paired samples at visits 1 (V1)
and 2 (V2). Pie charts generated using SPICE. Statistical analysis was performed on paired samples using Wilcoxon matched pairs signed-rank test.

Figure 6.10: CD8+ cytokine producing T cells before and after measles vaccination

(A) Proportions of CD8+ cytokine producing cells following 8-hour stimulation with PMA/Ionomycin in all participants at baseline (Visit 1) and two weeks after MV (Visit 2). PBMCs were stained for intracellular cytokines IL-2 and IFN; and analysed using Flowjo. Boolean gating was used to determine the frequencies of cytokine producing cells. The red slice shows the proportion of cells producing IL-2 alone, the green slice is the proportion of cells producing both IL-2 and IFN-γ, and the blue slice is the proportion of cells producing IFN-γ alone. (B) Absolute frequencies of cytokine producing CD8+ T cells. The red dots show the frequency of cells producing IL-2 alone; green dots show the frequency of cells producing both IL-2 and IFN-γ; blue dots show the frequency of cells producing IFN-γ alone. Data represents 10 paired samples at Visit 1 and Visit 2.
visits 1 (V1) and 2 (V2). Pie charts generated using SPICE. Statistical analysis was performed on paired samples using Wilcoxon matched pairs signed-rank test.

6.5.11 Measles vaccination is associated with a down-regulation of the transcription factor STAT4 by CD4 T cells, but not GATA3, Tbet or NFATc1

IL-2 has been shown to be required for Treg function *in-vitro* (De la Rosa et al, 2004). Furthermore, TNFR2+ Tregs have been shown to be maximally suppressive (Minigo et al., 2009), with exposure to TNF shown to enhance the expression of TNFR2 by Tregs (Valencia et al., 2006). We therefore cultured the cells with IL-2 and TNF in order to preferentially stimulate and expand the Tregs, in particular the TNFR2-expressing Tregs. PBMCs were stimulated with PMA/ionomycin and cultured for 3 days in either IL-2 alone or IL-2 + TNF. We did not observe any significant change in the frequencies of the Tregs when the PBMCs were cultured in IL-2 alone and/or with TNF. The expression of the proliferative marker Ki67 by the Tregs also remained unchanged in the cultured cells (Tables 6.7 and 6.8).

We next analysed for the expression of key transcription factors STAT4 (required for Th1 cell development), GATA3 (Th2 biasing), T-bet (influences IFN-γ production by Th1 cells) and NFATc1 (important for IL-2 production by Th1 cells). In the IL-2 alone cultured cells there was no change in expression of any of the transcription factors by CD4+ T cells after MV (Figure 6.11). By contrast, there was a significant down-regulation of the STAT4 expression by CD4+ T cells when the cells were cultured with IL-2 and TNF (p=0.0391), but no change in GATA3 or NFATc1 expression (Figure
6.12). Nevertheless, the reduced expression of STAT4 on the CD4+ T cells following vaccination was not significant when adjusted for multiple comparisons (adjusted $p=0.124$).

**Figure 6.11: Frequencies of CD4+ T cells expressing transcription factors in cultures with IL-2**

Frequencies of CD4+ T cells expressing GATA3, NFATc1, STAT4 AND T-bet after culture for 3 days with IL-2 alone. Each dot represents a single individual with the lines connecting paired sample points. Paired data for $n=8$ to 10 infants are shown. $P$ values were calculated using a matched-paired two-tailed Wilcoxon Test.

$STAT4 = $ Signal transducer and activator of transcription 4; $NFATc1 = $ Nuclear factor of activated T-cells, cytoplasmic 1; $GATA3 = $ Gata-binding protein 3; $T-bet=T-box$ transcription factor.
Table 6.7: Phenotyping of markers of interest following the STCL with IL-2

The median percentage of the gated cells expressing markers of interest following 3 day culture with the addition of IL-2 is shown at the top with the 25th-75th quartiles shown in the brackets below for both study visits. N = no. of paired samples tested, NS = not significant. P values derived from Wilcoxon matched-pairs signed rank tests.

Figure 6.12: Cd4+ T cells expressing transcription factors in IL-2 plus TNF cultures

Frequencies of CD4+ T cells expressing GATA3, NFATc1, STAT4 AND T-bet after culture for 3 days with IL-2 plus TNF. Paired data for n = 8-10 infants are shown. Each dot represents a single individual with the lines connecting paired sample points. P values were calculated using a matched-paired two-tailed Wilcoxon Test. STAT4 = Signal transducer and activator of transcription 4; NFATc1 = Nuclear factor of activated T-cells, cytoplasmic 1; GATA3 = Gata-binding protein 3; Tbet= T-box
transcription factor. The Benjamini-Hochberg method was used at a false discovery rate (FDR) of 0.05 to correct for 4 comparisons.

<table>
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<th>Median % (25th-75th Quartile) at Visit 1</th>
<th>Median % (25th-75th Quartile) at Visit 2</th>
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</tr>
</thead>
<tbody>
<tr>
<td>CD25hiFOXP3+(CD4)</td>
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<td>0.555 (0.2525-0.8575)</td>
<td>0.555 (0.3058-0.855)</td>
<td>NS</td>
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<tr>
<td>Ki67 of CD25hiFOXP3+(CD4)</td>
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<td>16.65 (6.818-33.48)</td>
<td>13.8 (0-34.98)</td>
<td>NS</td>
</tr>
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Table 6. 8: Phenotyping of markers of interest following the STCL with IL-2 and TNF

The median percentage of the gated cells expressing markers of interest following 3 day culture with the addition of IL-2 + TNF is shown at the top with the 25th-75th quartiles shown in the brackets below for both study visits. N = no. of paired samples tested, NS = not significant. P values derived from Wilcoxon matched-pairs signed rank tests.

6.5.10 No evidence that CD4+CD25hiFOXP3+ and TNFR2 expressing cells within the CD4+CD25hiFOXP3+ subset influence the CD4+ and CD8+ cytokine responses

We investigated for a correlation between the circulating CD4+CD25hiFOXP3+ Tregs and the CD4 and CD8 T cell cytokine responses to PMA/ionomycin in the 3-day cultures. There were no significant correlations between the Tregs and the pro-inflammatory CD4 and CD8 T cell cytokine responses either at baseline or post-MV. There was also no significant correlation between ex-vivo Tregs and the proliferating CD4 and CD8 T cell response in the 3-day STCL. To investigate whether functional
Tregs are induced in the cultures, we then correlated the Tregs in cultures to the pro-inflammatory and proliferating CD4 and CD8 T cell responses (Table 6.9). We observed strong significant positive correlations between the CD4+CD25hiFOXP3+Tregs and the CD4+IL-2+ (r=0.786, p=0.028), CD8+IL-2+ (r=0.881, p=0.007) and CD8+IFN-γ+ (r=0.786, p=0.028) responses in the IL-2+TNF cultures at Visit 1. Following correction for multiple testing, none of these correlations remained significant (adjusted p=0.087). The CD4+Ki67+ response also positively correlated with the CD4+CD25hiFOXP3+ Treg subset at the same time point (r=0.786, p=0.028). We did not observe any significant correlation between the TNFR2-expressing CD4+CD25hiFOXP3+ Treg subset and any of the pro-inflammatory or proliferative CD4 and CD8 T cell responses.
Table 6. 9: Correlation between Treg frequencies at baseline and T cell cytokine responses before and after vaccination

The r values for the association between TNFR2-expressing CD4+CD25hiFOXP3+ (TNFR2), CD4+CD25hiFOXP3+ (CD25hi) Treg frequencies, and antigen-specific CD4 and CD8 T cell cytokine responses before (Visit 1) and after (Visit 2) measles vaccination following 3-day cultures are shown. Cells were cultured with either IL-2 alone or IL2 and TNF. Significant r values are highlighted in green (inverse correlation) and red (positive correlation). Data from n = 10 infants shown. Comparison was done using Spearman’s Rank Correlation Coefficient. The Benjamini-Hochberg method was used at a false discovery rate (FDR) of 0.05 to correct for 80 comparisons. None of the correlations remained significant when adjusted for multiple testing.
6.6 DISCUSSION

From the phenotypic analysis of the CD4+CD25hiFOXP3+ Treg subset in this chapter, we observe both naïve and TEMRA Treg phenotypes, with no significant change following MV. This is consistent with cord blood studies that have shown Tregs to be predominantly of the naïve phenotype (Kanegane, Miyawaki et al. 1991, Wing, Ekmark et al. 2002, Ly, Ruiz-Perez et al. 2009, Flanagan, Halliday et al. 2010), and presumably they gradually progress towards a predominant memory phenotype throughout life.

Using CCR7 expression, we distinguished the memory Tregs into effector memory (EM) and central memory (CM) populations at both time-points, as described previously (Tosello, Odunsi et al. 2008). There was a reduction in the expression of the lymph-node homing marker CCR7 following measles vaccination thus suggesting an increase in effector memory Tregs, as might be expected shortly after vaccine administration. This finding was borderline significant, which is likely to be due to the small sample size.

Miyara et al. described 2 subpopulations of Tregs, which are phenotypically and functionally different; namely CD45RA+FOXP3lo resting Treg cells (rTreg cells) and CD45RA-FOXP3hi activated Treg cells (aTreg cells) (Miyara, Yoshioka et al. 2009). They found that the CD45RA-FOXP3hi activated Tregs rapidly die whereas the CD45RA+FOXP3lo resting Tregs (rTregs) proliferate and convert into activated Tregs (aTregs). From their microarray analysis, they also saw that aTregs were more
involved in the transcription of IL-10 and less involved in the transcription of TGF-β as compared to the rTregs. Whether the subsets use other mechanisms of suppression is yet to be determined. Using cord blood, Miyara et al., found a small population of proliferating CD45RAloFOXP3hi Tregs that resemble CD45RA-FOXP3hi aTregs in adults (Miyara, Yoshioka et al. 2009). Although the frequency of this subset was very low in the cord blood in the Miyara study, its’ presence nevertheless suggests that rTregs can become activated memory Tregs even in the very young. In our study infants, we compared FOXP3hi (activated) and FOXP3lo (resting) populations within the CD4+CD25hiFOXP3+ Tregs, and evaluated the four different functional T cell subsets using co-expression of CD45RA and CCR7. Interestingly, the FOXP3lo Tregs comprised comparable subsets to that observed in the conventional T cells, where we observe higher numbers of CD45RA+ cells (both Naïve and TEMRA). In the FOXP3hi Tregs, the majority of the cells were CCR7+ TCM cells. This suggests that Tregs, particularly the FOXP3hi population, undergo preferential differentiation from the CD45RA+ to the CD45RA- memory phenotype compared to the conventional T cells. A previous study conducted using cord blood and adult peripheral blood also observed preferential differentiation of the Tregs into memory cells but mainly of the EM rather than the CM phenotype (Santner-Nanan, Seddiki et al. 2008). A published PhD thesis similarly found that the CM subset was more pronounced in the Tregs of healthy adult donors, as compared to the conventional T cells (Hong-Lei 2014). Nevertheless, when we compared the functional subsets before and after measles vaccination, we found no evidence that MV alters the proportion of these Treg populations.
Of particular note was the increased proportion of IL-10 producing CD4+ T cells following MV, the majority of which were FOXP3 negative and CD25hi negative, suggesting that they may represent the Tr1 subset of Tregs (Levings and Roncarolo 2000). There was no increase in IL-10-producing CD4+CD25hiFOXP3+ Tregs in our study. IL-10 was first described as cytokine synthesis inhibitory factor (CSIF), shown to be produced by mouse Th2 cells inhibiting Th1 cell activation and cytokine production; however more recent studies also describe a subset of Th1 cells which co-express IL-10 and IFN-γ (reviewed by (Cope, Le Friec et al. 2011). Our data however shows that the IL-10 producing CD4+ T cells were mainly IFN-γ negative and IL-4 negative, further supporting our suggestion that this subset may be Tr1 Tregs. Following measles infection in children in sub-Saharan Africa, there were elevated levels of IL-10 for weeks, which was suggested to contribute to the impaired cellular immune response and increased hypersensitivity observed following infection (Moss, Ryon et al. 2002). Whether measles vaccination causes an increase in IL-10 producing CD4+ T cells in young infants is not clear. Nevertheless, an increase in IL-10 producing Tregs following neonatal vaccination with BCG has been previously reported (Akkoc, Aydogan et al. 2010). Further studies are required to determine whether the increase in IL-10 by the CD4+ T cells was a real effect as it was not significant after correcting for multiple testing.

We found that the proportions of IL-2 and IFN-γ producing CD4+ T cells in culture did not change following MV in males or females. By contrast, there was an increase in the proportion of IFN-γ producing CD8+ T cells following vaccination for males and
females either combined or analysed separately. However, the absolute frequencies of the single and multiple cytokine producing cells did not alter significantly following measles vaccination. Analysis for an association between the circulating pre-vaccination Treg frequencies and the cytokine effector readouts showed no significant correlations for both the 8-hour and the 3-day cultures.

Two measles virus receptors, CD46 and CD150, have been previously described, the latter also known as signaling lymphocyte activation molecule (SLAM). Wild-type measles virus strains use human SLAM as a cellular receptor (Tatsuo, Ono et al. 2000), while vaccine strains such as the Edmonston strain used in our study can use both SLAM and CD46 as a receptor (Naniche, Varior-Krishnan et al. 1993). CD46 is not only a pathogen receptor, but also a co-stimulatory molecule expressed on all nucleated cells (Astier, Trescol-Biemont et al. 2000). It was interesting that although there was no significant change in the expression of CD150/SLAM by the CD4+ T cells, there was a significant increase in CD46 expression. The latter has a significant role in Th1 immunity as underscored by the inability of CD46 deficient CD4+ T cells to mount a sufficient Th1 response (Couzi, Contin-Bordes et al. 2008), while Th2 responses were not affected. Signalling through the CD46 receptor has also been shown to cause a switch to IL-10 producing Tregs when Th1 cytokines accumulate as a homeostatic control (Cardone, Le Friec et al. 2011, Cope, Le Friec et al. 2011). Furthermore, CD46 co-stimulation in the presence of interleukin-2 (IL-2) and T cell receptor (TCR) stimulation has been shown to induce the production of IL-10 by cells, which resemble Tr1 Tregs, and suppress effector T cell proliferation (Kemper,
Chan et al. 2003). Thus the induction of CD46 on CD4 T cells following MV is likely to bias towards a mixed Th1 and Tr1 response, which is consistent with the CD4+IL-10+ cell increase, alongside the IFN-γ skewing of the CD8 T cell population.

