MSc (Clinical Immunology)

CD4 and CD8 T-Cell Responses to Acellular Pertussis and Rotavirus Vaccination in Breast-fed HIV exposed, uninfected infants

TRISHANA NUNDALALL

NNDTRI001

Supervisor : Dr. Heather Jaspan

Co-supervisor : Prof Clive Gray

Division of Immunology,

Department of Pathology,

Faculty of Health Sciences

University Of Cape Town

South Africa

DECLARATION

I, Trishana Nundalall hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ACKNOWLEDGEMENTS

I would like to thank all the people who contributed in some way to the work described in this thesis. Firstly, I thank my academic advisors, Dr.Heather Jaspan and Prof. Clive Gray for all the patients, advice and guidance. Under their watchful eyes I have been able to significantly improve my professionally and scientific skills.

Secondly, the results in this thesis would not have been possible without efforts from my fellow lab-mates; Sopie Beer and Jerome Wendoh who spent a lot of time in the flow room, acquiring data that contributed to this thesis. A special thanks to Dr.Selena Ferrian and Agano Kiravu for all the time they spent training me, without their labors my job would have undoubtedly been more difficult.

I am also very appreciative of the entire Infants study team and all the mums and babies who made this study possible, by being pricked and poked in the name of science.

Finally, I would like to acknowledge my family and friends who supported me throughout this time. Thank you to my mum, dad and brother for all the love and unyeielding encouragement. I could not have got to this point in my life without my amazing family. A sincere thanks to Nobomi Donsa, Berenice Alinde, Munadia Ansarie and Nazila Ghodsi Gharab for listening to all my complaints and making my experience as a student one of fun and friendship.

Lastly, I owe a huge thanks to Prashanthan Naidoo, who loved, encouraged and took care of me when I was to busy to do so for myself. I am extremely grateful of all time spent cooking and cleaning while I sat in front of my laptop procrastinating, and occasionally working.

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ABBREVIATIONS

A)
aP: Acellular pertussis

B)
BCR: B-cell receptor
BFA: Brefeldin A
BP: Bordetella pertussis antigen
BSA: Bovine serum albumin

D)
DMEM: Dulbecco's modified eagle medium
DNA: Deoxyribonucleic acid
dsRNA: double stranded ribonucleic acid
DTaP: Dipteria, Tetanus and acellular Pertussis

E)
EBF: HIV exposed breast fed
E.coli: Escherichia coil
ED: Early differentiated
EFF: HIV exposed formula fed
EMEM: Eagles minimum essential medium
EPI: Expanded Program of Immunization

G)
GCP: Good laboratory practices
GIT: Gastrointestinal tract

H)
Hep B: Hepatitis B
HEU: HIV exposed uninfected
Hib: Haemophilus Influenza B
HIV: Human Immuno-deficiency Virus
HIV-: HIV negative
HIV+: HIV positive
HU: HIV unexposed

I)
IgA: Immunoglobulin A
IgG: Immunoglobulin G
IFNα: Interferon alpha
IFNγ: Interferon gamma
IL-5: Interlukin 5

IL-6: Interlukin 6
IL- 10: Interlukin 10
IL-12: Interlukin 12
IL-23: Interlukin 23
IL-1β: Interlukin 1 beta

L)
LD: Late differentiated
LPS: Lipopolysacchrides

M)
MDSC: Myeloid derived suppressor cells
MHC: Major histocompatibility complex

N)
NSP4: Non-structural protein 4

O)
OPV: Oral Polio virus

P)
PAMP: Pattern associated molecular patterns
PBMC: Peripheral blood mononuclear cells
PBS: Phosphate buffer saline
PCR: Polymerase chain reaction
PHA: Phytohemagglutinin
PMTCT: Prevention of mother to child transmission

R)
RBC: Red blood cell
RET: recent thymic emigrants
RNA: Ribonucleic acid
rpm: Revolutions per minute
RPMI: Roswell Park Memorial Institue medium
RT: room temperature

S)
SEB: Staphylococcal enterotoxin B

T)
TB: Tuberculosis
TD: Terminally differentiated
TH1: T-helper 1
TH2: T-helper 2

TH-17: T-helper 17
TLR: Toll like receptors
TNFα: Tumor necrosis factor alpha
Treg: Regulatory T-helper

U)
UBF: Unexposed breast-fed

W)
WBA: Whole blood assay
WHO: World Health Organization
wP: Whole cell pertuss

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ABSTRACT

Introduction

Vaccination is one of the most efficient ways to prevent infectious diseases, however due to the naivety and regulation of immunity found in infants, induction of vaccine-mediated immunity is challenging. Respiratory and diarrheal diseases are major contributors to infant mortality. Additionally, Human Immunodeficiency Virus-1 (HIV) infections increase the risk of mortality. Current advances in Prevention of Mother-to-Child Transmission (PMTCT) have prevented HIV infections in almost 97% of infants being born to HIV-infected mothers. As a result there is an increasing number of HIV exposed, uninfected infants (HEU). HEU infants have a higher rate of infectious disease related mortality and morbidity compared to unexposed infants, the underling causes of these differences are still not understood. In this dissertation, responses to two childhood vaccines, live, attenuated rotaviral vaccine (Rotarix) and acellular Pertussis (aP), were analyzed in HEU infants, with specific focus on T-cell responses to Rotarix and aP, due to the current lack of published data on T-cell responses. Additionally, the influence of feeding mode, that is breast or formula feeding, was also assessed as it is well established that breast fed infants contract fewer infections compared to formula fed infants.

Methods

This dissertation included infants from a larger cohort which includes three groups of infants; HIV unexposed breast fed (UBF), HIV exposed breast-fed (EBF) and HIV exposed formula fed (EFF) infants. Infants were recruited at birth and followed up until 36 weeks of age. As no Rotavirus vaccine T-cell assay was previously published, multiple techniques were utilized to attempt to optimize an assay capable of detecting Rotavirus (RV) vaccine-specific T-cell responses. To determine T-cell responses to *Bordertella pertussis* (BP), blood was collected from infants at each time-point and 200ul was stimulated with BP antigen in a 12-hour whole blood assay. Cells from all assays were fixed and stained for flow cytometric analysis of CD4 and CD8 T-cell responses. The markers used included live/ dead, CD3, CD4 and CD8 for identification of T-cell populations, IFNγ, IL-2 and TNFα cytokines, HLA-DR and Ki67 for activation and proliferation, and CD45RA and CD27 memory differentiation. Data analysis was then completed using Microsoft Excel, Flow.Jo V9, GraphPad prism V6, Pestle 1.7 and Spice V5.33 software packages.

Results

Despite multiple attemps it was not possible to optimise an assay capable of consistently detecting Rotavirus vaccine specific responses. This was partly due to interferance from contaminating agents in the protein antigens used, and difficulty in culturing and purification of whole virus. Assessment of aP spcific CD4+ T-cell memory demonstrated an overall increase in terminally differentiated (TD) memory cells accross time. This mirrored the ontogeny of the total T cell pool which showed an overall decrease in naïve T-cell frequencies with a consequent increase in late and terminally differentiated CD4 and CD8 T-cell populations over time through the first months of life. Both total and aP specific CD4+ early differentiated (ED) memory T-cells remained unchanged over time. ED CD8+ memory T-cells peaked at week 15 in EBF infants. A similar observation was found in UBF infants but at a non-significant level. EFF infants had no significant changes in CD8+ naive, ED and late differentiated (LD) memory populations over time. Additionally all infants demonstrated high levels of Ki67 expression at D4-7, which is prior to vaccination and maintained this level of proliferation after vaccination. HEU infants had higher levels of activation compared to HU infants in the first week of life but this normalised to HU infant levels by week 7. Furthermore EFF infants had peak T-cell activation at week 7 as compared to week 15 in EBF infants. In addition HU infants had better cytokine responses than HEU infants at week 7 but similar responses at week 15 and 36. In Addition, EFF infants also had increased vaccine specific CD4+ responses at week 7 and week 36 compared to EBF infants. This was true for overall cytokine expressing CD4 T-cells and single TNFα expressing CD4+ T-cells.