There was a significant reduction in the expression of STAT4 by the CD4+ T cells in the IL-2+TNF STCL cultures, two weeks after the administration of MV compared to baseline responses. Previous studies have suggested that TNF impairs Treg function (Valencia, Stephens et al. 2006), however more recent studies show the opposite with TNF enhancing the regulatory capacity of the Tregs (Chen, Wu et al. 2013, Zaragoza, Chen et al. 2016). Interestingly, a very recent study in which anti-TNF antibody, adalimumab, was expected to block Treg function; it was unexpectedly shown to enhance the expression of TNF and augment its’ binding to the TNFR2 receptor expressed on the Tregs in rheumatoid arthritis patients, therefore resulting in Treg expansion (Nguyen and Ehrenstein 2016). The mechanism behind the observed expansion of the Tregs was shown to be driven by IL-2/STAT5 signalling mediated by the expression of the TNFR2 receptor. However, we did not find an expansion of Tregs in cultures containing IL-2+TNF, nor any evidence that the Tregs caused suppression of cytokine responses in these cultures. In contrast, we observed positive correlations between the Tregs in culture and the CD4 and CD8 pro-inflammatory cytokine responses and Ki67 expression. This suggests that these may be activated effector cells rather than induced Tregs.

We recognise certain limitations in this part of the study. When compared to a healthy adult donor, the PBMCs collected from the study infants had less distinct
CD4 and CD8 populations with very low proportion of CD8+ T cells. The plots show an intermediate population, which is not observed in the adult donor. These may possibly be CD4+CD8+ double positive cells, expressing the co-receptors CD4 and CD8 simultaneously, which accounts for around 1% of all T cells within PBMCs (Quandi, 2014). There are reports of premature release of thymocytes in infants in response to environmental stress such as maternal undernutrition and this results in relatively high levels of CD4+CD8+ double positive T cells. Double-positive T cells have been described in individuals with chronic viral infections (Macchi, Graziani et al. 1993, Giraldo, Bolanos et al. 2011, Durand, Buckheit et al. 2015) (Macchi 1993; Giraldo 2011; Durand 2015). Some of the observed CD3+CD4-CD8- T cells may also possibly be the gamma-delta T cells. In Gambian infants, Sarah Burl observed two populations of double positive T cells; the CD4hiCD8int population which were mainly seen in the ex-vivo population and consisted of 1-2% of the lymphocyte population, and the CD4intCD8hi population which observed following SEB stimulation and consisted of 27-37% of the lymphocyte population. From the latter, around 25% expressed FOXP3 (Burl 2009). Whether some of the double positive cells observed in our cohort are FOXP3+ Tregs is yet to be determined.

However, using the same cohort in Chapter 5, we also observe low proportions of CD8 but not as low as in this chapter. It may be that the CD8 antibody does not stain as well as it does on adult cells. Another plausible explanation is that the cryopreservation procedure may possibly cause a downregulation of CD8 expression, or affect the staining of the CD8 antibody.
Although we used a healthy adult control to ensure that the assay worked, we should have included a bead control to standardize the staining between the different panels. Another limitation is that only a small number of participants were tested in this study due to logistic reasons. The number of participants used in each panel varied as panels 1 and 4 were given priorities. Only 8 out of the 10 donors had enough cell numbers for all the panels. With such small numbers, it is unlikely that significant effects of vaccination would be observed.
CHAPTER 7 GENERAL DISCUSSION

This thesis presents an attempt to determine the role that Tregs play in attenuating the immune response to measles vaccination. We found evidence of Treg mediated suppression and go on to propose a possible mechanism of action used by Tregs to suppress MV immunogenicity. The significance and implications of our results will be discussed herein.

7.1 EVIDENCE FOR CD4+FOXP3+ TREG MEDIATED SUPPRESSION OF MEASLES VACCINE, BUT NOT DTP VACCINE ANTIBODY RESPONSES

In Chapter 3, two of the study participants had post-vaccination measles HAI titres that were below a \( \log_2 \) titre of 3 (125mIU), which is defined as the protective level (Samb, Aaby et al. 1995). A recent study reports cases of measles vaccinated individuals who fail to respond even after receiving two doses of MMR vaccine (Fiebelkorn, Coleman et al. 2016). The main risk factor was having low/negative measles antibody levels at baseline therefore suggesting that inherent biology may have a role to play in individuals who did not have protective measles antibody titres despite being vaccinated. This may possibly explain why the two donors in our cohort failed to respond to measles vaccination at 9 months of age.
We then correlated the antibody response with the Treg frequencies but found no evidence that Tregs affect secondary antibody responses to DTP vaccination. In contrast, our key finding in several of the chapters is the significant inverse correlation between baseline CD4+FOXP3+CD127lo or CD4+CD25hiFOXP3+ Tregs, and subsequent measles antibody responses (Chapters 3 and 5, respectively). It may be such that the dynamics of the Treg response following DTP differs from the response following measles vaccination. The timing of the blood sample collection could have missed the peak Treg response following DTP vaccination.

I thereby propose that circulating CD4+CD25hiFOXP3+CD127lo Tregs at the time of vaccination suppress the primary antibody response to measles vaccination. Several mechanisms for Treg mediated suppression of antibody responses have been proposed in the literature. These include suppression of helper T cells required for antibody responses (Lim, Hillsamer et al. 2004) and directly B cell suppression via cell-to-cell contact mechanisms (Lim, Hillsamer et al. 2005) (Figure. 7.1). Which method is responsible for the suppression of antibody responses observed in our study is yet to be determined.
A. Direct Suppression of B cells

CD4+CD25hiFOXP3+
CD127lo Treg

B. Suppression of B cells via T helper cells

CD4+CD25hiFOXP3+
CD127lo Treg

Tfh

B cell

Figure 7. 1: Proposed mechanisms of action used by CD4+CD25hiFOXP3+CD127lo Tregs to suppress measles vaccine antibody responses

Baseline CD4+FOXP3+CD127lo and CD4+CD25hiFOXPE+ Tregs suppress measles antibody response either (A) by directly suppressing B cell response via cell-to-cell contact mechanism as has been described (Lim et al., 2005); or (B) indirectly by suppressing the T helper cells (Lim et al., 2004). Tfh - follicular helper T cells.

This suggests that Tregs could provide a functional target for enhancing vaccine antibody responses in infancy, the target age group for many vaccines (Levy 2007). Understanding the mechanisms used by these Tregs to regulate vaccine responses would therefore contribute to the knowledge base required to improve the efficacy of current childhood vaccines. To our knowledge, this is the first evidence that
functional peripheral Tregs negatively regulate functional effector readouts to measles vaccination in infants.

7.2 LITTLE EVIDENCE FOR TREG MEDIATED SUPPRESSION OF MEASLES VACCINE CELL MEDIATED IMMUNE RESPONSES

The CMI response is important for the recovery from measles virus infection (Griffin 2010). Although earlier studies used the humoral response as a read-out of vaccine immunogenicity, the importance of CMI in measles virus clearance has now been recognized (Griffin, Lin et al. 2012). The data showed little evidence that circulating Tregs at the time of vaccination have any effect on subsequent vaccine-specific cellular immunity. This is beneficial as with the high Treg numbers in our cohort, it would be deleterious if induction of vaccine specific cellular immunity were suppressed.

In the co-culture suppression assay in Chapter 4 however, we do observe suppression in ELISpot IFN-γ response upon the addition of the isolated Tregs to the PBMCs in two out of the ten study participants (20%). This was not observed in the remaining donors but may suggest that measles-specific suppressive Tregs might have been induced. When we correlated the Treg frequencies with the antigen-specific cytokine response postvaccination, the correlation was not robust and was rather inconsistent. Although some suppression is detected in some of the donors in the co-culture suppression assay, it may be that suppression was not evident when correlation was used as a readout of Treg suppression. In the ELISpot assay, the IFN-γ may be possibly produced by different cell types and not just the CD4+ and CD8+ T
cells as assessed by the ICS assay. More recently, B cells have been shown to induce both antigen-specific and non-specific Tregs, as observed in both in vivo and in vitro models (Chien and Chiang 2017). Thus, other cell types in the PBMC fraction, such as the B cells may possibly induce suppressive Tregs in the co-culture suppression assay.

Another plausible explanation is that we may have missed the opportunity as we took samples at a single timepoint following measles vaccination. When cytokine responses were monitored over a period of six months in rhesus macaques, the cytokine responses peaked at various timepoints (Nelson, Putnam et al. 2017). In the same study they showed that both IFN-γ and IL-17 peaked at various timepoints; with IFN-γ being produced by both the CD4 and CD8 T cells in early response. In the late response, CD8 T cells were the main source of IFN-γ whilst IL-17 was produced by both CD8 and CD8 T cells, and was produced in the later rather than early stages of infection. The IL-17 response is important role in viral clearance (Xie, Chen et al. 2011), however, we did not investigate the IL-17 response in our vaccinated infants. This highlights the dynamics of the CMI during measles infection, and underscores the need to assess for varying cytokines at different timepoints rather than a single timepoint when investigating vaccine-induced responses.

Furthermore, the role of Tregs in suppressing immune activation induced by vaccination is not known, but previous studies show that Tregs protect individuals from the exaggerated immune activation in individuals with HIV infection (Belkaid and Rouse 2005). In the present study, there was no evidence that Tregs played a
role in the altered immune activation and proliferation following measles or DTP vaccination.

7.3 LITTLE EVIDENCE FOR AN IMMUNOSUPPRESSIVE ROLE FOR TNFR2+ TREGS IN INFANCY

Although not extensively studied as the CD4+CD25hi FOXP3+ Treg subset, the TNFR2-expressing Tregs have been shown to be maximally suppressive in adults (Minigo, Woodberry et al. 2009, Govindaraj, Madondo et al. 2014). Nevertheless, its role in the suppression of immune responses in infants remains to be explored.

Contrary to our hypothesis, there was no evidence for the upregulation of TNFR2+ Tregs nor for the suppression of vaccine-induced cell mediated and antibody-mediated immune responses. We mainly observed a positive association between the Tregs and vaccine-induced T cells responses. This is similar to what is observed in some inflammatory diseases (Lord, Valliant-Saunders et al. 2012, Lecendreux, Churlaud et al. 2017).

7.4 EVIDENCE OF SEX-DIFFERENTIAL IMMUNE RESPONSES IN OUR COHORT

In Chapter 3 we showed an increase in CD4+FOXP3+CD127lo Tregs in measles vaccinated females, and a decline in DTP-vaccinated males. This decline in Tregs may potentially allow for a greater inflammatory response in DTP vaccinated males compared to females, as evidenced by the significantly greater Th1 cytokine response to T cell stimulation among males compared to females in our published
study from this cohort (Noho-Konteh, Adetifa et al. 2016). A plausible explanation is that DTP may cause a preferential expansion of conventional T cells in males, thereby resulting in the subsequent drop in the Tregs. Sex differences in immunity (Klein and Flanagan 2016), and more specifically vaccine responses (Klein, Jedlicka et al. 2010) have been well described in the literature. Amongst a range of age groups, the sex-differences in vaccination with regards to vaccine-induced immune responses, side effects and vaccine efficacy (Flanagan and Plebanski 2017). Although males are more likely to receive vaccines, following vaccination, females typically develop higher antibody responses and report more adverse effects of vaccination than do males but are not usually considered in pre-clinical studies. However this is the first description of sex-differential changes in Treg frequencies following infant vaccination.

There are a number of described mechanisms whereby immune responses may differ in males and females (Klein and Flanagan 2016). These include the immunological effects of sex hormones, and differing expression levels of sex-linked immune response genes (Fish 2008) and microRNAs (Pinheiro, Dejager et al. 2011). The intracellular expression of FOXP3 may differ by sex as it is encoded on the X-chromosome (Fish 2008). Additionally, several immune cells express sex-hormone receptors, the levels of which differ in males and females even in early life (Flanagan 2015). Androgens such as testosterone are shown to be immunosuppressive, whilst oestrogens are generally pro-inflammatory. Although females have slightly higher levels of oestrogen, we observe an opposing effect with males generally exhibiting a
decline in the immunoregulatory response. This may possibly be that by 9 months of age, the hormones have levelled out (Flanagan 2015).

However, we were not able to replicate these findings in Chapter 5 where we looked CD4+CD25hiFOX3, TNFR2-expressing CD4+CD25hiFOX3+ and CD4+FOX3+TNFR2+ Treg populations following measles vaccination. There are several potential reasons for this discrepancy. One is that the increase in females in our first study was not significant after correcting for multiple testing and therefore may not have been a real effect. Another factor is that we measured responses at 2 weeks in the cohort in Chapter 5 and not the 4 weeks post-MV in the first cohort (Chapter 3). Jaye et al. found that the measles-specific effector T cell responses following measles vaccination were comparable to that seen following natural infection (Jaye, Magnusen et al. 1998). In measles infected individuals, the measles-specific effector T cell responses were stronger in the earlier weeks following which, they wane and the regulatory response kicks in. The dynamics may be such that two weeks may be too earlier to investigate the Treg response, and may peak later than two weeks to counteract the early effector T cell response.

Nevertheless, the humoral response was comparable in males and females in all vaccine groups; i.e. when MV and DTwP were administered alone or at the same time. In the present study, we did not observe any significant sex-differences in the humoral responses using both the HAI neutralization and measles IgG ELISA assays.
7.5 Evidence that IL-10 producing Tregs are induced following infant measles vaccination

The studies in this thesis were primarily designed to analyse for the regulatory function of FOXP3+ Tregs. However, we observed an induction of IL-10 producing CD4+ T cells which resemble Tr1 cells following measles vaccination in our cohort.

Measles-virus induced immunosuppression has been associated with increased IL-10 production (Moss, Ryon et al. 2002, Griffin 2010) by Th2 and Treg cells. Whether the live attenuated vaccine strain similarly causes immunosuppression by inducing Tregs remains unclear.

In humans, Immune tolerance is vital for immune homeostasis, with tTregs mediating central tolerance and pTregs regulating peripheral tolerance. Unlike the classical FOXP3+ and CD25+ Tregs, IL-10 producing Tr1 Tregs do not normally express CD25 or FOXP3. They have been characterized as expressing LAG3 and CD49b, and confer suppression of T cells by producing IL-10 (Zheng, 2015). Furthermore, crosslinking of CD46 on T cells has been shown to induce Tregs and potent production of IL-10 (Kemper, Chan et al. 2003).

Based on these previous reports, coupled with our phenotyping results, we propose a possible mechanism by which Tr1 cells modulate CD4+ T cells in measles vaccinated 9 month-old infants, leading to vaccine-induced IL-10 producing Tr1 cell upregulation. Measles vaccination is known to induce a pro-inflammatory CD8+ T
cell IFN-γ response, and we propose that this causes an upregulation of CD46 by CD4+ T cells, which facilitates entry of the virus. The CD46 receptor expression by CD4 T cells then causes a switch from a Th1 phenotype into IL-10 producing Tr1 regulatory T cells when pro-inflammatory cytokines accumulate. The induced Tr1 Tregs may downregulate STAT4 expression in a negative feedback homeostatic loop resulting in the reduction in CD4+IFN-γ+ T cells (Figure 7.2). We therefore propose that Tr1 Tregs may regulate measles vaccine responses in infants by the production of the inhibitory cytokine IL-10. Whether this actually happens in vivo would need to be studied and confirmed.

Measles vaccine enters immune cells via the CD46 receptor expressed by immune cells and induces a pro-inflammatory response, predominantly from CD8 T cells with increased production of IFN-γ (A). The increased IFN-γ leads to up-regulated CD46...
expression which facilitates further measles virus entry (B) but also causes a switch to
IL-10 producing Tr1 Tregs. The IL-10 from the Tr1 cells causes a negative feedback
loop, causing reduced STAT4 expression by CD4 T cells (C)

7.6 STUDY LIMITATIONS

7.6.1 Limited cell numbers for functional assays

Due to the limited amount of peripheral blood collected from our study infants,
combined with the relatively low numbers of regulatory T cells in peripheral blood,
we were not able to perform consistent and adequate functional assays. We
therefore opted to evaluate Treg function by analysing for associations between the
Tregs and the effector responses as a surrogate marker of suppressive function. Such
studies can only describe associations but not confirm Treg mediated suppression.

7.6.2 Variability in assays to measure measles-specific responses

For the first cohort (Chapter 3), a haemagglutinin measles peptide pool was used for
analysing measles-specific responses, whereas in the second cohort, the live measles
virus was used. We found limited reactivity to the live measles virus in vitro, and
although it is widely used to test for measles-specific immunity, it is known to have
immunosuppressive properties. In Chapter 6, we were unable to use the live measles
virus to evaluate for measles-specific responses due to health and safety precautions
at Monash University, and therefore non-specific stimulation was used instead.

For the first cohort, the measles HAI neutralization assay was used to measure the
measles titres, which is a functional assay. In the second cohort, the measles IgG
ELISA kit was used. This kit, however, has an excellent correlation with the
neutralization assay, and we found evidence of Treg suppression of measles Abs measured by both assays.

7.6.3 Sample size limitations

For most of the thesis we had robust numbers of infant samples. However, for the detailed phenotyping of the Tregs and effector T cells, samples from only 10 participants were used due to financial and time constraints. This limited our ability to make some significant conclusions.

7.6.4 A single bleeding time point

We only analysed at a single post-vaccination time point, and thus do not know the dynamics of the effector T cell or Treg response following vaccination. This single snapshot may lead us to make erroneous conclusions or miss important changes that occur over time following vaccination.

7.6.5 Other methodological issues

Different Treg phenotypes were analysed in the first and second studies (CD4+FOXP3+CD127lo/- and CD4+CD25hiFOXP3+ respectively). This was due to a change in emphasis on particular Treg phenotypes during the course of the study. However, both phenotypes have been shown to be functionally suppressive in humans and are often used interchangeably and the Tregs in the second study were confirmed to be predominantly CD127lo/neg.

For the first part of the study (Chapter 3), a live/dead marker was not included in the flow cytometry panels due to limitations in the number of markers I could analyse,
thus I could not gate out the dead cells. Having said that, all the samples used were stained fresh from whole blood within hours of collection, which usually yields good viability rates while the second study utilised thawed PBMC which are more susceptible to apoptosis and cell death. In this latter study I included the live/dead marker to gate the dead cells out.

7.7 IMPLICATIONS OF THE RESULTS OF THIS THESIS

The immune response within the first year of life is compromised compared to older children and adults. Previous studies have shown that infant Treg levels are high and cord blood Tregs are known to be functional (Wing, Ekmark et al. 2002, Godfrey, Spoden et al. 2005), thus it is important to investigate the implication of this with regards to infant vaccine responses. As Tregs decline in the second year of life, it is tempting to suggest that delaying measles vaccination until after 12 months may result in enhanced vaccine-induced immunity, or alternatively offering a second dose at a later age. In our cohort, two of the study participants had post-vaccination measles HAI titres below the protective levels; hence there is a need to explore this further, especially in infants who fail to achieve protective measles antibody levels. It might also be important to consider whether using vaccine formulations, which modulate the function and/or numbers of the Tregs in this age group, could improve the suboptimal responses to certain vaccines observed in infancy.

Studies also show that previously induced Tregs can suppress responses to an unrelated antigen resulting in a ‘bystander’ or ‘heterologous’ suppression effect which can compromise immune responses (Joosten and Ottenhoff 2008). An
example of this is observed in helminth infections, where Tregs have been shown to be induced, and vaccination in helminth-infected children can compromise the immunogenicity of the BCG and tetanus vaccines (van Riet, Hartgers et al. 2007). Our cohort of infants were healthy and recruited from a region where helminth infections are uncommon (Noho-Konteh, Adetifa et al. 2016), so this is unlikely to have played a role in our study, but other factors which alter the Treg profile could potentially affect vaccine immunogenicity.

Previous studies have observed high Tregs in malaria-infected individuals (Torres, Villasis et al. 2014), more so in patients with severe malaria. The Tregs also correlated with levels of parasitaemia. One of the ethnic groups in The Gambia, known as the Fula, have reduced expression of Treg markers such as FOXP3 and CTLA-4 and are relatively resistant to the *P. falciparum* malaria parasite (Torcia, Santarlasci et al. 2008). The Gambia is a malaria-endemic country and in some parts of The Gambia, it is one of the leading causes of death in infants with a mortality rate of 4.11 per 1,000 person-years [95% CI: 3.09–5.47] (Jasseh, Howie et al. 2014). Prior exposure to malaria or being infected with malaria at the time of vaccination may affect the vaccine response and with high Tregs in infancy; this should be considered.

The live-attenuated BCG vaccine has been shown to induce IL-10 producing Tregs (Akkoc, Aydogan et al. 2010). BCG vaccination has also been associated with protection against several conditions including allergy and asthma, diabetes and melanoma. Whether Treg induction plays a role in this altered disease susceptibility
is not known. However, if measles vaccination induces Tr1 Tregs, then in the future it could similarly be used to prevent certain inflammatory conditions.

TLR agonists are currently being investigated as potential vaccine adjuvants in young children (Levy, Suter et al. 2006). Interestingly, TLRs have been shown to modulate the immunosuppressive effects of Tregs directly as Tregs express TLRs, and indirectly through APCs (Liu, Zhang et al. 2010). BCG has been shown to be ‘self-adjuvanted’ by being able to stimulate various TLRs, possibly explaining why adult-like Th1 responses to BCG are seen in infants (Marchant, Goetghebuer et al. 1999). Modulating Treg function in vaccinated neonates and young infants is therefore a promising approach to improving vaccine immunogenicity, permitting potent immune responses despite poorly developed adaptive immunity at this age (Levy, Suter et al. 2006, Burl, Townend et al. 2011).

Currently, a number of agents that transiently deplete Tregs at the site of vaccination are being used in human trials in order to enhance vaccine efficacy (Ndure and Flanagan 2014). Monoclonal antibodies such as Basiliximab and Daclizumab are anti-human CD25 MAbs. By blocking IL-2 signalling, these MAbs modulate the number and function of Tregs (Mitchell, Cui et al. 2011). Another MAb Ipilimumab has been approved by the FDA to be used for treatment of melanoma patients, and it modulates Treg function by blocking CTLA-4 (Peggs, Quezada et al. 2009).

Another approach is to alter the effector T cell: Treg ratio by preferentially expanding the conventional T cells over Tregs (Ndure and Flanagan 2014). Examples of these include innate agonists such as Poly (I:C) and the CpG-ODN which are TLR3
and TLR6 agonists respectively. A monoclonal antibody, a member of the TNFR superfamily OX40, augments effector T cell function. Indeed, OX40 clones have been humanized and are used as vaccine adjuvants (Voo, Bover et al. 2013). All these offer promising prospects of manipulating Tregs in the future to improve vaccine immunogenicity.

7.8 FUTURE WORK

This thesis hints at an immunosuppressive role for Tregs in the context of measles vaccination. To further investigate our suggested models of Treg suppression, a subsequent study with a larger cohort, additional time points, and higher cell numbers to conduct the suppression assays with optimal measles immunogenicity readouts would be ideal. We propose the studies below to continue the line of research presented in this thesis.

7.8.1 In vitro studies in humans

My particular interest would be to analyse for the effect of isolated CD4+CD25hiFOXP3+CD127lo/- Tregs on B cell function in vitro to determine whether suppression occurs via the direct action of Tregs on B cells or via helper T cell modulation. To do this I would isolate B cells, T cells and APCs and perform in vitro experiments of B cell function using the B cell ELISpot for example. We can perform transwell and antibody blockade experiments to investigate the role of cell-to-cell contact, and different cytokines and Treg markers.
I would also like to investigate whether modulation/depletion of Tregs (CD4+CD25hiFOXP3+CD127lo/- and Tr1) in vitro augments vaccine-induced effector responses. I would particularly like to investigate blocking Tr1 cells in vitro by using monoclonal antibodies against Tr1 specific markers LAG-3 and CD49b to determine whether this results in increased CD4+ and CD8+ T cell IFN-γ responses.

### 7.8.2 In-vivo studies using the murine model

Studies have shown that transient modulation/depletion of Tregs in BCG-vaccinated mice (Ho, Wei et al. 2010) are associated with improved vaccine responses. Due to ethical and safety concerns, using animal models would provide the most suitable tool to better understand the mechanism employed by Tregs within the context of measles vaccination. I would therefore propose to use the humanized mouse model to deplete the CD25hi Tregs in-vivo by using low concentration of CD25 monoclonal antibody Daclizumab to investigate whether the transient depletion of CD25hi Tregs result in increased cellular or antibody responses to measles vaccination.

We observed a significant decline in the CD46 receptor following measles vaccination in our cohort. As we propose that the CD46 receptor may be involved in the activation of Tr1 Tregs following vaccination, I would like to investigate using the mouse model whether blocking CD46/IL-10 results in an increase in IFN-γ production. This can be done using CD46 transgenic mice as previously described (Greig, Buckley et al. 2009).
7.8.3 Gene expression profiling studies

RNA can be extracted from sorted Tregs to do a targeted assessment of the Treg transcriptome profile following measles vaccination compared to the pre-vaccination. Technology is now available to do this at the single cell level. The full Treg gene expression profiles before and after vaccination would provide unprecedented insights into Treg function and mechanisms of action, which could then be explored in further in vitro and in-vivo experiments.

7.9 CLOSING REMARKS

Our results support a role for Tregs in regulating measles vaccine responses. Transient modulation of Tregs has been proposed as a therapeutic intervention to improve vaccine responses, possibly by using TLRs as adjuvants or adding small doses of anti-CD25, but this research is still in its infancy. Tregs are an essential component of the immune system and are required to prevent an over exuberant response and bring the immune response back to normalcy. Thus this approach to improving vaccine immunogenicity is complex and much more knowledge is required before it can be applied to infant vaccination strategies.

This study provides a rationale and basis for future studies of the role of Tregs in infant vaccine responses. A time may come when Tregs can be manipulated in early life to improve vaccine responses. The prospect of modulating Treg function in neonates and young infants in order to improve vaccine immunogenicity is an exciting one, and we hope the results presented in this thesis will galvanize others into investigating this important question.
8.0 REFERENCES


9.0 APPENDIX

Blood Processing Sheet

<table>
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<th>Study No.: TREG</th>
<th>Protocol: SCC1230 version:</th>
</tr>
</thead>
</table>

Collection date (dd/mm/yy): | |
Time (hh:mm): |

Processing date (dd/mm/yy): | |
Time (hh:mm): |

Processed by: _______________________________ Signature: _______________

<table>
<thead>
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<th>Processed whole blood volume</th>
<th>mL</th>
</tr>
</thead>
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<tr>
<td>Plasma Volume</td>
<td>ml</td>
</tr>
<tr>
<td>Storage location (-20 °C)</td>
<td></td>
</tr>
<tr>
<td>Freezer no.</td>
<td></td>
</tr>
<tr>
<td>Rack</td>
<td></td>
</tr>
<tr>
<td>Box</td>
<td></td>
</tr>
<tr>
<td>Position</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell counts</th>
<th>Live</th>
<th>Dead</th>
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</thead>
<tbody>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<p>| Viability: live/total no of cells x 100 (%) | 100% |
| Dilution factor (D):                       | 10   |
| Correction factor (F):                      | 10^-4|
| Resuspension volume (R):                    | mL   |
| Total no of viable cells: viable cells x D x F x R | x10^6|
| Aliquot 1 x 10^6 for purity analysis by FACS (Y/N) |   |</p>
<table>
<thead>
<tr>
<th>Description</th>
<th>Units</th>
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<tr>
<td>Aliquot 2 x 10^6 cells for co-culture suppression assay (Y/N)</td>
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</tr>
<tr>
<td>No of cells remaining pre-isolation</td>
<td>x10^6</td>
</tr>
<tr>
<td>No of cells recovered post-isolation (TREG population)</td>
<td>x10^6</td>
</tr>
<tr>
<td>Aliquot 0.1 x10^6 cells for purity analysis by FACS (Y/N)</td>
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</tr>
<tr>
<td>Enough cells to set up ELISpot? (Y/N)</td>
<td>If Yes calculate Resuspension volume</td>
</tr>
<tr>
<td></td>
<td>ml</td>
</tr>
<tr>
<td>Study No.</td>
<td>TREG</td>
</tr>
<tr>
<td>-----------</td>
<td>------</td>
</tr>
</tbody>
</table>

**Child’s Initials**

**Date of Birth**

**Visit date**

**Has the study been explained to parent?**

**Has information sheet been given to parent?**

**Has parent given consent to participate in the study?**

**Has child received all EPI vaccines as scheduled to date?**

**Interim Medical History**

**Does the infant have any of the following?**

- Cough
- Wheeze
- Fever
- Diarrhoea
- Refusal to feed
- Other

**Vital signs and Anthropometry**

- Temp
- Resp Rate
- Pulse rate
- Weight
- Length
- Left mid upper arm circumference

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### Routine EPI Vaccines administered to date

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<th>Age in Weeks</th>
<th>Comments</th>
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<tr>
<td>HBV</td>
<td>__ / __ / __</td>
<td>__ / __ / __</td>
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<tr>
<td>OPV</td>
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</tr>
<tr>
<td>PENTAVALENT 1 PCV-7 + OPV</td>
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</tr>
<tr>
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</tr>
<tr>
<td>PENTAVALENT 3 PCV-7 + OPV</td>
<td>__ / __ / __</td>
<td>__ / __ / __</td>
<td></td>
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</tbody>
</table>