Disscussion

Given the important role T-cells play in the clearance of Rotavirus, it is important that an assay capable of detecting RV vaccine specific T-cell responses be developed. Furthermore, T cells play a role in providing help for antibody responses to BP and for killing of intracellular bacteria. Our findings regarding immunity to aP suggest that all infants, regardless of HIV exposure status and feeding mode, are able to mount a T cell response to aP vaccination. However the differing ontogeny of responses seen in all three groups of infants lends some insight on the complex determinants of vaccine T -cell immunogenicity. In this case, age since vaccination, HIV exposure, and feeding mode resulted in apparent changes in vaccine responses as well as T cell differetiation and activation.

CHAPTER 1:

LITERATURE REVIEW

SECTION 1.1:

INTRODUCTION

The burden of infectious diseases in infants is a major area of concern. According to the World Health Organization (WHO), an estimated 7.1 million infants die yearly due to infectious diseases (World Health Organization, 2004). In developing areas like South Asia and Sub Saharan Africa, the burden is even higher, with approximately 1 million newborn deaths occurring every year (R. E. Black et al., 2010). Major contributors of infant death are respiratory and diarrheal diseases. In addition, HIV, TB and malaria infections increase the risk of mortality (Ganatra & Zaidi, 2010).

Neonates are very susceptible to infectious diseases due to the naïve state of their immune system and inadequately developed skin barrier (Wynn & Levy, 2010). Infants have a compromised innate immune system, which is the first line of defense against infections, resulting in a decreased production of pro-inflammatory cytokines (Wynn & Levy, 2010). Neonatal antigen presenting cells exhibit deficits in phagocytic and bactericidal function, in pathogen recognition, as well as activation post stimulation (Velilla, Rugeles, & Chougnet, 2006). Neonatal antigen presenting cells also have a limited antigen presenting ability, resulting in poor activation of the adaptive immune system (Wynn & Levy, 2010). In addition, infants have a compromised adaptive immune system, with TH2 skewed T-cell responses and a reduced antibody production relative to adults (Ganatra & Zaidi, 2010; Levy, 2007).

In addition to the naturally vulnerable state of neonatal immune system, infants in developing countries have an added risk of infections due to poor hygiene, limited access to medical facilities and exposure to wide spread diseases in the population, such as that of HIV (Dauby, Goetghebuer, Kollmann, Levy, & Marchant, 2012). There are approximately 40 million people infected with HIV worldwide. In Sub-Saharan Africa, women of child-bearing age seem to be disproportionately infected (Centers for Disease Control and Prevention (CDC), 2006). The current advances in prevention of mother to child transmission (PMTCT), have led to preventing HIV infection in almost 97% of infants being born to HIV+ mothers. As a result there is an increasing population of HIV exposed uninfected (HEU) infants (Agangi, Thorne, Newell, & European Collaborative Study, 2005; Connor et al., 1994).

Despite being HIV-negative at birth, infants born to HIV+ mothers continue to be at risk of HIV acquisition through breast-feeding. These infants are exposed to roughly 700,000 cell-free and cell-associated particles of HIV in breast milk daily (Kourtis & Bulterys, 2012). However the transmission rate of HIV from mother to child via breast milk is highly inefficient. It was estimated that 14% of infants would become infected if there is a complete lack of medical treatment while exclusively breast-feeding (Kourtis & Bulterys, 2012). Thus in developing countries, with poor sanitation, exclusive breast feeding is recommended due to low risk of transmission and the high association between formula feeding and malnutrition and exposure to other antigens that may be deleterious.

It is well documented that HEU infants have higher rates of infectious mortality and morbidity compared to HIV unexposed (HU) infants (Filteau, 2009; Marinda et al., 2007; Mussi-Pinhata et al., 2007). It is also established that HEU infants have poor responses to standard childhood vaccines. However, the underlying causes of this phenomenon are still not understood (Reikie et al., 2013). Vaccination is one of the main mechanisms for preventing infections, but vaccine-induced protection is sub-optimal in this vulnerable population (Abramczuk et al., 2011). Hence, investigating the responses of HEU infants to childhood vaccines is an important area for investigation. In this present study, Rotavirus and acellular Pertussis (aP) vaccines were assessed.

Rotavirus is a mucosal pathogen and the primary cause of infantile gastroenteritis (Parashar et al., 2009). Protection from natural infection is most likely provided by serotype-specific neutralizing antibodies (Chan et al., 2011). Breast or formula feeding practices, in infants, may have implications on immune activation in the gut and, in turn, may alter mucosal vaccine responses, such as to Rotavirus vaccination. Thus assessing the impact of breast or formula feeding on Rotavirus vaccine responses will assist in characterizing the relationship between mucosal immunity and feeding mode. This information is important, especially for HEU infants, as altered gut activation in this population may not only affect vaccine responses, but may also alter susceptibility to HIV itself (Peltier et al., 2009).

Bordetella Pertussis (BP) is a bacterial respiratory pathogen causing severe illness in infants (Kilgore, Salim, Zervos, & Schmitt, 2016). A study previously conducted on a

South African infant cohort showed that HEU infants had better immune responses to Bordetella Pertussis vaccination, developing protective one year earlier than HU infants (Reikie et al., 2013). Another study also demonstrated that HEU infants have better antibody responses than HU infants, not only to BP vaccination but to pneumococcus vaccines as well (Jones et al., 2011). Given that HEU infant BP humoral responses were better then HU infants, assessing the cellular responses further may provide crucial insight on what is require to stimulate a successful vaccine response in HEU infants. Hence, one of the main aims in this study was to assess how HIV exposure and different feeding modes affect infant T cellular immune responses to Bordetella pertussis vaccination.

The remainder of this chapter provides a more detailed overview of all the factors, which contribute to vaccine responses in infants. It describes the infant immune system and the different components, including HIV exposure and breast-feeding, which contribute to the protection against and susceptibility to infectious diseases in infancy. In addition, the main aims, objectives and hypotheses of this study will be addressed.

SECTION 1.2:

GENERAL VACCINE RESPONSES

Under most circumstances, infants are able to mount immune responses to vaccines or pathogen stimulation. These responses do, however, vary widely depending on a multitude of factors including; the development of the immune system, dietary intake including breast or formula feeding, exposure to pathogens like HIV, and specific characteristics of the vaccine, such as the dosage interval and active antigen in the vaccine.

Vaccine responses may be broadly classified as T-cell dependent or T-cell independent (Wilson & Kollmann, 2008). In most cases however, the success of a vaccine usually relies on the production of adequate protective antibodies, which prevent future symptomatic infections. Although, some data suggest that in the case of viral pathogens, T-cell immunity may be a better correlate of protection than antibodies (PrabhuDas et al., 2011). Vaccines such as those against Rotavirus infection may require both the production of protective antibodies as well as CD8+ T-cell responses to provide complete protection.

Vaccine mediated protection is relatively slow and multiple boosters are required for successful protection from all vaccines except BCG (Siegrist, 2001). This is evident with vaccines like Hib and Pertussis, where infants who do not complete vaccination with all boosters are still at risk of disease acquisition (Bisgard et al., 1998; Centers for Disease Control and Prevention (CDC, 1995). In addition, the large number of naïve immune cells and increased number of regulatory cells found in infant immune systems results in poorer vaccine outcomes (Gervassi & Horton, 2014; Siegrist, 2001).