### Comments

________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________

Name (Physician/Nurse/Field Worker)  Signature  Date
**STUDY NO. TREG**  |__|__|__| ⇒ cont. Visit 1 Form

**To be completed by the Study Physician or Delegated Person**

### Inclusion Criteria

| Healthy infant aged 9 months | Yes |__| No |__| |
| Weight for age z-scores within 2 standard deviations of normal | Yes |__| No |__| |
| Has received all standard EPI immunizations | Yes |__| |

| Date of informed Consent | dd | / | mm | / | yy |__|__|__| |

### Exclusion Criteria

| Acute disease at the time of vaccination (defined as the presence of a moderate or severe illness with or without fever) including invasive bacterial infection (e.g. pneumonia, meningitis) | Yes |__| No |__| |
| Axillary temperature of ≥37.5 °C at the current visit | Yes |__| No |__| |
| History of allergic disease or reactions likely to be exacerbated by any component of the vaccine e.g. egg products, previous allergic vaccine reaction | Yes |__| No |__| |
| Presence of any underlying disease that might compromise evaluation of response to the vaccine, or on-going chronic illness requiring hospital specialist supervision | Yes |__| No |__| |
| Research Physician's assessment of lack of willingness by parents to participate | Yes |__| No |__| |
| Any history of anaphylaxis in reaction to vaccination | Yes |__| No |__| |
| Likelihood of travel away from the study area | Yes |__| |

Is the child eligible for the study? | Yes |__| |

| _________________________________ | ______________________ | ______________________ |

---

305
<table>
<thead>
<tr>
<th>Name (Physician/Nurse/Field Worker)</th>
<th>Signature</th>
<th>Date</th>
</tr>
</thead>
</table>


STUDY NO. TREG | | | | ⇒ cont. Visit 1 Form
If not eligible
Do not randomise
If eligible
Randomise to a group by selecting next available envelope
Yes | | No

Randomised to which group?
Group 1 | |
Group 2 | | Yes | | No | |
5ml of Blood collected
Yes | | No | |
Sample Collection Form completed?
Yes | | No | |
Sensitisation number entered in the Vaccine Dispensing log
Yes | | No | |

Group 1: MV GIVEN
Yes | | No

MV
Date Given (dd/mm/yy)
Age (weeks)
Batch
Site
LA = left arm
RA = right arm
Rout
e
IM = Intra
musc
ularly

Given by:
Nurse’s/FW’s Name
Signature
Date

Group 2: DO NOT GIVE ANY VACCINE

Has participant been given an appointment
to come back in 2 weeks? Yes | | No | |
Date of next appointment
| | / | | / | |
dd / mm / yy

STUDY NO. TREG | | | | ⇒ cont.
Visit 1 Form

Comments

_______________________________

_____________________________
<table>
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<tr>
<th>Name (Physician/Nurse/Field Worker)</th>
<th>Signature</th>
<th>Date</th>
</tr>
</thead>
</table>

---
| STUDY NO. TREG |   |   |   |   |   |   | IWC NO |   |   |   |   |   |   |
| Child’s Initials |   |   |   |   |   |   | SEX (M/F) |   |
| Date of Birth | dd | / | mm | / | yy | Age |   | weeks |
| Visit date | dd | / | mm | / | yy |
| Date of blood collection | dd | / | mm | / | yy |

**Blood collection 5 mL**

Purple top (EDTA 5 mL) Yes |   | ............ mL

**Comments**

________________________________________________________

Physician/Nurse/Field Worker Name __________________________ Signature __________________________ Date __________________________
### Infant Visit 2 Form – (2 weeks Post-Vaccination)

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<table>
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<tr>
<th>Visit date</th>
<th>Allocated field worker (initials)</th>
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<table>
<thead>
<tr>
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<table>
<thead>
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<th>Continued willingness to participate in the study?</th>
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<tbody>
<tr>
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### Interim Medical History

<table>
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<th>Does the infant have any of the following?</th>
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<tbody>
<tr>
<td>Cough</td>
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<tr>
<td>Wheeze</td>
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<tr>
<td>Fever</td>
</tr>
<tr>
<td>Diarrhoea</td>
</tr>
<tr>
<td>Refusal to feed</td>
</tr>
<tr>
<td>Other :</td>
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<table>
<thead>
<tr>
<th>Any changes to medical condition since last visit?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
</tr>
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*If ‘Yes’, please record below*

---

<table>
<thead>
<tr>
<th>Any medications taken since last visit?</th>
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<tbody>
<tr>
<td>Yes</td>
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</table>

*If ‘Yes’ please record names and times of medication(number of days) below*

---

---

310
### Vital signs and Anthropometry

<table>
<thead>
<tr>
<th>Temp</th>
<th>Resp Rate</th>
<th>Pulse rate</th>
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<th>Weight</th>
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<tbody>
<tr>
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<td></td>
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</table>

5ml of Blood collected: Yes [ ], No [ ]

Sample Collection Form completed?: Yes [ ], No [ ]

**Randomisation Group**

- 1 [ ]
- 2 [ ]

#### If Group 1, administer the vaccines below:

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### Comments

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Name (Physician/Nurse/Field Worker) 
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# Sample Collection Form Visit 2

**Post-Vaccination Bleed**

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## Blood collection 5 mL

Purple top (EDTA 5 mL) Yes | mL

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# Study Termination Form

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**If No state**

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312
| Subject died, | Yes | No | __| / | __| / |
|-------------|-----|----|----|-----|---|
| Physicin/PI's decision | Yes | No | __| / | __| / |
| Other reason | Yes | No | __| / | __| / |

Reason

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Has the child received all his/her due vaccinations to date? Yes | No |
Termination Form

If No give any due vaccinations and complete table below to show what given:

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Given by: ____________________________ ______________________
Name Signature

Study termination

Has parent/guardian been told that this study has ended
Yes ___ No ___
Has the sticker been removed from the infant welfare card
Yes ___ No ___
Has the parent/guardian been told to receive subsequent EPI vaccines from health centre?
Yes ___ No ___

Comments

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I certify that I have carefully examined all the entries on the case report forms and that all information entered on these pages by myself or associates is correct.

___________________________ ______________________
Study Physician’s Name Signature

Date

MV/Treg SCC1230
Regulatory T Cells and Vaccines

PARTICIPANT INFORMATION SHEET
Version 3.0   Date 19/05/11

Vaccines in The Gambia
Vaccines protect your child from diseases that they might otherwise become seriously ill with, or even die from. Vaccines work by teaching your immune system to recognise and fight an infection the next time you come into contact with it. All children in The Gambia are recommended to receive routine vaccinations in the form of the Expanded Programme on Immunization (EPI). This vaccination programme is very important for the health of your child and has saved millions of lives worldwide since it was introduced. How the immune system responds to vaccination is not very well understood, but this is important because we want to make better and more effective vaccines.

What are Regulatory T Cells?
When your body recognises a foreign particle, an immune response occurs. Regulatory t cells ensure that this immune response is controlled and does not become over-activated.

What is the aim of the study?
We would like to know if these regulatory t cells affect vaccines in any way. This is important because it will help in understanding how vaccines work, and also with the design of better and more effective vaccines for the future.

What happens if you agree to participate?
We would like to take a 5mL (1 teaspoon) venous blood sample from your child. We will then give your child another dose of measles vaccine and ask your child to come back 2 weeks later when we will take another 5mL blood sample.

If your child is unwell when they present for vaccination or blood sampling then the vaccine will be delayed / blood sample will not be taken. You may be given an appointment for a later date. There are no risks involved with taking the two 5mL blood samples from your child, and the measles vaccination is safe and will improve protection against measles.

Part of the sample collected from your child will be stored for future use, and may be sent abroad for tests we lack the facility to do locally.

Your rights
Your child will receive free health care at Sukuta Health Centre for one more year. We will compensate your travel costs to the clinic for each study visit. Should your child require health care outside of the Sukuta Health Centre for any reason the MRC will refer your child to the appropriate centres.

The blood will not be used for any other study without your consent. All information that you give us will be treated as confidential. You are free to leave the study at any time you wish without giving a reason for doing so. This will not affect your access to normal medical care.

MRC Contacts
Dr Katie Flanagan  MRC, Fajara  Office 4495442/3 ext 5003
Dr Jane Adetifa  Sukuta Health Centre
If you are willing to help us with this study then please sign or fingerprint the consent form.

THANK YOU.
I have read the information sheet fully, and / or it has been explained to me, and I have had a chance to ask questions about the study and had them answered.

I willingly and voluntarily agree for my child to participate in this study.

I understand that part of the samples will be stored for future use, and may be sent abroad for further tests that cannot be done locally.

I understand I am free to leave the study at any time, and that this will not affect my access to standard normal medical care.

Name of child _____________________________________________________

Study No: TREG[___|___|___]

Name of Parent or Guardian ____________________________________________

Mother / Father / Guardian (delete as appropriate)

Signature or thumbprint ________________________________________________

Date ___|___|___|___|___|___|___|2|0|___|___|___|___|___|___|___|

I _______________________________________________________(name), fieldworker, declare that I have explained the study to the above participant, that as far as I am aware they have understood what participation entails, and that they freely and willingly give their consent to participate.

Signature _________________________________________________________

Date ___|___|___|___|___|___|___|2|0|___|___|___|___|___|___|___|
Negative Correlation between Circulating CD4+FOXP3+CD127− Regulatory T Cells and Subsequent Antibody Responses to Infant Measles Vaccine but Not Diphtheria–Tetanus–Pertussis Vaccine Implies a Regulatory Role

Jorjoh Ndure1, Fatou Noho-Konteh1, Jane U. Adetifa1, Momodou Cox1, Francis Barker1, My Thanh Le1, Lady C. Sanyang1, Adboulie Drammeh1, Hilton C. Whittle1,2, Ed Clarke1, Magdalena Plebanski3, Sarah L. Rowland-Jones4 and Katie L. Flanagan1,3*

1 Infant Immunology Group, Vaccines and Immunity Theme, MRC Unit, Fajara, Gambia, 2 Department of Clinical Research, London School of Hygiene and Tropical Medicine, London, United Kingdom, 3 Department of Immunology and Pathology, Monash University, Prahran, VIC, Australia, 4 Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom

Regulatory T cells (Tregs) play a key homeostatic role by suppressing immune responses. They have been targeted in mouse and human cancer studies to improve vaccine immunogenicity and tumor clearance. A number of commercially available drugs and experimental vaccine adjuvants have been shown to target Tregs. Infants have high numbers of Tregs and often have poor responses to vaccination, yet the role Tregs play in controlling vaccine immunogenicity has not been explored in this age group. Herein, we explore the role of CD4+FOXP3+CD127− Tregs in controlling immunity in infant males and females to vaccination with diphtheria–tetanus–whole cell pertussis (DTP) and/or measles vaccine (MV). We find correlative evidence that circulating Tregs at the time of vaccination suppress antibody responses to MV but not DTP; and Tregs 4 weeks after DTP vaccination may suppress vaccine-specific cellular immunity. This opens the exciting possibility that Tregs may provide a future target for improved vaccine responses in early life, including reducing the number of doses of vaccine required. Such an approach would need to be safe and the benefits outweigh the risks, thus further research in this area is required.

Keywords: vaccines, regulatory T cells, sex, beta-2 microglobulin, cytokines, immune activation, antibodies

INTRODUCTION

The human immune system has evolved multiple mechanisms to ensure the induction of protective immunity, whilst regulating damage from over exuberant or uncontrolled immune responses. Specialized T cell subsets known as regulatory T cells (Tregs) play a vital role in this process by restoring and maintaining a homeostatic environment following an inflammatory response to an immunogen (1).
Regulatory T cells were initially identified in mice as CD4+ T cells highly expressing CD25 (the alpha chain of the IL-2 receptor) (2); but the later discovery of the forkhead family transcription factor FOXP3 further defined Tregs, being central to their development and function (3, 4). Its importance is highlighted in Scurfy mice and humans with IPEX syndrome where FOXP3 mutation results in uncontrolled inflammation and death (5). In humans, CD25 is also expressed by activated T cells (3), and it is the CD25+ subset that is suppressive (6). Low-level expression of CD127 (the IL-7 receptor) further defines functional Tregs in humans (7). Approximately 1–5% of human CD4+ T cells are CD4+FOXP3+ Tregs (4) and are either thymus derived natural Tregs or peripherally derived pTregs, which are indistinguishable. Tregs act by several modes of action including the induction of inhibitory cytokines, modulation of dendritic cell function, cytolyis, and metabolic disruption (8, 9).

Neonates and young children have highly adapted immune systems as compared to adults (10). Tregs in early life are highly suppressive, are present in higher frequencies than in adults, and are more naïve and less differentiated (11–14). The role of Tregs in controlling inflammation in infectious diseases remains controversial, with studies reporting both beneficial effects of limiting immunopathology and detrimental effects with suppression of protective immunity allowing pathogen survival and persistence (15). Little is known about the role Tregs play in controlling vaccine immunogenicity in animals or humans (16). There is a need to determine whether circulating Tregs at the time of vaccination interfere with vaccine-induced responses, particularly in infants who suffer the greatest burden of infectious diseases, and where vaccine immunogenicity is often poor and Treg levels high. Understanding the homeostatic role of Tregs in infant vaccination may provide strategies to improve vaccine immunogenicity in this vulnerable age group.

We investigated for the first time whether preexisting circulating CD4+FOX3+CD127- Tregs influence antibody and cellular responses following vaccination with the live measles vaccine (MV) and killed diphtheria–tetanus–whole cell pertussis (DTP) vaccine in 9-month-old Gambian infants, and further to determine whether Treg functional capacity is altered by vaccination.

**MATERIALS AND METHODS**

**Study Design**

This study was nested into a larger randomized study investigating the immunological effects of measles vaccination, DTP vaccination, or giving both vaccines together to 9-month-old Gambian infants, some of the results of which have been published previously (17). In this study, we focused on the role that Tregs play in regulating immune responses to these vaccines. 302 infants were recruited at 4 months of age at Sokuta Health Centre, a peri-urban area 20 km from the coast of The Gambia. Eligibility criteria included being well with no history of chronic illness, apyrexial (<37.5ºC), normal weight-for-age, and all recommended vaccines received to date. Infants were randomized into one of three vaccine groups (Table 1). At 4 months of age, Group 1 received DTP3 as normal while Groups 2 and 3 had their third dose of DTP withheld. All three groups received oral polio vaccine and *Haemophilus influenzae* b vaccine at 4 months of age. At 9 months of age, Group 1 were given MV alone; Group 2 received DTP3 with MV; and Group 3 received DTP3 alone (Figure 1). Males and females were randomized separately.