There are many reasons why a single dose of vaccine is not sufficient to induce protection against most pathogens. Studies in foetal baboons, lambs and premature infants demonstrate that even though IgG antibody responses are induced in utero, the responses differ in quantity and quality from those responses generated later on in life (Stoll et al., 1993). In the first 12 months of age, both IgA and IgG responses to viral and bacterial pathogens are incompletely protective as, they have a shorter half-life and have lower affinity compared to adult antibodies (Brandenburg et al., 1997;

Samukawa, Yamanaka, Hollingshead, Klingman, & Faden, 2000). In many vaccine studies comparing vaccination visits earlier on in life to later show that, infants who are vaccinated later on in life develop higher numbers of protective antibodies compared to those vaccinated at an early age (Booy et al., 1992; Goldfarb et al., 1994; Olin, Hallander, Gustafsson, Barreto, & Podda, 1998; Tiru, Hallander, Gustafsson, Storsaeter, & Olin, 2000).

Another limitation of vaccine induced antibodies within the first year of life is that immunity wanes more so than in adulthood. Thus, even when protective immunity does developed, immunity wanes without boosters (Siegrist, 2001; Whittle et al., 1999). One reason for the waning of immunity is the lack of adult like T-cell responses (B. Adkins & Hamilton, 1992). Infants are able to mount T-cell responses as soon as 10 days after vaccination (Barrios et al., 1996). Similarly to antibody responses however, these T-cell responses differ from that of adult T-cells (B. Adkins & Hamilton, 1992).

According to the expanded program of immunization (EPI) in most countries, multiple vaccines are administered simultaneously in the first 6 months of life, to prevent infections from various infectious diseases. These vaccines contain different antigens and adjuvants designed to elicit a desired protective response against a specific pathogen (Ota, Idoko, Ogundare, & Afolabi, 2013). However, when administered simultaneously, vaccine-vaccine interference may occur, where one individual vaccine may inhibit or promote responses to concomitantly encountered vaccines and antigens (Ota et al., 2013). One study assessing the effects of concomitantly administered vaccines found that BCG has a desirable adjuvant effect, promoting TH1 and TH2 responses to Hepatitis B and oral Polio vaccines (Aaby et al., 2000). However, this is not a consistent finding, a study conducted by Blakney et al, found that BCG vaccination had minimal effects on T-cell responses to aP and tetanus vaccines (Blakney et al., 2015). This study was done in HEU infants, who have been shown to have altered CD4+ and CD8+ responses to vaccines, which could explain the inconsistencies in findings (Blakney et al., 2015). Another study (Knuf et al., 2010), assessed the effects of acellular pertussis vaccination given either at 2-5 days of life with Hep B, or at 2,4 and 6 months as DTaP with Hep B, inactivated polio and Hib. They found that acellular pertussis elicited more rapid antibody response when

given with the multitude of other vaccines at 2-5 days of life. Contrastingly, responses to Hep B and Hib had diminished responses due to being concomitantly administered with DTaP demonstrating the varying effects of concomitantly administered vaccines on one another.

SECTION 1.3:

INFANT IMMUNE RESPONSES

Like many other biological systems at birth, neonatal immune systems are different from those of adults, resulting in an increased risk of infection (Schelonka and Infante, 1998; Hodgins and Shewen, 2011; PrabhuDas et al., 2011). There are two dominating hypotheses, which may explain why infants have suboptimal immune responses. The first hypothesis suggests that infants have functionally immature cells, which prevent them from mounting an adult like immune response (Blosser & Randolph, 2013). The second hypothesis suggests that sub-optimal immune responses in infants are more likely attributed to active immune suppression, which is induced during pregnancy (Gervassi & Horton, 2014). While both hypotheses are different, they are not mutually exclusive. Thus, the distinct characteristics of infant immune systems are most likely due to a combination of functional immaturity and immune suppression, as explained below (PrabhuDas et al., 2011).

1.3.1 FUNCTIONAL IMMATURITY

THE INNATE IMMUNE RESPONSE

Having poor or no memory response to pathogens, results in infants been heavily dependent on their innate immune responses at birth. This is because the innate immune system is able to mount efficient immune responses against pathogens without prior engagement (Velilla et al., 2006). One essential component of the innate immune system, required for a successful immune response, is pattern recognition of pathogens by toll like receptors (TLR) of innate cells. In infants, despite having the ability to repond to TLRs, seem to have defective signaling cascades downstream of TLR-engagement, resulting in an altered immune response compared to adults (Chelvarajan et al., 2004; Levy et al., 2004; Maródi, 2006; Velilla et al., 2006).

Studies have demonstrated that TLR-mediated cytokine production by mononuclear cells differ with age (Levy et al., 2004). Compared to adult responses, infant blood monocytes have a reduced ability to produce TH1 cytokines such as IFN-γ, IFN-α and IL-12 (Corbett et al., 2010). This reduction is temporary, as there is rapid

increase in cytokine production of monocytes from birth, reaching adult levels around 1-2 years of age. Contrastingly, infant monocytes have a superior ability to express cytokines like IL-10, IL-6 and IL-23, which strongly promote TH2 and TH17 cell proliferation. Furthermore, even those cells that are successfully stimulated to produce cytokine are less polyfunctional than adult monocytes (Corbett et al., 2010).

Other studies have demonstrated that even upon stimulation by potent antigens like bacterial lipopolysacchrides (LPS) or by TLR ligands, monocytes and macrophages produce diminished levels of proinflammatory cytokines, such as TNFα, IL-1β or IL-12 (Chelvarajan et al., 2004; Upham et al., 2002). Neonatal mononuclear phagocytes also show decreased responsiveness to exogenous IFNγ stimulation, which results in reduced killing of intracellular pathogens (Marodi, Goda, Palicz, & Szabo, 2001). In addition to the decreased responsiveness and lack of cytokine production, infant macrophages also have defective phagocytic ability (Strunk et al., 2004). Moreover, cord blood studies have shown that dendritic cells have suboptimal antigen presenting function and exhibit low expression of CD86 and CD40 co-stimulatory molecules (Hunt, Huppertz, Jiang, & Petty, 1994).

Another important innate cell type that has altered cell function during infancy is the neutrophil (Schelonka & Infante, 1998). Newborns have a limited number of circulating neutrophils compared to adults (Christensen, Macfarlane, Taylor, Hill, & Rothstein, 1982). They are also less able to increase production of neutrophils from the bone marrow during times of infection or rapid utilization (Christensen & Rothstein, 1980). Furthermore, these cells have diminished ability to accumulate at sites of infection. This is most likely because infants generate less filamentous actin, a cytoskeleton molecule that is essential to locomotion (Harris, Shalit, & Southwick, 1993). Under most conditions, the infant neutrophil is able to engulf and destroy bacteria relatively well. However, during respiratory distress syndrome or sepsis infant neutrophils have diminished bactericidal ability (Shigeoka, Charette, Wyman, & Hill, 1981).

Additionally, the neonatal complement system is deficient; with approximately 60% lower component concentration relative to adult blood (Firth, Shewen, & Hodgins, 2005). The complement system is necessary for pathogen killing by membrane disruption as well as opsonisation of pathogens, so enabling phagocytosis and

antigen presentation. Certain proteins such as C9, which is an integral part of the complement cascade, may be as little as 10% the concentration found in adults (Firth et al., 2005). Moreover, the cleavage of complement protein C3, results in the production of products that enhance the adaptive immune system (Dempsey, Allison, Akkaraju, Goodnow, & Fearon, 1996). These cleaved products interact with complement receptors on follicular dendritic cells, circulatory dendritic cells and B cells. A study conducted by Pihlgren et al, demonstrated that one of the complement system products, C3d, had the ability to reduce the amount of antigen, required to stimulate antibody responses by up to 10000 fold. In addition, C3d also induced a 1000 fold Ca^{2+} ion flux of antigen-specific B cells (Pihlgren et al., 2004). Since B-cells and antibodies are essential to the success of certain vaccines, this lack of complement proteins may result in a major reduction of vaccine success.

Although innate immunity is seen as a primary line of defense against invading antigen, innate immune cells should also be considered as crucial role-players in shaping the adaptive immune system to mount an antigen-specific response. This is especially true for dendritic cells (Zaghouani, Hoeman, & Adkins, 2009). Dendritic cells engulf and digest microbes into peptide fragments, which are then displayed on the surface of the cell complexed with MHC molecules. The T-cell receptor (TCR) of T-cells bind to the displayed peptides bound within the MHC groove resulting in the T-cell becoming activated. Dendritic cells also secrete multiple cytokines that serve to activate other T-cells. Thus, defects in the functional ability of dendritic cells may also result in altered T-cell responses. A murine model has been used to show that, defects in dendritic cell function alters T-cell responses (Lee et al., 2008). In this study, adult T-cells were isolated, primed with antigen, and transferred to newborns, where they differentiated into TH1 subtypes that did not undergo apoptosis, as is common for T-cells that originate in newborns and undergo TH1 differentiation upon re-challenge with antigen. However, newborn T-cells were able to develop a TH1 response without apoptosis, when exogenous IL-12 was present during re-challenge. In infants the levels of exogenous IL-12 is very low (Li et al., 2001). Dendritic cells mainly produce IL-12 but infants have lower frequencies of dendritic cells than any other accessory cell (Goriely et al., 2004). In addition, the subtypes of dendritic cells that are present in newborns are of a CD8a-CD4- phenotype. This type of dendritic

cells inherently produces less IL-12 then other subtypes. The expansion of IL-12 producing dendritic cell subtypes increases with age (Lee et al., 2008).

The interaction between dendritic cells and T-cells is just one example of the inter-relationship between the innate and adaptive immunity and is required in infants for the development of protective vaccine responses early in life.

B-CELLS

Neonatal B-cells are functionally and phenotypically different from adult B-cells (Marshall-Clarke, Reen, Tasker, & Hassan, 2000). Most B-cells in infants are cells that have recently migrated from the bone marrow to the circulation. These cells are not completely matured and more functionally limited than mature B-cells. In infants under the age of 4 months, germinal center defects are exhibited, as well as suppressed expression of surface markers CD21, CD40, CD80 and CD86 on naïve B-cells (Siegrist & Aspinall, 2009). This predominance of B-cell that have recently migrated from the bone marrow, together with the immaturity of other immune cells results in altered antibody responses. Infant antibodies usually have a shorter half-life than adults do. Infants also have limited antibody-producing plasma cells in the bone marrow, resulting in a limited persistence of antibody responses. Additionally infant antibody responses have a delayed onset and a different distribution of immunoglobulin isotypes. When compared to adult antibodies these antibodies also exhibit a lower affinity for antigen (Siegrist & Aspinall, 2009).

Studies in mice have demonstrated that neonatal splenic B-cells have a selective inability to up regulate MHC II in response to BCR ligation (Tasker & Marshall-Clarke, 1997). However, this response is only induced by CD40 ligation or IL-4. Because antigenic peptides are presented largely on recently synthesized MHC II, the failure to hyper-express MHC II may result in reduced array of antigenic peptides presentation via MHC II (Brugnoni et al., 1994; Marshall-Clarke et al., 2000). Consequently, B-cell signaling of T-cells would be reduced. If these signals are reduced significantly, they will fail to activate T-cells and no CD40 ligand establishment of T-cell-B-cell interaction would occur. Additionally, diminished upregulation of CD40L on T-cells will compound this effect (Brugnoni et al., 1994).

The failure of B-cells to upregulate co-stimulatory molecules, which are required for T-cell interaction, can result in anergy towards antigens due to a lack of secondary signals required for T-cell function. That is, T-cells receiving poor signals due to low density peptide-loaded MHC II, in the absences of co-stimulatory molecules, rarely get activated (Marshall-Clarke et al., 2000). Even when neonatal T-cells have fully developed signaling abilities, anergy seems to be induced, as neonatal T-cells seem to require a higher density of co-stimulatory molecules than adult T-cells for activation. Low expression of MHC on B-cells has also been shown to favor a TH2 type differentiation and may be one factor contributing to the TH2 bias found in infants (Murray, 1998).

TH1, TH2 AND TH17 RESPONSES

At birth neonates are exposed to a vast number of antigens, some pathogenic, others benign. CD4+ T-cells play a central role in shaping the type of immune response that is directed againt an antigen, such as cellular immune responses, antibody responses and tolerance. T-cells are able to modulate the immune system by differentiating into different subsets of T-cells in response to different antigens. Each subset expresses distinguishing transcription factors, secretes characteristic cytokines and promotes distinctive immunological functions (Blosser & Randolph, 2013). However, there are also T-cells which have overlapping characteristics and may be classified into multiple subsets. A good example of this is TH1/TH17 cells, which produce both IL-17 and IFNγ (Annunziato et al., 2007; Lexberg et al., 2010). Figure 1.3.1 below shows the differences in transcription factors and cytokine production between the 4 most common types of T-cells. It also demonstrates the different effector functions of each subtype.

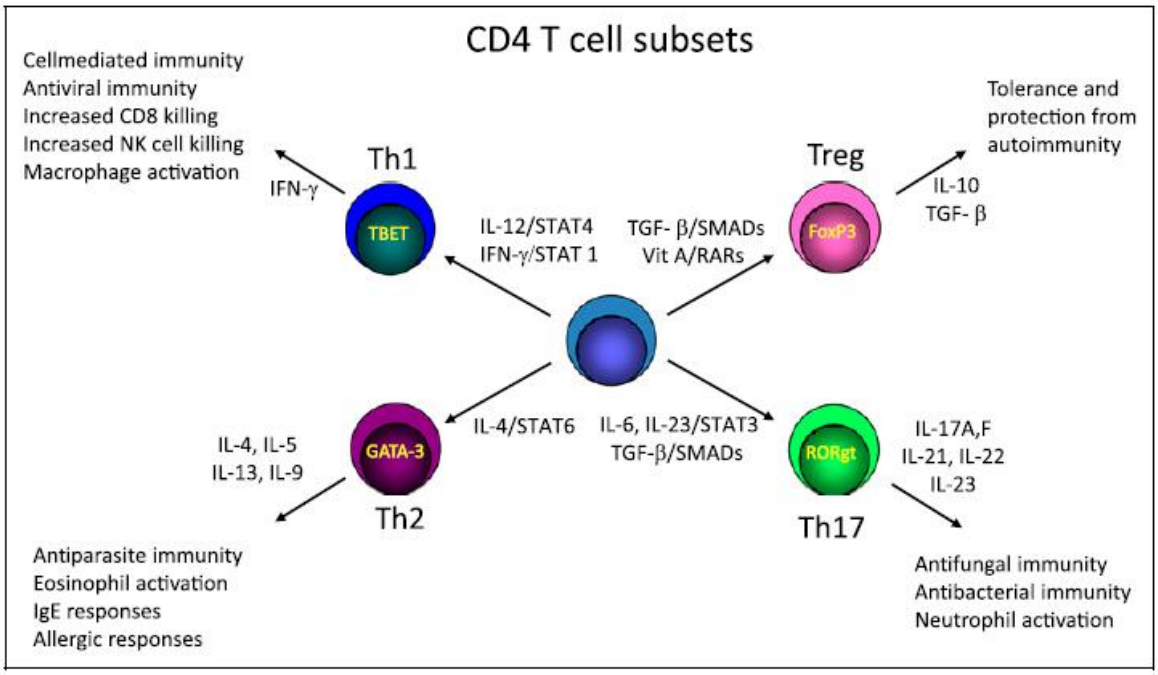


Figure 1.3.1: Development of CD4 effector/memory subsets from naive T cells. After activation of a naive cell by TCR stimulation, polarizing cytokines signaling through signal transduction molecules push the cell toward an effector lineage. Upregulation of a lineage-specific master regulator transcription factor stabilizes the cell phenotype. The differentiated cell then produces effector cytokines that promote a particular facet of the immune system (Blosser & Randolph, 2013)

TH1 and TH2 subsets were the first two subsets of T-cells to be described (COFFMAN, 1986). TH1 cells promote natural killer cell and CD8+ cytolytic functions as well as encourage macrophage phagocytosis, all of which are important in cellular immunity and allow for the clearance of intracellular infections. TH2 cells are involved in promoting eosinophil and mast cell functions as well as stimulating humoral responses. By 2006 a third subset of helper T-cells now known as TH17 cells were discovered (Thomas Korn,1 Estelle Bettelli,2 Mohamed Oukka,3 and Vijay K. Kuchroo, 2009). TH17 cells encourage neutrophil activity and are vital in antibacterial and antifungal immune responses, especially at mucosal surfaces (Blosser & Randolph, 2013; Yamane & Paul, 2013). Since then many more T-helper subsets have been discovered, such as TH9, T-follicular helper cells, TH 23 and T-regs. (Bluestone & Abbas, 2003; Nurieva et al., 2008; Stassen, Schmitt, & Bopp, 2012; Yamane & Paul, 2013). In most healthy adults, there is a fine balance between the many subsets of T-cells, which allow for optimal functioning of the immune system (Blosser & Randolph, 2013).