**Ethics Statement**

The study protocol was approved by the Joint Gambia Government/MRC Ethics Committee (project number SCC1085) and the London School of Hygiene and Tropical Medicine Ethics Committee. Written informed consent was provided by a parent/guardian of all participating infants.

**Blood Sampling**

Blood samples were taken at 9 months of age prior to vaccine administration, and 4 weeks later (10 months). Four and a half milliliters of venous blood was collected into heparin tubes and transported to the laboratory within 4 h of collection.

**Ex Vivo Flow Cytometric Analysis for Tregs, T Cell Memory, and Function**

50 µL whole blood was stained with cocktails of fluorochrome-conjugated surface antibodies to analyze for CD4 (CD4 APC-Cy7 or PerCP) and CD8 (CD8 Pacific blue) T cells expressing markers of Tregs (CD127 PE, FOXP3 APC); memory (CD45RO PerCP and CD38 PE-Cy7); terminal differentiation (CD57 FITC); activation (HLADR PerCP and CD38 PE-Cy7), proliferation (Ki67 FITC), and perforin production (Perforin PE) [Becton-Dickinson (BD) for all fluorochromes except for CD8 PB, FOXP3 APC, and CD62L PE-Cy7, which were from E-biosciences]. Red blood cells were lysed, and cells washed and incubated with surface antibodies for 30 min at 4°C. Cells for Treg analysis were then washed in 200 µL permeabilization buffer (E-biosciences); incubated with normal rat serum (1:50 dilution) for 15 min, 4°C in the dark, then FOXP3 added and incubated for 30 min.
Figure 1 | Study flowchart. 368 infants were assessed for eligibility and 302 (141 females and 161 males) randomized to one of the three vaccine groups; of whom 286 received the vaccine intervention at 9 months of age; and 32 were lost to follow-up at 10 months of age. Blood samples were collected prior to vaccination at 9 months and 4 weeks postvaccination at 10 months of age.

at 4°C. Cells for Ki67 and perforin intracellular staining were washed with BD permeabilization buffer, Ki67 and perforin fluorochromes were added, and cells incubated for 30 min at 4°C in the dark. Stained cells were resuspended in 150 µL fix buffer (1% formalin in PBS), then acquired on the Cyan Flow cytometer (CyanADP). Flow cytometry data were analyzed using FlowJo software (Treestar, CA, USA) according to the gating strategies described (Figure 2).

Vaccine Antibody Titers
The measles IgG hemagglutination inhibition assay (HAI) was performed using monkey red blood cells as described previously (18). Results are expressed as log₂ units, the minimum detection level being 31.2 mIU, and a protective level defined as ≥125 mIU (log₂ titer ≥3). A multiplex microsphere-based fluorescent immunoassay for IgG antibodies to diphtheria toxoid (Dtx), tetanus toxoid (Ttx), and four pertussis antigens [pertussis toxoid (Ptx), fimbriae, pertaxin, and filamentous hemagglutinin] was performed at the National Institute of Public Health and the Environment, Netherlands using published protocols (19). Protective levels for Dtx and Ttx are ≥0.1 IU/mL, but there is no established protective level for the pertussis antibodies.

Beta-2 Microglobulin Assay
The plasma beta-2 microglobulin (β2m) levels in milligrams per milliliter were measured by an automated microparticle enzyme immunoassay using an AxSYM automated machine (Abbott Laboratories, Wiesbaden, Germany) according to the manufacturer’s instructions.

Whole Blood Cultures
Heparinized whole blood was cultured in 100 µL aliquots in 96 well U-bottom plates with tetanus toxoid (TT) (10 µg/mL, Sanofi Pasteur, France); a measles peptide pool of 122 15mer peptides overlapping by 10 amino acids spanning the measles protein hemagglutinin (all 1 µg/mL final concentration, Sigma-Genosys, UK); anti-CD3 (αCD3) (5 µg/mL, BD) plus anti-CD28 (αCD28) (5 µg/mL, E-biosciences) as a positive control T cell stimulus; and
**RESULTS**

**No Convincing Effect of Vaccination on Circulating CD4+FOXP3+CD127− Treg Frequencies**

We first analyzed whether circulating Treg frequencies were altered following vaccination with MV, DTP, or both vaccines combined. When male and female donors were analyzed together, there was a significant decline in Tregs in the DTP-vaccinated group ($p = 0.039$), but no change in the other two groups (Figure 3A). When males and females were analyzed separately, there was an increase in circulating Tregs in measles-vaccinated females ($p = 0.040$) and a decrease in DTP-vaccinated males ($p = 0.029$) (Figure 3A). However, none of these changes remained significant after Bonferroni correcting for multiple testing and were therefore not considered convincing evidence of changes in Tregs postvaccination.

**Vaccination Affects Immune Activation and T Cell Function**

Beta-2 microglobulin levels increased after MV + DTP vaccination when all infants were analyzed together ($p = 0.035$), but not when analyzed by sex (Figure 3B). The frequency of CD38+HLADR+-activated CD4 T cells increased in the females.
FIGURE 3 | Regulatory T cells (Tregs), plasma β2m, and activated CD4 and CD8 T cell levels before and after vaccination. Results for CD4^+FOXP3^+CD127^− Treg frequencies (A), plasma β2m levels (B), activated CD4 T cells (C), and activated CD8 T cells (D) in the three vaccines groups at baseline (Pre) and 4 weeks after vaccination (post) for all infants combined, females and males separately. The bars show the median value in milligrams per liter; the error bars indicate the 95% confidence interval. Data were analyzed by Mann–Whitney U test, *p ≤ 0.05, **p ≤ 0.01, ^, no longer significant after correcting for multiple testing. Data are shown for Tregs: n = 194 infants, measles vaccine (MV) group n = 68 (31F, 37M), MV + DTP group n = 77 (43F, 34M), DTP n = 49 (26F, 23M); β2m: n = 259 infants, MV group n = 91 (39F, 52M), MV + DTP group n = 99 (47F, 52M), DTP n = 69 (35F, 36M); activated T cells: n = 198 infants, MV group n = 61 (26F, 33M), MV + DTP group n = 63 (32F, 31M), DTP n = 34 (19F, 15M).
who received MV + DTP simultaneously ($p = 0.0073$), but no other group (Figure 3C). By contrast CD38+HLADR+ CD8 T cells declined in infants following MV ($p = 0.0403$), but not the other vaccine groups (Figure 3D). CD4+ T cell proliferation determined by Ki67 expression also increased in the MV + DTP group (all infants $p = 0.0038$; females $p = 0.0017$) (Figure 4A), while levels of proliferating CD4 and CD8 T cells declined in the DTP-vaccinated females ($p = 0.0137$ and 0.0353, respectively), but not males (Figures 4A,B). After Bonferroni correction for multiple testing only the above changes in CD4 T cells proved to be statistically significant, namely, the altered activated and proliferating CD4 T cells (Figures 3C and 4A).

CD8+ T cell perforin production increased in the MV + DTP group (all infants $p = 0.008$; males $p = 0.0376$) but declined in the DTP-vaccinated groups (all infants $p = 0.0077$; males $p = 0.0224$) (Figure 4C). After correcting for multiple testing, males and females combined had an increase in perforin+ CD8 T cells after MV + DTP and a decline after DTP alone.

None of the above effector readouts of proliferation, perforin production, or immune activation correlated negatively with circulating Tregs either at baseline or 4 weeks after vaccination.

![Figure 4](https://example.com/figure4.png)

**FIGURE 4** | Proliferating and perforin producing T cell frequencies before and after vaccination. Results for CD4 T cell proliferation (A), CD8 T cell proliferation (B), and intracellular perforin levels (C) in the three vaccines groups at baseline (pre) and 4 weeks after vaccination (post) for all infants combined, females and males separately. The bars show the median value; the error bars indicate the 95% confidence interval. Data were analyzed by Mann–Whitney U test, *$p \leq 0.05$, **$p \leq 0.01$, ^, no longer significant after correcting for multiple testing. Data are shown for $n = 158$ infants, measles vaccine (MV) group $n = 61$ (28F, 33M), MV + DTP group $n = 63$ (32F, 31M), and DTP $n = 34$ (19F, 15M).
Altered Memory T Cell Populations in Dual Vaccinated Infants

We analyzed for effects on ex vivo circulating memory T cell populations. It was striking that the only significant changes occurred in the group that received the MV and DTP vaccines at the same time, with no changes in the single vaccine groups. The CD4 naïve (CD4+CD45RO−CD62L+) population declined in the MV + DTP group for all infants (p = 0.0001), males (p = 0.0024), and females (p < 0.0001) (Figure 5A); while the T cell effector memory (TEM) (CD4+CD45RO+CD62L−) increased in this group (TEM: all infants p = 0.0001, females p = 0.0006, males p = 0.0337; TEMRA: all infants p < 0.0001, females p = 0.012, males p = 0.0009) (Figures 5B,C). The decreased CD4 naïve T cell frequency in MV infants (p = 0.0292) was not significant after correcting for multiple testing (Figure 5A), nor was the TEM increase in males (Figure 5B). The CD8 population was less affected with only the naïve subset declining significantly in the MV + DTP group for all infants (p = 0.0038) and females (p = 0.0087) (Figure 5D). There was no change in central memory (TCM) (CD45RO+CD62L+) frequencies for CD4 or CD8 T cells for any vaccine group. The frequency of terminally differentiated T cells was examined by CD57 expression, hinting at a decline in MV + DTP-vaccinated infants for both CD4 and CD8 populations (p = 0.0455 and 0.0464, respectively), although neither were significant after correcting for multiple testing (not shown).

Baseline CD4+FOXP3+CD127− Tregs Negatively Correlate with Antibody Responses to MV but Not DTP

We previously showed that measles antibody titers 4 weeks after vaccination were not affected by simultaneous administration of DTP; nor were tetanus toxoid (Ttx), diphtheria toxoid (Dtx), and Ptx titers post-DTP vaccination altered by giving MV at the same time (17). To increase statistical power, we combined the MV and Ptx titers post-DTP vaccination altered by giving MV at the same time (17). To increase statistical power, we combined the MV and DTP; nor were tetanus toxoid (Ttx), diphtheria toxoid (Dtx), and Ptx titers post-DTP vaccination altered by giving MV at the same time (17). To increase statistical power, we combined the MV and DTP groups to analyze for measles Ab correlations between baseline circulating Tregs and vaccine antibody levels 4 weeks later; and the DTP and MV + DTP groups to analyze for DTP Ab correlations. This showed a significant negative correlation between baseline Tregs and measles Ab titers in infants who received MV (MV and MV + DTP groups combined) (r = −0.208, p = 0.048) (Figure 6A); but no correlation with any of the antibody readouts for DTP-vaccinated infants (Figures 6B–D). There were no significant correlations between Tregs and vaccine antibodies when males and females were analyzed separately.

No Correlations between Baseline Tregs and Postvaccination Cellular Responses

We could not perform functional Treg assays in this study for logistic reasons including the multiple assays being conducted, small blood volumes, and lack of flow cytometry cell sorting facilities in The Gambia. We thus chose to analyze for correlations between CD4+FOXP3+CD127− Tregs and readouts of vaccine-specific cellular immunity. We questioned whether baseline Tregs on the day of vaccination correlated negatively with subsequent vaccine-specific pro-inflammatory (IL-1β, TNF), Th1 (TNF, IFN-γ) or Th2 (IL-4) cellular responses 2 weeks after vaccination, or with the immunosuppressive cytokine IL-10, which can also be produced by activated Th1 and Th2 cells. There was no evidence for any significant correlation between baseline Tregs and subsequent cytokine responses to measles peptide or TT stimulation to suggest an immunoregulatory role for circulating Tregs at the time of vaccination.

Functionally Suppressive Tregs Post-DTP Vaccination

We next analyzed for a correlation between the circulating Tregs 4 weeks postvaccination and in vitro cytokine responses to the measles peptide pool, TT, PPD, and αCD3/αCD28 at the same time point. There was a negative correlation between postvaccination Tregs and the IFN-γ:IL-10 ratio in TT cultures (p = 0.0202, r = −0.471) and measles peptide cultures (p = 0.0475, r = −0.4001) in the DTP group only (Figures 7A,B). There was no correlation between postvaccination Tregs and cytokine responses to the T cell stimulus αCD3/αCD28 or the unrelated antigen PPD for any vaccine group to support an immunoregulatory role.

DISCUSSION

Several studies suggest that FOXP3+ Tregs suppress the generation of immune responses to vaccination (16). The DEREG mouse model is a useful tool in this respect allowing Tregs depletion in vivo (20). Treg depletion using this model showed enhanced vaccine antitumor responses in a melanoma vaccine trial (21) and enhanced vaccine-induced solid tumor immune responses (22). In the latter study, Treg depletion was associated with enhanced CD8 activation and IFN-γ production, and increased NK cells. In mice, the administration of anti-CD25 mAbs to deplete Tregs enhanced CD4 and CD8 T cell responses to BCG and hepatitis B vaccines (23), and preexisting CD4+CD25+ Tregs suppressed BCG responses in vitro and in vivo (24).

Little is known about the role of Tregs in controlling human vaccine responses for any age group. Furthermore, translating the mouse into human studies may not be appropriate since their Tregs are phenotypically distinct. A human study showed marked increases in CD4+CD25+FOXP3+ Tregs following vaccination with a DC-based HIV vaccine, and enhanced T cell immunity in vitro following Treg depletion (25). We have previously shown that infant BCG vaccination induces CD4+CD25+FOXP3+ Tregs, but they did not negatively correlate with IFN-γ reactivity to PPD to support a regulatory role (26). By contrast, the TB vaccine MVA85A was associated with a small decrease in CD4+CD25+CD39+ Tregs (27) decreased TGF-β mRNA and serum levels up to 12 weeks after vaccination (28).

The changes in Treg frequencies after vaccination were not significant after correcting for multiple testing, thus the question of whether MV or DTP vaccination alters Treg frequencies remains unresolved and further studies are needed. Tregs remained stable in the group that received MV + DTP; but this group had increased CD8 T cell perforin production; and increased CD4 T cell activation and proliferation among females but not males.
Figure 5 | Effect of vaccination on naïve and memory T cell frequencies. Results for CD4 naïve T cell frequencies (A), CD4 TEM (CD4\(^+\)CD45RO\(^+\)CD62L\(^-\)) cells (B), CD4 TEMRA (CD4\(^+\)CD45RO\(^-\)CD62L\(^-\)) cells (C), and CD8 naïve T cell frequencies (D) in the three vaccines groups at baseline (pre) and 4 weeks after vaccination (post) for all infants combined, females and males separately. The bars show the median value; the error bars indicate the 95% confidence interval. Data were analyzed by Mann–Whitney U test, *\(p \leq 0.05\), **\(p \leq 0.01\), ***\(p \leq 0.001\), ****\(p \leq 0.0001\), ^, no longer significant after correcting for multiple testing. Data are shown for \(n = 230\) infants, measles vaccine (MV) group \(n = 83\) (37F, 46M), MV + DTP group \(n = 91\) (43F, 48M), and DTP \(n = 56\) (27F, 29M).
Correlations between baseline circulating regulatory T cells (Tregs) at the time of vaccination and vaccine antibodies 4 weeks later. The frequency of Tregs at 9 months of age on the day of vaccination is shown on the x-axis, and the IgG antibody titers at 10 months are shown on the y-axis. (A) There was a significant inverse correlation between baseline Tregs and measles HAI titers for all measles-vaccinated infants [measles vaccine (MV) and MV + DTP groups combined]. Diphtheria toxoid (Dtx) titers (b), Ttx titers (c), and pertussis toxoid (Ptx) titers (D) for DTP vaccine recipients (DTP and MV + DTP groups) failed to correlate with baseline Tregs. The line indicates the best-fit correlation using Spearman’s rank correlation coefficient, and the correlation coefficient (r value) and p value for the correlation are shown. Data for 97 measles-vaccinated (47F, 50M) and 81 DTP-vaccinated (45F, 36M) infants are shown.

Correlation between regulatory T cells (Tregs) and vaccine-specific cytokine responses 4 weeks after vaccination. There was a significant inverse correlation between postvaccination Tregs and interferon-gamma (IFN-γ):IL-10 in TT culture supernatants at the same time point for DTP-vaccinated infants (A). There was also a significant inverse correlation for IFN-γ:IL-10 in measles peptide pool cultures in the DTP group (B). The line indicates the best-fit correlation using Spearman’s rank correlation coefficient, and the correlation coefficient (r value) and p value for the correlation are shown. Data for 25 DTP-vaccinated infants (18F, 7M) are shown.
The MV + DTP group also experienced a decline in naïve CD4 and CD8 T cell frequencies, and increased CD4 T EM and CD4 T_{EMRA} populations following vaccination, with some sex differences in this effect. These results are consistent with our previous findings that combining MV with DTP vaccination has immune suppressing effects on PPD cellular responses and RNA expression in males, but the opposite effect in females, compared to giving DTP or MV alone (17).

Sex differences have been described for antibody and cellular responses to many vaccines, with females generally mounting higher responses, and also suffering greater adverse events (29–32). Females are also generally more susceptible to the non-targeted heterologous effects of vaccines, whereby vaccination alters the host's response to subsequent exposure to unrelated (heterologous) organisms or vaccines (33, 34). Thus the sex differences described in this study are to be expected, but are often overlooked in immunological studies because they are not specifically analyzed for. Sex differences in Treg frequencies have not been described in infants although males have higher levels later in life (32), and we found no convincing evidence of a sex difference in our study either before or after vaccination.

In a small study, baseline regulatory cytokine gene expression (TGF-β and IL-10) at the time of vaccination with the malaria vaccines RTS,S/AS02A and MVA-CS showed an inverse correlation with subsequent antibody responses (35). Our results similarly suggest that Tregs at the time of vaccination suppress primary antibody responses to measles vaccination, but had no effect on secondary DTP vaccine antibody responses. We have since repeated this finding of a significant negative correlation between baseline Tregs and MV antibodies using a different Treg definition (CD4+CD25hiFOXP3+) and different time point (2 weeks post-measles vaccination) in a separate prospective cohort (in preparation). Potential mechanisms include direct suppression of B cells via cell-contact mechanisms involving TGF-β and CTLA-4 (36); and suppression of T cell help essential for B cell activation and expansion (37). If confirmed, Tregs could provide a functional target for enhancing vaccine antibody responses in infancy.

We analyzed for associations between Tregs and key CMI readouts as an indication of Treg function. We hypothesized that baseline Tregs at vaccination might suppress subsequent CMI to vaccination, and postvaccination Tregs might be functionally suppressive. We further speculated that the live and killed vaccines might have different effects in these respects. We found no evidence that baseline Tregs affected subsequent ex vivo plasma cytokines or β2m, activated or proliferating CD4 or CD8 T cells, CD8 T cell perforin production, or measles and TT-specific cytokine responses.

The inverse correlation between the circulating CD4+FOX3+CD127+ Tregs post-DTP vaccination and the postvaccination IFN-γ:IL-10 ratio in TT and measles peptide cultures, but not PPD or αCD3/αCD28, may support a vaccine-specific immunoregulatory role. No such correlations were observed in the MV or MV + DTP groups. However, given the multiple factors analyzed in these correlations these findings may well be type 1 error and further studies are required. If true, it would suggest that some vaccines, but not others, may induce suppressive Tregs. Indeed, we have found that mice immunized with aluminum adjuvant preferentially expand CD4+CD25+FOX3+TNFR2+ Tregs in draining lymph nodes, providing a potential mechanism whereby Tregs may be more functionally suppressive following vaccination with the aluminum adjuvanted DTP vaccine (unpublished). We previously showed suppressed type 1 immunity to αCD3/αCD28 stimulation in DTP-vaccinated females but not males in this cohort (17), but found no significant correlations between Tregs and αCD3/αCD28 cytokine responses to suggest Tregs were responsible for this.

There are several limitations to this study. We only had one postvaccination time point that does not reveal the dynamics of the immune factors analyzed. Power was reduced due to multiple comparisons, but corrections for multiple testing were done to allow for this. The Treg function conclusions are based on correlations rather than functional suppressive assays, although many studies use this approach. Nevertheless the results offer potential insights into the immunoregulatory role that circulating CD4+FOX3+CD127+ Tregs, either at the time of vaccination or postvaccination, may play in controlling vaccine immunogenicity in infants.

Certain vaccine adjuvants preferentially expand Teff over Tregs, e.g., the TLR3 agonist Poly(I:C) and the TLR9 agonist CpG-ODN; whereas others favor Treg expansion, e.g., the TLR7 agonist imiquimod (38). Thus adjuvants might be selected for future infant vaccines that allow for optimal Teff responses. Chemokine receptor 4 (CCR4) antagonists have been used as vaccine adjuvants to target and decrease local recruitment of CCR4+ Tregs to amplify vaccine responses at the immunization site (39). The mAb to OX40, part of the TNFR superfamily, increases Teff function while blocking Treg function; and humanized OX40 clones have been generated which enhance the immunogenicity of vaccines against infectious diseases (40). There are also a number of therapeutic agents that can manipulate Tregs in vivo. For example, low dose cyclophosphamide transiently decreases Treg frequencies while preserving effector T cell (Teff) function, permitting enhanced vaccine immunogenicity in mouse and human cancer vaccine trials (41, 42). Treg depletion with anti-CD25 monoclonal antibodies enhanced vaccine efficacy in mouse melanoma (43) and pancreatic carcinoma (44). The antihuman CD25 mAbs basiliximab and daclizumab decrease Treg number and function by blocking IL-2 signaling (45, 46); and daclizumab has been used to deplete Tregs and improve effector responses in human breast cancer vaccine trials (47, 48). The human mAb, ipilimumab, inhibits Tregs by blocking CTLA-4 and is FDA approved for use in melanoma patients (49).

While the above therapeutic approaches are not currently appropriate for infant vaccination strategies where any immune benefits will likely be outweighed by adverse effects, they demonstrate the future potential for Treg manipulation to improve vaccine immunogenicity. Transient Treg depletion would be preferable, since prolonged depletion could lead to increased immune pathology and autoimmunity. By contrast, the adjuvant approaches discussed above are already available and could be used to improve vaccine immunogenicity in vulnerable
populations such as neonates and infants. To do this, we need to understand the role that Tregs play in controlling responses to early life vaccines. We hope our results will galvanize further research in this dynamic and evolving field.

ETHICS STATEMENT

The study protocol was approved by the Joint Gambia Government/MRC Ethics Committee (project number SCC1085) and the London School of Hygiene and Tropical Medicine Ethics Committee. Written informed consent was provided by a parent/guardian of all participating infants.

AUTHOR CONTRIBUTIONS

HW, KF, and SR-J designed the study; JA oversaw infant recruitment/vaccination/bleeding/clinical assessment; JN, FN-K, MC, FB, ML, LS, and AD did the laboratory assays; JN, KF, MP, and EC did the analysis and interpreted the data; all the authors critically revised and approved the manuscript and are accountable for the accuracy and integrity of the work.

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Targeting regulatory T cells to improve vaccine immunogenicity in early life

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INTRODUCTION

The infant immune system is uniquely adapted to meet the challenges of early life (Kollmann et al., 2012). The newborn emerges from an immune-protected environment into a world where they constantly encounter new antigens. There is therefore a need to have a series of immunoregulatory mechanisms in place in order to prevent excessive inflammation and tissue damage. At the same time the infant needs to develop immune memory upon pathogen encounter in order to be protected against future challenge. The newborn has little immunological memory, and neonates and infants are heavily reliant on innate immunity to protect them against antigenic challenge as discussed in a series of comprehensive review articles (Levy, 2007; Ghazal et al., 2013; Levy and Wynn, 2014).

In this review we discuss the regulatory factors that infants employ to suppress or control their developing immunity. We will focus on regulatory T cells (Tregs) in particular, and the potential role they play in suppressing or controlling vaccine-induced immunity in early life. We will explore the mechanisms of action used by Tregs to confer suppression, and differences in the phenotypic and functional characteristics between Tregs in infants and adults. We will discuss the role of Tregs in malaria, HIV, and hepatitis C virus (HCV) infections; and briefly describe the results of clinical trials in human infants of vaccines against these three infections. A detailed understanding of the immunoregulatory factors controlling vaccine immunogenicity in early life may provide potential strategies for improving vaccine efficacy in this vulnerable age group. We will discuss immunotherapeutic agents and vaccine adjuvants developed for use in humans that can down-modulate Treg activity and thus enhance vaccine efficacy, demonstrating that this approach is a viable option for the future.

THE INFANT IMMUNE SYSTEM

INNATE IMMUNITY

The innate immune system which acts as the first line of defense against infection is suboptimal at birth, and does not reach full capacity until teenage years. Innate cells express pattern recognition receptors (PRRs) which detect highly conserved pattern associate molecular patterns (PAMPs) expressed by invading pathogens or vaccines, including Toll-like receptors (TLRs) and NOD-like receptors (NLRs). Newborns and young infants have similar levels of expression of these PRRs as adults (Kollmann et al., 2012), however, responses to PRR stimulation are low at birth in part due to diminished innate signaling pathways such as IRF7 translocation (Danis et al., 2008) and TLR3 and 4 signaling (Aksoy et al., 2007). Reactivity to certain PRR agonists, e.g., TLR4 and TLR5 are acquired rapidly, and reactivity of the viral ssRNA sensing TLR7 and TLR8 receptors is robust from birth (Burl et al., 2011), hence TLR7/8 agonists are being investigated.
as possible adjuvants to boost immune responses to neonatal vaccines (Dowling et al., 2013), Th2 (IL-6, IL-10) and Th17 (IL-6, IL-23) polarizing cytokines dominate the innate response in early life, while TNF-α and IL-1β responses rise in the first few years of life as the former cytokines decline (Belderbos et al., 2009; Kollmann et al., 2009; Nguyen et al., 2010; Burl et al., 2011). Infant dendritic cell (DC) function is also suboptimal (De Wit et al., 2004; Goriely et al., 2004; Aksoy et al., 2007), and NK cells (Guilmot et al., 2011) and neutrophil functions (Carr, 2000) are less potent than in adults. Low complement levels in neonatal plasma are thought to increase susceptibility to certain bacterial infections, and lead to impaired adaptive immunity (Levy, 2007).

ADAPTIVE IMMUNITY IN INFANCY

Infant T cell immunity

The adaptive immune system is characterized by minimal immunological memory at birth, since the newborn has been relatively protected from antigenic exposure in utero, and most of their T cells are of a naïve phenotype. Furthermore, high levels of TGF-β, progesterone and prostaglandin E2 in utero required to prevent the mother developing Th1 alloreactivity to her fetus (Philbin and Levy, 2009), alongside poor innate Th1 support (Langrish et al., 2002), result in the newborn having intrinsically skewed Th2-type immunity from birth. Additionally, the Th17 biased innate immunity in infants also results in a Th17 adaptive bias. This bias against Th1 immunity results in an increased vulnerability to microbial infections and suboptimal reactivity to many vaccines. Despite this, infants have been shown to stimulate adult level Th1 type immune responses to BCG vaccination (Marchant et al., 1999) and are thus capable of robust Th1 immunity. However, neonatal BCG vaccination results in a Th17 biased mycobacterial response compared to those receiving BCG at 4½ months of age (Burl et al., 2010), in keeping with the Th17 bias described above.

Infant B cell immunity

Newborn infants acquire IgG antibodies transplacentally from their mothers which provide protection against infections encountered in early life, while the other immunoglobulin subclasses are unable to cross the maternal–placenta interface. The maternally acquired antibody (MAb) levels wane over the first 6 months of life and are usually absent by 1 year of age. Several studies suggest that MAbs inhibit humoral responses to infant vaccines; including live measles vaccine (Albrecht et al., 1977) and oral poliovirus vaccines. Despite this, infants have been shown to stimulate adult level Th1 type immune responses to BCG vaccination (Marchant et al., 1999) and are thus capable of robust Th1 immunity. However, neonatal BCG vaccination results in a Th17 biased mycobacterial response compared to those receiving BCG at 4½ months of age (Burl et al., 2010), in keeping with the Th17 bias described above.

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IMMUNOREGULATORY FACTORS IN NEONATAL AND INFANT PLASMA

Neonatal and infant plasma contain a number of immunoregulatory factors that serve to maintain Th2 polarization, and limit pro-inflammatory innate and adaptive immunity. Newborns and infants have high levels of plasma adenosine, an endogenous purine metabolite with immunosuppressive properties. Adenosine causes mononuclear cells to produce cAMP, which acts as a second messenger to inhibit TLR-stimulated production of pro-inflammatory cytokines while polarizing toward IL-10 and Th17 cytokine production (Levy et al., 2006; Power Coombs et al., 2011; Philbin et al., 2012). Neonatal monocytes have increased sensitivity to these effects of adenosine via their adenosine A3 receptors, thus modulation of this system could potentially be used to enhance innate and therefore adaptive pro-inflammatory responses.

Several studies have shown that there are high levels of the immunosuppressive cytokine IL-10 in cord blood (CB; De Wit et al., 2004; Belderbos et al., 2009; Nguyen et al., 2010). IL-10 can be produced by most cell types of the immune system, including antigen presenting cells (APCs), granulocytes, and Th1, Th2 and many regulatory T cell subsets. IL-10 acts at a number of stages of an immune response in order to control inflammation. It inhibits the production of pro-inflammatory cytokines and chemokines by monocytes, macrophages and DCs, leading to increased IL-10 production by various T cell subsets. It suppresses both Th1 and the more recently described “Th1+Th17” cells, while enhancing CD4+FOXP3+ (forkhead box P3) regulatory T cell survival and activity, and promoting IgG and IgA class switching by B cells (Banchereau et al., 2012).