Neonatal immune systems are commonly characterized by a lack of effective T-helper 1 (TH1) cell responses and excessive T-helper 2 (TH2) responses (Van den Biggelaar, Anita HJ et al., 2011). During pregnancy, the maternal immune system has to have a dampened TH1 response, thus preventing an inflammatory response towards the fetus, as it is an allograft within the pregnant mother (Wegmann, Lin, Guilbert, & Mosmann, 1993). The placenta produces soluble factors, which are responsible for skewing maternal responses away from a TH1 response to a TH2 response, thus protecting the fetus. However, these factors seem to have a similar effect on the growing fetus's immune system, resulting in a skewed TH2 response and dampened TH1 response at birth (Morein, Blomqvist, & Hu, 2007; Wegmann et al., 1993).

Another factor that contributes to the TH2 skewing is a lack of CD154 expression (Lewis et al., 2006). CD154 on T-cell surfaces interact with CD40 on myeloid dendritic cells resulting in the release of IL-23 and IL-12 by dendritic cells. It also upregulates expression of CD80 and CD86, which are involved in T-cell activation (O'Sullivan & Thomas, 2003). IL-12, as previously mentioned, together with IL-23 is essential to the development of TH1 type CD4 memory cells. CD154 is also important in enhancing the microbicidal activity of mononuclear phagocytes (Lewis et al., 2006).

In addition to the imbalance of TH1 and TH2 cells, infants lack effector/memory Th17 cells and are therefore not fully protected against bacterial and fungal infections (Katz, Benoist, & Mathis, 1995). With the exception of T-reg cells, when compared to most other T-cell subtypes, naive infant T-cells preferentially differentiate into effector TH17 cells (A. Black, Bhaumik, Kirkman, Weaver, & Randolph, 2012). This contrasts with naïve adult T-cells and implies that infants are capable of rapidly developing TH17 responses upon stimulation by a pathogen.

Furthermore, infants have a larger proportion of naïve T-cells compared to adults (Thome et al., 2016). In the blood, spleen, lymph nodes, and colon of children under the age of 4 years, approximately 70-95% of CD4+ and CD8+ T-cells are naïve. In contrast, fewer than 50% of adult cells are naïve. Aside from a lack of antigenic exposure, infant naïve T-cells are functionally different from adult naïve T-cells. Poor T-cell responses were postulated to be caused by low numbers of T-cells been

present in the infant spleen compared to lymph nodes, where faster maturation is thought to occur (B. Adkins & Du, 1998). A study looking specifically at recent thymic emigrants (RTE) found that these cells are actually present in high quantities in the infant periphery, but had a diminished ability to perform TH1 functions (Boursalian, Golob, Soper, Cooper, & Fink, 2004). This is because RTE, which have recently emerged from the thymus and entered the lymphatic system, have had very little time to mature. Their maturation is very subtle and they still express naïve markers until they encounter their corresponding antigen. These RTE favor a TH2 differentiation, and express reduced amounts of IL-2 when activated compared to adult T-cells. The amount of RTE in circulation decreases with age (Bhaumik et al., 2013; Yamane & Paul, 2013). This is most likely beneficial against the development of allergic responses in early life, as infants are detecting multiple antigens for the first time, most of which are harmless and should be tolerated.

1.3.2) IMMUNE SUPPRESSION

One of the most important subset of T-cells is the regulatory T-helper cell (T-reg) subset. As shown in figure 1.3.1, these cells are important in tolerance and protection from autoimmunity. At birth, infants show a dominant anti-inflammatory T-reg cytokine profile. This seems to be induced during pregnancy to prevent reactivity with maternal and environmental antigens (Michaelsson, Mold, McCune, & Nixon, 2006). The amount of thymic T-reg cells in blood decreases with gestational age but seems to be the only subset of T-cells present in significant numbers at birth (Mayer et al., 2012; Takahata et al., 2004). These cells are strong suppressors of allogeneic T-cell proliferation. They are essential in preventing autoimmunity and inducing tolerance to benign environmental antigens (Godfrey et al., 2005; Mold et al., 2008). Studies in mice have shown that during development, the thymus expresses high levels of milk antigens (Anderson et al., 2002). This suggests that neonates are born with a repertoire of T-reg cells capable of inducing tolerance to one of the first antigens infants are exposed to, mothers' milk content. Moreover, in contrast with adult responses, when infant CD4 T-cells are stimulated through the T-cell receptor by any type of antigen, 70% of T-cells will preferentially differentiate into Tregs rather than effector CD4 T-cells (G. Wang et al., 2010).

Another subset of suppressor cells found in infants, myeloid derived suppressor cells (MDSC), have also been shown to skew immunity towards a TH2 response or dampen TH1/Th17 responses (Poschke et al., 2012). There are 2 types of MDSC's, granulocytic-like (G-MDSC) and monocytic-like (M-MDSC) (Ioannou et al., 2012; Tacke et al., 2012; Vollbrecht et al., 2012). Healthy neonates have and increased frequency of G-MDSC from birth to about 6 weeks of age (Gervassi et al). Neonatal MDSC have been shown to decrease IL-5, IL-17 and IFNγ secretion by T-cells, as well as drastically suppress T-cell proliferation (Rieber et al., 2013).

In contrast to the immaturity hypothesis, these findings collectively suggest that infants have fully functional T-cells but that these cells are actively suppressed preventing adult-like immune responses. While both the 'immaturity' and 'active immune suppression' hypotheses suggests that infants are unable to respond optimally to infections, recent evidence shows that these suboptimal responses are actually necessary for the maintenance of homeostasis (Kollmann, Kampmann, Mazmanian, Marchant, & Levy, 2017). This is mainly because infants are exposed to rapidly shifting environmental and microbial exposures, which relentlessly stimulate the immune system in the post-natal period (MacGillivray & Kollmann, 2014). Thus, intense immune regulatory mechanisms are required to prevent excessive inflammation and maintain homeostasis (MacGillivray & Kollmann, 2014). Given the unique demands forced upon infants to maintain homeostasis and simultaneously prevent infections, the infant immune system is not considered immature or suppressed but rather it is designed to perform what is necessary at that particular age of life. Ghazal et al, provided evidence for this by demonstrating that infants are capable of mounting immune responses of substantial magnitudes (Ghazal, Dickinson, & Smith, 2013). However, it seems that the threshold of stimuli needed to initiate such a responses is much higher than those of adults. This is likely due to the fact that once initiated, the high magnitude immune responses found in infants result in high immune pathology, which partially explain the elevated risk of morbidity found in early life compared to adult life (Iwasaki and Medzhitov, 2015). While the infant immune system is required to function suboptimally in order to maintain homeostasis, it does leave infants more susceptible to infections. However, there are naturally occurring compensatory practices, such as breast-feeding which may reduce some of this susceptibility.