HUMAN REGULATORY T CELL SUBTYPES AND THEIR MODES OF ACTION

Regulatory T cells are unique subpopulations of T cells that play a major role in immune homeostasis and tolerance (Sakaguchi, 2006; Belkaid et al., 2002; Mills and Meguerd, 2004; Belkaid, 2007). Although Tregs have been shown to be beneficial in preventing an over-exuberant response and immune pathology following encounter with pathogens (Belkaid, 2008; Belkaid and Tarbell, 2009), they have also been shown to limit the favorable effector responses required for sterilizing immunity, thus allowing pathogen persistence (Kao et al., 2010).
recently a group of experts in the Treg field introduced a consensus nomenclature for FOXP3+ Tregs. They suggest replacing previously used terms which they describe as being to some extent “inaccurate and ambiguous” (Abbas et al., 2013). They recommend that the subset of FOXP3+ Tregs of thymic origin, which are also known as natural Tregs (nTregs), should be called thymus-derived Tregs (tTregs); while FOXP3+ Tregs that differentiate in the periphery should be called peripherally derived Tregs (pTregs) rather than the previous term inducible Tregs (iTregs). The pTregs are induced in the periphery in response to antigenic stimulation, and possess identical characteristics to tTregs, and therefore both subsets will be described together.

### Phenotype of human CD4+ FOXP3+ Tregs

CD4+ FOXP3+ Tregs are the most widely studied Treg subset. They were first described as a subset of CD4+ T cells which constitutively express the interleukin 2 (IL-2) receptor alpha-chains (CD25) and can prevent the development of autoimmunity in mice (Sakaguchi et al., 1995).

Determining the precise phenotype of human CD4+ FOXP3+ Tregs has proven difficult with many conflicting studies. Since CD25 is transiently expressed on conventional T cells (Hatakeyama et al., 1989), the CD25hi subset is described as a more reliable marker of CD4+ FOXP3+ Tregs in humans (Schmetterer et al., 2012). Human FOXP3+ Tregs also tend to express low levels of the IL-7 receptor CD127 (Liu et al., 2006; Seddiki et al., 2006). Therefore the most commonly analyzed phenotypes in human studies are CD4+CD25+FOXP3+ or CD4+CD25−CD127hi. Furthermore, human CD4+ FOXP3+ Tregs generally express high levels of the co-inhibitory receptor cytotoxic T lymphocyte antigen 4 (CTLA4; Sansom and Walker, 2006). More recently the chemokine markers CCR4, CCR6, CXC3R, and CXCR3 have been proposed to define four distinct populations of human tTregs, each with distinct functional characteristics (Duhen et al., 2012). Each of these four Treg subsets are thought to co-localize in vivo with and regulate a distinct TH subset (Th1, Th2, Th17, Th22) expressing the same chemokine receptors.

CD45RA expression can be used to distinguish Tregs that are naive or resting (rTregs; FOXP3hiCD45RA−) from the memory subset described as activated Tregs (aTregs; FOXP3hiCD45RA+; Miyara et al., 2009). The memory Tregs can be further subdivided into central memory (TregCM) and effector memory (TregEM) similarly to TH cells, based on the expression of chemokine receptor 7 (CCR7; Sallusto et al., 1999; Tosello et al., 2008).

Certain subpopulations among CD4+ FOXP3+ Tregs are more suppressive than others. For example, Tregs expressing the tumor necrosis factor receptor 2 (TNFRII) are thought to represent a highly suppressive CD4+ FOXP3+ Treg subset (Minigo et al., 2009). Those CD4+ CD25+ FOXP3+ Tregs expressing the transmembrane cyclic ADP ribose hydrolase CD38 (mainly thymic derived and in the spleen) have particularly high suppressive activity in a murine model (Patton et al., 2011). CD38 is part of a cascade involved in the production of the immunosuppressive factor adenosine from NAD+ (Horenstein et al., 2013) which can have immunoregulatory properties as discussed earlier. Interestingly, the majority of infant T cells express CD38 (Scalzo-Inguanti and Plebanski, 2011), and as previously mentioned infants also have high plasma levels of adenosine, but a link between these two factors has yet to be explored in neonates and infants.

### Mechanisms of CD4+ FOXP3+ Treg mediated suppression

CD4+ FOXP3+ Tregs can suppress the proliferation and activation of a multitude of immune cell types including T cells, NK and NKT cells, monocytes, macrophages, B cells, DCs, and eosinophils. They employ a variety of mechanisms to mediate this suppression, and are thought to be flexible in this respect by adapting their mechanism according to their local environment (reviewed by Wing and Sakaguchi, 2012). Both IL-2 and CTLA-4-dependent mechanisms have been described, with CD25 and CTLA-4 knockout mice having a similar phenotype to Foxp3 deficient mice (Wing and Sakaguchi, 2012). It is thought that the constitutive expression of CD25 by CD4+ FOXP3+ Tregs allows them to consume the available IL-2, depriving effector T cells (Teffs) and leading to effector cell death (De La Rosa et al., 2004). Those Tregs expressing CTLA-4 can suppress T cell responses via down-modulation of CD28 signaling (Walker, 2013), and reduced co-stimulatory capacity of CD80/86 expressed by DCs (Wing et al., 2011).

A commonly used mechanism of Treg action is the production of soluble inhibitory factors, including either membrane bound or released immunosuppressive cytokines IL-10, TGF-β, and IL-35 (Collison et al., 2007). FOXP3+ Tregs can also generate high concentrations of adenosine (Mandapathil et al., 2010) which binds to the A2a receptor on immune cells activating an immunoinhibitory loop (Sitkowsky and Ohta, 2005) which results in inhibition of T cell proliferation and cytokine production (Rasko et al., 2005).

### Type 1 Regulatory T Cells (Tr1)

The Tr1 Tregs are a unique Treg subset that do not rely on the expression of high levels of CD25 or FOXP3 for their function (Levings and Roncarolo, 2000). They are activated in the periphery following antigenic stimulation in the presence of IL-10 (Groux et al., 1997; Vieira et al., 2004). Recently, lymphocyte-activation gene 3 (LAG3) and CD49b have been described to represent specific markers for Tr1 cells (Gagliani et al., 2013).

The Tr1 Tregs are known to produce high levels of the immunosuppressive cytokines IL-10 and TGF-β, some IL-5, low levels of IFN-γ and IL-2, and no IL-4 (Groux et al., 1997). The secretion of IL-10 is the main mechanism by which Tr1 cells are thought to mediate suppression. The IL-10 can be either free or membrane bound, and has been shown to suppress Teff proliferation/activation both directly
and indirectly via a modulation of APC function (Roncarolo et al., 2006). They have also been shown to use cell–cell contact mechanisms (Gregori et al., 2012) and the production of granzyme B and perforin (Gregori et al., 2010) to mediate suppression.

**T HELPER TYPE 3 CELLS (Th3)**

This unique subset of TGF-β producing Tregs was identified in early studies investigating oral tolerance. They have been shown to suppress the proliferation and activation of Th1 cells and suppress the development of autoimmunity in the mouse model of multiple sclerosis (Chen et al., 1994). They become activated in the periphery upon encounter with a specific antigen, and suppress via the production of the inhibitory cytokine TGF-β. Some studies show that Th3 cells may have a role to play in controlling autoimmunity and allergy in humans (Andersson et al., 2002; Perez-Machado et al., 2003), but the role that this subset plays in the maintenance of immune tolerance in humans is still not clearly defined.

**CD8⁺ Tregs**

While CD4⁺ Tregs have been widely studied in humans, CD8⁺ Tregs have not received the same attention. However, there is increasing evidence that subsets of CD8⁺ Tregs also play important immunoregulatory roles, and impaired CD8⁺ Treg function may lead to autoimmunity (Hu et al., 2004; Lu et al., 2008). The most widely described phenotype for CD8⁺ Tregs is CD25⁺CD28⁻ (Ciubotariu et al., 1998; Filaci et al., 2004). Other markers include CD122, CTLA-4, GITR, CD38, CD103, and CD8αα (Uss et al., 2006; Simone et al., 2008; Smith and Kumar, 2008; Liu et al., 2014); a host of different CD8 Treg subsets have been described in humans expressing various combinations of these markers (Suzuki et al., 2012). While FOXP3 expression has been described in many CD8 Treg subsets, it may also represent an activation marker rather than acting as a regulatory factor since CD8⁺ T reg subsets, it may also represent an activation marker (Godfrey et al., 2005; Wing et al., 2005). Fan et al. (2012) found that CD4⁺CD25⁻ CB Tregs had a stronger immunosuppressive function than adult blood Tregs following two cycles of polyclonal stimulation. Mayer et al. (2012) found that CD4⁺CD25⁺ CB Tregs had a stronger immunosuppressive function than adult blood Tregs following two cycles of polyclonal stimulation. Mayer et al. (2012) found that CB CD4⁺CD25⁺ Tregs failed to suppress upon TCR activation whereas these freshly purified from adult blood, but CB Tregs became strongly suppressive after antigen-specific stimulation. Another study found that low FOXP3 expression levels by CB Tregs correlated with minimal suppressive activity, but following expansion there was a significant increase in the suppressive activity of these CB Tregs with a shift from the CD45RA⁺ to the CD45RA⁻ phenotype (Fujimaki et al., 2008). It has recently been shown that CB Tregs can be expanded more easily than adult peripheral blood Tregs, and CB Tregs are better suppressors in allogeneic mixed lymphocyte reactions than their adult counterparts (Lin et al., 2014).

**PHENOTYPIC AND FUNCTIONAL DIFFERENCES BETWEEN Tregs IN INFANTS COMPARED TO ADULTS**

Distinct qualitative and quantitative differences have been identified between the Tregs in adults and those of infants. Most of the studies in infants have analyzed Tregs in neonatal CB for comparison with adults, and different phenotypic markers have been used to characterize the Tregs in these studies contributing to some discrepancies in the results.

FOXP3⁺ Tregs have been found in much higher levels at birth compared to adults, whether defined as CD4⁺CD25⁺CD127lo (Nettenstrom et al., 2013) or CD4⁺CD25⁺FOXP3⁺ (Flanagan et al., 2010). Preterm infants have been shown to have higher levels still (Luciano et al., 2014). However, a study comparing CD4⁺CD25⁺CD127lo Tregs at different age groups, showed slight increases in Treg frequencies with age: 6.10% in CB; 7.22% in adults aged 20–25 years; and 7.5% in adults over the age of 60 years (Santner-Nanan et al., 2008); and another study found that neonates had similar number of cells expressing FOXP3 compared to their mothers, and a lower number of CD4⁺CD25brighth cells (Ly et al., 2009). The reason for these conflicting results is not known.

Cord blood Tregs have been shown to be predominantly of the CD45RA⁺CD45RO⁻ naïve phenotype in several studies (Kanegane et al., 1991; Wing et al., 2002; Takahata et al., 2004; Ly et al., 2009; Flanagan et al., 2010). Other phenotypic differences between cord and adult Tregs include the observation that CB Tregs are mostly CD27⁺ and thus at an earlier differentiation state than their mothers; they have a lower apoptotic potential as evidenced by lower CD95/Fas expression than their mothers; and less CD62L suggesting less of a T regCM lymph node homing phenotype (Ly et al., 2009). The reason for these conflicting results is not known.

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**In vitro** Treg suppression assays are difficult to perform in infants due to the lack of availability of large volumes of blood, combined with the low Treg frequencies in peripheral blood. Studies using CB are easier since large volumes are available for study. Several studies have shown that newborn CB Tregs are highly functional whereby they suppress T cell proliferation and IFN-γ production, further deviating from a Th1 response (Godfrey et al., 2005; Wing et al., 2005). Fan et al. (2012) found that CD4⁺CD25⁺ CB Tregs had a stronger immunosuppressive function than adult blood Tregs following two cycles of polyclonal stimulation. Mayer et al. (2012) found that CB CD4⁺CD25⁺ cells failed to suppress upon TCR activation whereas these freshly purified from adult blood did, but CB Tregs became strongly suppressive after antigen-specific stimulation. Another study found that low FOXP3 expression levels by CB Tregs correlated with minimal suppressive activity, but following expansion there was a significant increase in the suppressive activity of these CB Tregs with a shift from the CD45RA⁺ to the CD45RA⁻ phenotype (Fujimaki et al., 2008). It has recently been shown that CB Tregs can be expanded more easily than adult peripheral blood Tregs, and CB Tregs are better suppressors in allogeneic mixed lymphocyte reactions than their adult counterparts (Lin et al., 2014).

Taken together these studies suggest distinct differences between infant and adult Tregs. Overall they seem to be present in higher frequencies than in adults, are more naïve and less differentiated, and are highly suppressive; all supporting an active immunoregulatory role in early life.
THE ROLE OF Tregs IN REGULATING IMMUNITY TO MALARIA, HIV, AND HEPATITIS C VIRUS

Regulatory T cells have been implicated with an immunoregulatory role in both murine and human malaria infections (Scholzen et al., 2010). In mice, ablation of Foxp3+ Tregs led to increased T cell activation and decreased parasitaemia (Abel et al., 2012). 

In vivo depletion of Tregs protected mice from experimental cerebral malaria in a Plasmodium berghei model of infection (Wu et al., 2010). A FOXP3 promoter polymorphism in children has been associated with significant parasitaemia in a Congolese study suggesting a Treg role (Koukouikila-Koussounda et al., 2013). Malaria infected red blood cells (iRBCs) induced CD4+CD25hiFOXP3+ Tregs in vitro in healthy human volunteers (Scholzen et al., 2009). In human malaria sporozoite challenge experiments, Tregs have been shown to be induced rapidly after infection, and linked to lower pro-inflammatory cytokines and increased TGF-β production (Walther et al., 2005). Another study showed that malaria antigens can activate latent TGF-β on the surface of aTregs (Clemente et al., 2011). A study of 112 subjects in Kenya (infants to adults) found a correlation between the frequency of CD4+CD25hi T cells and increased risk of clinical malaria, suggesting Tregs may negatively affect natural immunity to malaria in humans (Todryk et al., 2008). In naturally exposed Gambians CD4+FOXP3+CD127lo Tregs during acute infection were inversely correlated with memory responses at 28 days, suggesting suppression of immune memory. In the same study, a CD4+FOXP3+CD45RO+ T cell population co-producing IFN-γ and IL-10 was more prevalent among children with uncomplicated malaria than those with severe disease, suggesting a beneficial immunoregulatory role for this IL-10 producing subset, presumably by limiting excessive inflammation (Walther et al., 2009). A role for the highly suppressive TNFRII+ Tregs in malaria parasite survival has been implicated in a study of Indonesian school children (Wammes et al., 2013). Overall, these data support an induction of Tregs during acute malaria infection which can limit the generation of immune memory and increase susceptibility to infection, but also control immunopathology and disease severity.