SECTION 1.4:

BREAST MILK

All neonates receive passive maternal immune protection during gestation in the form of antibodies. These antibodies are capable of protecting the infant from pathogens for weeks to months after birth. As infants are unable to produce antibodies that are as effective at pathogen clearance as adult antibodies, passive immunity transferred through breast-feeding after birth plays an important role in protection of the infant.

It has long been known that infants who are breast-fed contract fewer infections compared to those that are formula fed (Newman, 1995). This is in part because formula milk has to be mixed with water and delivered in containers, both of which may not be sterile and thus may expose the infant to pathogens. In addition, breast milk contains a host of bioactive factors, capable of immunological, hormonal and enzymatic activity that actively protects the infant from infection (Hamosh, 2001). Breast milk also has an abundance of immune cells that are capable of modulating the infant's immune system (Xanthou, 1997).

1.4.1) BIOACTIVE FACTORS IN BREAST MILK

Within the gut, indigestible milk components like sialylated and fucosylated oligosaccharides directly protect against infectious diarrhea (Coppa et al., 2006). These oligosaccharides contain bacterial binding site and accomplish protection by binding to bacterial molecules forming complexes, which the infant excretes (Andersson, Porras, Hanson, Lagergard, & Svanborg-Eden, 1986; Cravioto et al., 1991; Ruiz-Palacios et al., 1990). Similarly, to this, mucins in breast milk adhere to different bacteria and viruses, eliminating them from the infant's body (Newman, 1995).

Other valuable functions performed by breast milk may be attributed to the presences of the protein lactoferrin (Lönnerdal, 2010). Lactoferrin has an affinity for iron, where each lactoferrin molecule is able to bind to two iron molecules. Many pathogenic bacteria require iron for proliferation. Thus, lactoferrin halts the spread of such bacteria by limiting the supply of iron (Wakabayashi, Yamauchi, & Takase, 2008). Lactoferrin also limits the growth of bacteria by disrupting the mechanism by which

bacteria ingest carbohydrates. Additionally, a study done by Arnold et al demonstrated the iron free-forms of lactoferrin, which are the most abundant type of lactoferrin found in breast milk, can kill multiple pathogenic bacteria (Arnold, Brewer, & Gauthier, 1980).

Multiple other factors contribute to the protection conferred by breast milk. B12 binding-protein haptocorrin, for example, binds to b12 depriving microorganisms, such as e.coli, of the vitamin (Y. Adkins & Lonnerdal, 2003). Bifidus factor is the oldest known disease-preventing factor found in milk and promotes the growth Bifidobacteria, known to be important in regulating immunity (Soto et al., 2014) Additionally, free fatty acids in milk can damage the membranes of certain enveloped viruses such as Chicken pox virus (Newman, 1995).

Human colostrum, which is the milk produced during the first few days after birth, is the most concentrated source of maternally derived immune protective factors (Rodriguez, 2013; Uruakpa, Ismond, & Akobundu, 2002). Fibronectin, which is present in large quantities, enhances the function of phagocytes, allowing them to attack microbes, which are not targeted by antibodies (Golinelli, Del Aguila, Paschoalin, Silva, & Conte-Junior, 2014). Fibronectin also limits inflammation and assists in repairing inflammation induced tissue damage (Newman, 1995).

Colostrum also contains many cytokines, some of which include; Interferon gamma, transforming growth factor alpha and beta, interleukins 6, 8 and 10 and tumor necrosis factor alpha (Bocci et al., 1993; Goldman, Chheda, Garofalo, & Schmalstieg, 1996; Tomičić et al., 2010). These cytokines modulate the infant immune system but their exact mechanisms are not yet fully understood. TGFβ levels in colostrum have been shown to correlate with infant serum IgA concentrations at 1 month of age (Ogawa et al., 2004).

1.4.2) BREAST MILK CELLS

Cellular components are also found in colostrum and breast milk. The most abundant cell types found in milk are macrophages, neutrophils and lymphocytes (Lawrence & Lawrence, 2010). Macrophages are the most copious leukocyte in breast milk, followed by neutrophils (Hassiotou, Geddes, & Hartmann, 2013). Macrophages are active and phagocytic within the infant gut, where they are also more motile that blood monocytes (Özkaragöz et al., 1988). They manufacture lysozymes and release them into the infants' intestinal tract. These lysozymes are enzymes, which kill bacteria by destroying the cell wall of bacteria (Pitt, 1979). The function of neutrophils in milk is not yet clear, it was suggested that these cells are present to simply protect the breast from infection rather than be beneficial to the infant (Gershwin, German, & Keen, 1999)

Around 80% of breast milk lymphocytes are T-cells, the other 20% are antibody-producing plasma cells (Xanthou, 1997). Most of these cells are of memory phenotype, and are able to eliminate infectious agents (Bertotto et al., 1990). They also modulate other aspects of the immune system through chemical signaling (Newman, 1995). They manufacture chemokines and cytokines that may strengthen the infant's own immune response. Although these traits are similar to blood cells, breast milk lymphocytes are different from blood lymphocytes in certain aspects. In the presences of E.coli, bacteria that may cause life-threatening infections in infants, milk lymphocytes proliferate at a superior rate to their blood counterparts (Newman, 1995).

Animal studies have shown that breast milk leucocytes are able to reach organs other than the GIT in their offspring. Jain et al found that human milk leucocytes that were transferred to neonatal baboons via breast milk were in high concentrations within the stomach and intestinal lumen of the neonate (Jain et al., 1989). Additionally, minor concentrations of leucocytes were found in the baboons' spleen and liver. In humans, it was found that activated and memory T-cells could pass through the stomach and intestine of infants, especially in the first few days of life when gastric pH has not yet become acidic (Wirt, Adkins, Palkowetz, Schmalstieg, & Goldman, 1992). Being activated, motile, and interactive, suggests that breast milk leucocytes may confer active immunity to infants (Smith & Goldman, 1968; Smith &

Goldman, 1970; Wirt et al., 1992). However, it is still to be determined whether these cells are capable of surviving the acidic pH of the stomach in older infants.

1.4.3 BREAST MILK ANTIBODIES

Arguably, the most influential aspect of breast-feeding on infant immunity is the transfer of passive antibodies from mother to child. Particularly well known for its protective role in neonates is breast milk IgA. IgA2 is found in much higher concentrations in breast milk than in plasma. This subset of IgA is resistant to digestion by enteric enzymes and peptic acid in the stomach (Chirico, Marzollo, Cortinovis, Fonte, & Gasparoni, 2008). Like many other milk components, IgA binds to microorganisms preventing them from interacting with the infants' gut mucosa.

Most passively transferred antibodies made by the maternal immune system, would be directed against environmental pathogens. Since, infants' most likely share the same environment as their mothers, they are likely to encounter the same pathogens and be protected by passively transferred antibodies (Chirico et al., 2008). In addition, while infant derived antibodies may still be developing, and thus have lower affinities for their target pathogens, maternal antibodies are highly specific. Consequently, maternal antibodies provide critical protection against pathogens whilst infant antibodies are still developing (Newman, 1995).

Although passively transferred maternal antibodies are crucial for the protection of infants against infections, they may also have negative impacts on vaccine responses. In general, there is a negative association between passive antibody titers and the magnitude of active antibody responses to vaccination in infants (S. S. Moon et al., 2010; PLOTKIN, KATZ, BROWN, & Pagano, 1966). This association is most apparent in modified live vaccine responses, like Rotavirus, OPV and Measles. In many cases, maternal neutralizing antibodies reduce replication of the vaccine virus resulting in a reduced dose of vaccine antigen available for stimulation of the infant immune system (Hodgins & Shewen, 2012).

SECTION 1.5:

HIV EXPOSURE

Another potential consequence of breast-feeding by HIV-infected mothers is the potential for transfer of HIV to the infant via breast milk. At birth and during the first few months of life, 20-100% of HEU infants have detectable proliferative and cytotoxic T-cell responses against numerous viral antigens, which may contribute to the risk of acquisition (Aldhous, Watret, Mok, Bird, & Froebel, 1994; Cheynier et al., 1992; Kuhn, Meddows-Taylor, Gray, & Tiemessen, 2002; Rowland-Jones et al., 1993). Activated CD4 T-cells are target cells for HIV infection. Thus an increase in proliferation or activation of CD8+ T-cell, which may results in activation of CD4+ T-cells, is likely to increase the risk of HIV acquisition.