The role of Tregs in HIV infection remains poorly understood and the data are conflicting, in part due to the different phenotypes used to define Tregs in the various studies. However, the studies do support a regulatory role. For example, combination anti-retroviral therapy (cART) non-responders with persistent CD4 counts <200 cells/μL on therapy had higher peripheral blood Tregs and aTregs than cART responders, with higher IL-10+ Tregs and lower FOXP3 in lymphoid tissue (Gaardbo et al., 2014). Another longitudinal study also found higher numbers of Tregs associated with immunological non-responders defined as CD4 <500/μL (Saison et al., 2014). A study analyzing multiple Treg phenotypes in HIV infected individuals found evidence of Treg redistribution depending on HIV status (Serana et al., 2014). Untreated viraemic patients with stable CD4 counts had higher proportions of naive Tregs with decreased TregCM compared to those on cART and healthy controls (Serana et al., 2014). The study suggests that effective cART restores Treg homeostasis since Treg subpopulations in the cART group were similar to those of healthy donors. Increased proportions and decreased numbers of Tregs associate with progression of HIV (Wang et al., 2013). Treg depletion in a murine chronic retrovirus infection model resulted in reduced viral loads (Dietze et al., 2013). In combination, the data suggest that Tregs may suppress HIV-specific immunity leading to lower CD4 counts and viral persistence.

Hepatitis C virus is characterized by its ability to establish chronic infection in the majority of those infected, and an immunoregulatory role for Tregs in this process has been well described. Chronic HCV patients have increased levels of CD4+ and Tr1 Tregs in peripheral blood which are thought to suppress anti-viral T cell responses leading to viral persistence (Chang, 2007). Certain HCV epitope variants have been shown to induce Tregs in HCV-infected patients (Cusick et al., 2011). Chronic HCV patients have more serum IL-10 than those with resolved infection, which is proposed to play a role in the induction of CD4+FOXP3+ Tregs in chronic HCV infection (Macdonald et al., 2002; Cusick et al., 2013); and CD49b, a marker for IL-10 producing Tr1 Treg cells, is lower in those who respond to viral therapy, thus suggesting a regulatory role for Tr1 Tregs too (Fabien et al., 2014). Indoleamine 2,3-dioxygenase (IDO) production by stimulated monocyte derived DCs was higher in HCV patients compared to healthy controls, and these DCs were more able to induce Tregs, suggesting a role for this Treg induction pathway in chronic HCV (Higashitani et al., 2013). Expression of the inhibitory signaling pathway molecule T cell immunoglobulin and mucin-domain-protein-3 (Tim-3) is upregulated on both Teff and CD4+CD25+FOXP3+ Tregs in chronic HCV, correlating with increased Treg and decreased Teff proliferation, suggesting that the Tim-3 pathway controls the Treg/Teff balance in chronic HCV (Moorman et al., 2012). Viral persistence following acute HCV infection is accompanied by increased plasma Galectin-9 (Gal-9) which is the ligand for Tim-3, alongside expanded Gal-9 expressing Tregs and increased expression of Tim-3 and CTLA-4 on HCV-specific CD8+ T cells (Kared et al., 2013). Thus high levels of Tregs likely contribute to viral persistence in HCV infection, and both FOXP3+ and Tr1 Tregs have been implicated. Mechanisms of Treg induction in HCV may be multifactorial but include HCV antigen driven induction, IL-10, IDO, Gal-9/Tim-3, and CTLA-4.

ROLE OF Tregs IN CONTROLLING VACCINE IMMUNOGENICITY

The role that Tregs play in controlling or limiting vaccine immunogenicity remains to be fully determined. Given that Tregs are induced by natural infections to regulate the inflammatory response, it makes sense that Tregs would be induced as part of the immune response to vaccination, particularly for live attenuated vaccines. One might predict that their induction would play a beneficial immunoregulatory role by preventing an overexuberant immune response to the vaccine. However, most studies suggest that Tregs can interfere with the generation of vaccine-induced immunity. Thus, depletion of Tregs pre-vaccination in murine models has been shown to enhance immune responses to some vaccines. In a DEREG mouse model, which allows for in vivo depletion of Foxp3+ Treg cells at any point during an immune response using diphtheria toxin (Lahl and Sparwasser,
2011), Treg depletion led to an enhanced anti-tumor response to vaccination against an established melanoma (Klages et al., 2010). A more recent study showed that the short term depletion of Tregs in DEREG mice greatly enhanced vaccine-induced immunity against a solid tumor; increasing NK cells and CD8 T cell activation and IFN-γ production (Mattarollo et al., 2013). Administration of vaccines with anti-CD25 monoclonal Ab has been shown to induce more durable immunity in mice compared to when the vaccine is administered alone, for both BCG and hepatitis B vaccines, which has been attributed to a depletion of CD25+ Tregs (Moore et al., 2005). Ho et al. (2010) showed that antigen-specific Tregs induced by environmental mycobacteria suppress Th1 immune responses, thus compromising the response to BCG vaccination in mice. They also showed a correlation between the pre-existing Tregs and the subsequent vaccine response. Murine studies of Parkinson’s disease have shown that Tregs are induced by BCG vaccination (Lacan et al., 2013).

It is difficult to translate these studies into primates and humans since murine Tregs are not phenotypically identical, and in vivo depletion of FOXP3+ Tregs in healthy humans presents logistic and ethical challenges. An oral vaccine against simian immunodeficiency virus (SIV) based on a Lactobacillus commensal that favors immune tolerance induction was used to induce T cell tolerance to SIV antigens in macaques (Lu et al., 2012). The vaccine-induced CD8+ Tregs that suppressed CD4+ T cell activation and ex vivo SIV replication, and provided sterile protection against an intrarectal SIV challenge in 15 of 16 vaccinated macaques. This strategy is thought to work because CD8+ T cell activation drives the initial phase of viral replication, and provides the proof-of-concept that an oral Treg inducing vaccine could prevent the establishment of HIV infection.

Using a DC-based vaccine in HIV patients undergoing cART, it was shown that depletion of the Tregs in vitro significantly enhanced the vaccine-induced anti-HIV-1-specific polyfunctional T cell response, suggesting that Tregs can dampen vaccine-induced immunity (Macatangay et al., 2010). This study also showed a marked increase in the CD4+CD25+FOXP3+ Treg numbers following vaccination, however, this increase did not correlate with the effector CD8+ T cell vaccine-induced response. Increased FOXP3 mRNA expression has been demonstrated in malaria vaccinated adults; however, the authors concluded that this might be attributed to the participants being naturally exposed to the malaria parasite rather than as a result of vaccination per se (Mwacharo et al., 2009).

Very few studies have looked at the role that Tregs play in controlling vaccine immunogenicity in infants. Our group found no correlation between PPD-specific CD4+CD25+FOXP3+ Tregs or CD4+IL-10+ Tregs, or PPD stimulated total IL-10 production on the day of BCG vaccination of Gambian infants, and subsequent IFN-γ responses to PPD (Burl et al., 2010). No functional Treg assays were conducted in this study. In another study we found that placental associated malaria (PAM) infection is associated with increased malaria-specific CD4+CD25+FOXP3+ Tregs (Flanagan et al., 2010) and that PAM also correlates with decreased immunogenicity of BCG vaccination as evidenced by poorer PPD reactivity persisting to 1 year of age compared to PAM negative children (Walther et al., 2012). Whether the Tregs are the cause of this attenuation of BCG responses is not known.

TARGETING Tregs IN VIVO TO ENHANCE VACCINE IMMUNOGENICITY

The cancer research field has made considerable advances in dissecting the role that Tregs play in cancer progression and their role in suppressing responses to cancer vaccines. Moreover, trials conducted in animal models and humans have demonstrated that certain drugs and immunotherapies can transiently decrease Treg frequencies in vivo leading to improved anti-tumor T eff functions, and in some cases reduced tumor load. Since Treg depletion can enhance inflammation and autoimmunity then such transient depletion, as opposed to long term effects, is desirable. In low doses, the agent cyclophosphamide transiently decreases Treg frequencies while T eff functions are preserved, leading to enhanced responses to vaccine antigens and improved vaccine immunogenicity in mouse and human cancer vaccine trials (Barbon et al., 2010; Le and Jaffee, 2012). Anti-CD25 monoclonal antibodies, which deplete Tregs in vivo, enhanced vaccine efficacy in mouse models of pancreatic carcinoma (Keenan et al., 2014) and melanoma (Tan et al., 2013). Basiliximab and Daclizumab are anti-human CD25 MAbs that cause both decreased number and decreased function of Tregs by blocking IL-2 signaling (Goebel et al., 2000; Kohm et al., 2006; Mitchell et al., 2011). Daclizumab has been used in several human breast cancer vaccine trials where it depleted Tregs and improved effector responses, and furthermore may reprogram naïve Tregs to become IFN-γ producers (Rech and Vonderheide, 2009; Rech et al., 2012). The human monoclonal antibody, Ipilimumab, inhibits Tregs by blocking CTLA-4; and was approved by the FDA in 2011 for use in melanoma patients (Peggs et al., 2009).

Certain innate agonists that are being used as vaccine adjuvants preferentially expand T eff over Tregs, e.g., the TLR3 agonist Poly(I:C) and the TLR9 agonist CpG-ODN; whereas others favor Treg expansion, e.g., the TLR7 agonist imiquimod (Perret et al., 2013). OX40 is part of the TNFR superfamily expressed by T eff and Tregs, and the monoclonal antibody increases T eff function while blocking Treg function. OX40 clones have been humanized as potential agents to enhance the immunogenicity of vaccines against infectious diseases (Voo et al., 2013).

An interesting approach is that of local depletion of Tregs at the site of injection of a vaccine. Chemokine receptor 4 (CCR4) antagonists can be used as vaccine adjuvants to target and decrease local recruitment of CCR4+ Tregs in order to amplify vaccine responses at the site of immunization (Bayry, 2014).

Therefore a number of agents that target Tregs are being used experimentally in humans in order to enhance vaccine efficacy. Some of these are non-toxic and safe for use in humans, offering the future prospect of using this approach to enhance immunogenicity of vaccines against infectious diseases including malaria, HIV, and HCV.

TRIALS OF NOVEL VACCINES AGAINST MALARIA, HIV, AND HCV IN INFANTS

Despite the multiple obstacles to successful infant vaccination discussed above, many vaccines are currently delivered in infancy
with good immunogenicity, including the live BCG, measles and yellow fever vaccines; and the inactivated diphtheria, tetanus, pertussis and hepatitis B vaccines, *H. influenzae b*, and pneumococcal conjugate vaccine. The RTS,S/AS01 malaria vaccine is the most advanced malaria vaccine in human clinical trials, having reached phase III testing in children and infants, with potential licensure in 2015. It reduced clinical and severe malaria by 56 and 47.3% respectively in children aged 5–17 months (Bejon et al., 2007; Olotu et al., 2011); but only 31.3 and 36.6% when administered in three doses with routine Expanded Program on Immunization (EPI) vaccines in the 6–12 week old age group (Rts et al., 2012). Follow up of the 5–17 month old vaccinated group over a 4 year period found protection waned to 16.8%; with waning greater among those with higher malaria exposure suggesting that natural immunity to malaria contributes to the waning (Olotu et al., 2011); but only 31.3 and 36.6% when administered 24 months (Kaleebu et al., 2014).

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Fowlpox and modified vaccinia Ankara (MVA) based malaria vaccines have been tested in 1–6 year olds with no evidence of protective efficacy (Bejon et al., 2007). A blood stage vaccine FM2P2.1/AS02A has been tested in Malian children aged 1–6 years with an efficacy of <10% (Laurens et al., 2013). The blood stage alum adjuvanted GMZ2 malaria vaccine elicited good inhibitory antibody levels in pre-school children (Jepsen et al., 2013). Prime-boost strategies based on chimp adenovirus vector priming followed by MVA boosting are being tested in children and infants in Africa, and while results of these trials are not yet available the adult studies have shown unprecedented immunogenicity for malaria exposed populations (Ogwang et al., 2013) and should stimulate good immunity in infants.

Only a few human HIV vaccine trials have been conducted in healthy uninfected and HIV-exposed infants. Immunogenicity was limited among healthy Gambian infants given a single dose of MVA.HIVA, but this was not surprising given that MVA alone is known to be poorly immunogenic (Afolabi et al., 2013). In a Ugandan trial, infants were vaccinated at birth, 4, 8, and 12 weeks of age with ALVAC-HIV vCP1521 (ALVAC) candidate HIV vaccine which induced low level CD4 and CD8 T cell responses at 24 months (Kaleebu et al., 2014).

The target population for HIV vaccines include intravenous drug abusers and health care professionals. However, HIV infection is common throughout the world and mother-to-child transmission is well described (Yeung et al., 2001). Thus an infant HIV vaccine would have its place, particularly in resource poor settings where the anti-viral therapies available are currently not affordable. Both therapeutic and prophylactic vaccines are being developed, and several have now entered phase I/II human clinical trials, mostly of therapeutic vaccines in chronically infected cohorts. Efficacy of recombinant protein, peptide, DNA, and vector-based vaccines aimed at producing robust anti-T cell responses (Halliday et al., 2011). As far as we are aware no HIV vaccine trials have been conducted in children.

**FUTURE PROSPECTS**

There is very little in the literature regarding the role of Tregs in infants in general, and even less in respect to vaccine immunogenicity. The fact that functional Tregs are present in high numbers in infancy and have potent suppressive activity, coupled with poor immunological responses to some vaccines in this vulnerable age group, supports a need to better understand the role they play in controlling the response to childhood vaccines in particular. The data available suggest that Tregs suppress immunity to vaccines, and that they can also be induced by vaccination. We have shown that malaria, HIV, and HCV all use Tregs to evade host immune responses, therefore vaccine-induced Tregs would be predicted to reduce the protective efficacy of vaccines against these infections. A better understanding of the role that Tregs and other immunoregulatory factors play in contributing to poor vaccine immunogenicity in childhood would help with the design of better vaccines. Studies in cancer patients have shown that transient Treg inactivation or depletion is a viable approach to enhancing vaccine efficacy. A number of Treg modifying agents are available for use in humans, therefore this approach is a very real prospect for the future and may be particularly applicable to neonates and infants.

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Targeting Tregs to improve vaccines


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