The implications of immunological differences found between HEU and HU infants on childhood vaccines were investigated in a South African based cohort (Jones et al., 2011). The study looked at maternal and infant specific antibody levels in response to numerous childhood vaccines. They found that antenatal HIV exposure was associated with lower specific antibody responses at birth but with more robust responses post vaccination. Further investigation on the effects of HIV exposure on an infant's immune response to vaccines is important. It may help us to understand why these infants are at higher risk for infections (Simani et al., 2014).

Furthermore, HEU infants have also been shown to have an elevated risk for mortality and morbidity when compared to infants born to HIV negative mothers (Mussi-Pinhata et al., 2007). Studies done in Sub-Saharan Africa found that HEU infants have an increased mortality risk due to infectious diseases, and this risk is associated with advanced maternal HIV/AIDS (Kuhn et al., 2005; Marinda et al., 2007). A large cohort study done in the Caribbean and Latin America found that HEU infants had at least one infection by the age of 6 months (Mussi-Pinhata et al., 2007). Lower respiratory infections were the most common. These infants had a risk of death that was associated to low maternal CD4 counts (Kuhn et al., 2005).

Additionally pathogens such as Mycobacterium tuberculosis, Pneumocystis jirovecii, together with group A Streptococcus and Cytomegalovirus have caused an unexpectedly high number of infections in HEU infants (Heresi, Caceres, Atkins,

Reuben, & Doyle, 1997; McNally et al., 2007; Slogrove, Cotton, & Esser, 2010). In Belgium it was reported that HEU have a 19 times higher incidences rate of group B Streptococcus infections then HU infants (Epalza et al., 2010). Within the same cohort, it was found that 25% of HEU infants with infectious diseases occurring in the first 12 months of life required hospitalization. The rate of hospitalization was associated with advance maternal HIV/AIDS. Collectively these studies suggest that, maternal factors associated with HIV infection may increase susceptibility of HEU infants to infection diseases.

Several maternal and external factors may contribute to this amplified susceptibility of HEU infants. HEU infants are exposed to increased numbers of pathogens due to maternal HIV-related disease (Dauby et al., 2012). Since most of the studies conducted were in developing countries, poor socio-economic factors may have an influence. Also, while PMTCT may prevent infants from getting HIV, the toxicity of the drugs may have a negative impact on the health of these infants as well (Bunders, Thorne, & Newell, 2005; Le Chenadec, Mayaux, Guihenneuc-Jouyaux, Blanche, & Enquete Perinatale Francaise Study Group, 2003; Pacheco et al., 2006)

Moreover, altered passive or actively acquired immunity has been suggested to hinder certain immunological functions in HEU infants. During the first months of life HEU infants have been shown to have lower than normal levels of CD4+ T-cells (Roberts & Yeung, 2002). This has been associated with a decrease in efficiency of clonal progenitors as well as reduced thymic output and increased serum levels of interleukin 7 (Desai et al., 2007; Woodburn et al., 2009). After in-vitro polyclonal stimulation of HEU infants, an altered T-cell activation with increased production of interleukin 10 and decreased interleukin 2 production was detected.

The mechanisms that cause these vast differences between the immune system of HEU infants and infants born to HIV negative mothers are only partially known. It may be attributed to placental transfer of non-infectious HIV molecules that have immunosuppressive abilities. Perhaps activation of the maternal immune system results in the transfer of immune cells and mediators across the placenta. Antiretroviral exposure may also contribute. It was previously shown that nucleotide reverse transcriptase inhibitors administered during pregnancy causes a decline in blood cell counts of various lineages (Bunders et al., 2005; Le Chenadec et al., 2003;

Pacheco et al., 2006). Although, increased morbidity has been detected, even in the absence of ART suggesting that, the increased morbidity is not caused by exposure to ART alone (Marinda et al., 2007; Thea et al., 1993). Moreover, with the introduction of ART, and therefore predominantly undetectable viral loads in pregnant mother, infants likely are not exposed to HIV-antigens. This implies that the immunologically differences is likely attributable to something other than direct exposure to HIV.

Assessing the effects of maternally-derived HIV/ART exposure on an infant's immune response to vaccines will help provide a marker of infant immune competence and aid in our understanding of why these infants are at higher risk of co-morbidities (Simani et al., 2014). A study conducted in Sub-Saharan Africa has shown that exposure to HIV results in altered T-cell responses to BCG and aP vaccination (Kidzeru et al., 2014). This was the first study to assess B. pertussis specific T-cell responses in HEU and HU infants. They found that HEU infant's cytokine responses to B. pertussis were less polyfunctional than HU infants. One of the main aims of this dissertation is to assess aP vaccine induced T-cell responses in a similar cohort of HEU infants.

SECTION 1.6:

AIMS AND HYPOTHESIS

Hypothesis 1: Infant Rotavirus specific T-cell responses will be detected against all rotavirus antigens regardless of HIV exposure and feeding mode.

Aim 1: To develop an in vitro assay to measure Rotavirus-specific T-cell responses in infants.

Objectives:

- To culture DS-1 strain of Rotavirus in Vero cells
- To remove LPS from E.coil derived VP4 and VP6
- To determine the optimal titration of DS-1 whole Rotavirus, VP4 and VP6 proteins, for the detection of peak CD4 and CD8 IFNg, IL-2 and TNFa rotavirus vaccine specific responses.
- To measure T-cell cytokine responses to Rotarix in infants.

Hypothesis 2: HIV Exposed but uninfected infants have compromised immunity, that will result in weaker vaccine responses compared to unexposed infants.

Hypothesis 3: HEU formula fed infants will have the most compromised immunity resulting in weaker vaccine responses to Bordetella pertussis vaccination compared to HEU breast fed infants.

As a measure of immunity, acellular Pertussis vaccine responses will be assessed in the three different populations of infants enrolled in this study, in order to test hypotheses 1 and 2 using the following aims and objectives.

Aim 2: To determine the kinetics of acellular Pertussis vaccine responses in infants

Objectives :

- To measure peak aP specific CD4+ and CD8+ IFNg, IL-2 and TNFa vaccine responses.
- To determine the peak activation (HLA-DR+) and proliferation (Ki67) time point of CD4+ and CD8+ Bordetella Pertussis vaccine specific responses.
- To assess the changes in memory phenotypes of aP specific CD4+ T-cells from pre-vaccination to week 36 of age.

Aim 3: To assess the effects of HIV exposure on Bordetella Pertussis Vaccine Responses.

Objective :

- Will compare aP specific CD4+ and CD8+ T-cell activation, proliferation, memory differentiation and cytokine responses between HEU and HU infants.

Aim 4: To assess the effects of feeding practice on Bordetella Pertussis vaccine responses in HIV exposed but uninfected infants.

Objective :

- Will compare aP specific CD4+ and CD8+ T-cell activation, proliferation, memory differentiation and cytokine responses between HEU breast and HEU formula infants.

Chapter 2:

STUDY DESIGN AND METHODOLOGY

SECTION 2.1:

STUDY DESIGN

STUDY SITE

300 mother-infant pairs were recruited from a larger parent study at the Midwife Obstetric Unit (MOU) at site B in Khayelitsha. The parent study entitled, “Innate, Adaptive and Mucosal Immune Responses in HIV-1 Exposed Uninfected Infants: A Human Model to Understand Correlates of Immune Protection”(UCT REC/REF 285/2012), seeks to identify correlates of protection against HIV conferred by exclusive breast feeding. Khayelitsha is an urban township situated 28 miles from the University of Cape Town, where the study laboratories are located. There is a population size of approximately 400 000 people in this area and unemployment and informal housings are highly prevalent. An estimated 30-32% of the deliveries are to HIV-infected mothers. Over the past 6 years breastfeeding has become a more frequent practice due to the 2010 WHO guidelines (World Health Organization, 2010), which supports breast-feeding with the use of ART. The current PMTCT and EPI programs in SA make this site suitable and feasible for the purposes of this study.

STUDY COHORT

Recruitment occurred at the MOU the morning after delivery. All women who met the eligibility criteria (Table 2.1.1) were invited to participate. In accordance with WHO guidelines, all women were encouraged to exclusively breast (or formula where applicable) feed their infant for 6 months. Trained counselors provided support and counseling to mothers, including information on the risks related to HIV transmission. All women and the infant's father (where applicable) signed a study informed consent form prior to any study procedures. The study was implemented in conjunction with the routine pediatric follow up and the infant EPI vaccination programs to enhance its feasibility and acceptability to study participants.

Table 2.1.2: South African EPI schedule for Healthy, Asymptomatic HIV Infected and HIV Exposed but Uninfected Infants

Inclusion Criteria	Exclusion Criteria
Maternal Factors	Maternal Factors
1) Age of mother ≥ 18 yrs 2) Mother is able and willing to do the follow up assessments	1) Complications during pregnancy and delivery such as chorioamnionitis and eclampsia
Infant Factors	Infant Factors
1) Gestational age ≥36 weeks 2) Birth weight ≥ 2.4kg	1) Hypoxic injury/ seizures/ sepsis/ Intrauterine growth retardation

VACCINATIONS

All infants have been vaccinated according to the South African EPI vaccination schedule, depicted in table 2.1.2 below.

Table 2.1.2: South African EPI schedule for Healthy and Asymptomatic HIV Infected Infants

Vaccine	At birth	Week 6	Week 10	Week 14
BCG	x			
OPV	x	x		
DTaP		x	x	x
Hep B		x	x	x
IPV		X	x	x
Rotarix		X		x

CLINICAL ASSESSMENTS

Clinical evaluations were undertaken at each scheduled study visit. Maternal baseline evaluations included a medical history and co-morbidities, physical, HIV disease history; ART treatment, most recent plasma HIV-RNA if available and CD4 count. In addition, obstetric information has also been derived from the medical charts. Infant baseline evaluations consisted of anthropometrics, physical, gestational age at birth and relevant birthing factors and complications. The status of maternal and infant health was monitored at each clinic visit using standardized

forms. Specific health factors evaluated at each visit included, height, weight, co-infections, thrush (infant), and medications including ART. In addition a breast exam was conducted at each visit to identify any clinical breast pathology (e.g. nipple cracks, mastitis).

FEEDING PRACTICES

Information on feeding practices was also collected using structured feeding questionnaires validated in similar settings (Donald, 2006). Importantly, research personnel administering the questionnaire were different from individuals that provided breastfeeding counseling to the mothers, in order to minimize bias.

ETHICAL CONSIDERATIONS

Prior to enrolling patients in the study, the proposed research had undergone ethical review and been approved by the human research ethics committee at the University of Cape Town and by the Western Cape Department of Health and Site B Clinic Facility manager, the medical clinic where the work was carried out. Approved consent forms were used to document all consent. Informed consent was obtained from the mother in their preferred language, isiXhosa for most participants. Infants were ineligible if mothers were less than 18 years of age. All research staff underwent Good Clinical Practice (GCP) training. The maximum volume of blood that can be drawn from an infant is 2.5ml. Blood volumes used for this study was well within the limits in this age group, The alternative to study participation was not to participate, and this was made clear that the choice not to participate would not affect their clinical care.

LABORATORY ASSESSMENTS

INFANT BLOOD:

Sodium heparin tubes were used for the collection of infant blood, which was transported to the lab for processing within 4 hours of collection. Approximately 1.5ml of blood was collected at birth and 5ml at week 4, 15 and 36. The blood was used for PBMC isolation as well as whole blood fixing with FACS lysing buffer (BD FACS Lysing Solution, Cat#349202) after the removal of plasma. Plasma was stored at -80^{o}C, fixed blood and PBMC's were stored in liquid nitrogen for later analysis in

the parent study. Additionally, whole blood assays were done using infant blood at all 4 time points to assess vaccine immunogenicity (Section 2.2 page 49).

MATERNAL BLOOD:

Maternal blood was collected on D4-7, weeks 4, 15 and 36 in ethylenediaminetetraacetic-acid (EDTA) tubes to prevent coagulation. Plasma and PBMC's were removed and stored as for infant blood.

HIV DNA PCR

Infants born to HIV+ mothers had HIV DNA PCR's performed in real time at birth and week 4. Those infants that were breast fed also had a dried blood spots collected for PCR at week 15 and 36 or, at 6 weeks post cessation of breast feeding, whichever came first.

SECTION 2.2:

EXPERIMENTAL ASSAYS USED IN DESSERTATION

Several different techniques were utilized to address the aims of this dissertation. For the optimization of a rotavirus T-cell detection assay three different antigens were tested, including, DS-1 whole Rotavirus and two viral proteins VP4 and VP6, in PBMC stimulation assays as well as a whole blood assay. Cytokine responses were assessed using intracellular staining and subsequent flow cytometry. To assess cellular immune responses to BP an already optimized whole blood assay and flow cytometry were utilized.

Table 2.2.1: Summary And Description Of Assays

Assay	Description
Vero cell culture	DS-1 rotavirus was grown in vero-cell lines
VP4 and VP6 protein derivation in E.coli	VP4 and VP6 were the antigens tested for optimal detection of RV CD4 and CD8 responses
Western Blot	Identification of protiens
Endotoxin removal	Endotoxin was removed from the E.coli derived VP4 and VP6
Endotoxin Quantification	The Limulus Amoebocyte Lysate chromogenic endotoxin quantification kit was used to determine the concentration of LPS pre and post endotoxin removal
Whole Blood Assay	Determination of antigen specific CD4+ and CD8+ T cell responses in infants
Flow Cytometery	Determination of antigen specific CD4+ and CD8+ T cell responses in infants

STIMULATION ANTIGENS

DS-1 ROTAVIRUS

DS-1 Rotavirus is a Group A Rotavirus, and one of the main strains which infect humans (Gentsch et al., 2005). Based on the assumption that most adults would have a response to DS-1 due to its ubiquitous nature, this strain was chosen as an antigen. DS-1 was cultured in Vero-cells, which are a lineage of cells derived from African Green Monkey kidney cells.

Vero cells were cultured in EMEM containing antibiotics and essential amino acids at 37^{o}C in 500ml culture flasks. When cells had reached 70% confluence, they were split. Vero cells are adherent, so 1-5ml of trypsin was added to culture for 10 minutes to loosen cells. Loosened cells were transferred to 50ml falcon screw cap tubes and centrifuged at 1500rpm for 10 minutes. Trypsin was discarded and the cells were resuspended in EMEM. Half the cells were transferred to a second culture flask. Cells were split multiple times to obtain a large volume of working stock prior to infection.

Once sufficient amounts of Vero cells were cultured, the cells were infected with unpurified DS-1 whole Rotavirus. For infection, the virus was activated with Trypsin at a concentration of 10ul to 1ml virus at 37oC for 45 minutes. Activation is necessary as Vero cells are only semi-permissive to Rotavirus infections. Trypsin has been shown to enhance infectivity (Estes, Graham, & Mason, 1981). A mixture of 1ul of trypsin and 5ml DMEM was added directly to Vero cells. Cell culture and 500ul of activated virus were then manually mixed by inversion of the tube for 1 minute, before being incubated at 37^{o}C with 5%CO_2. The virus-cell mixture was then observed daily to assess for a cytopathic effect (CPE). When >90% of cells were dead the mixture was stored in aliquots of 1ml at -80^{o}C until use. Half the virus-cell suspension was heat inactivated at 57 ^{o} C for 45 minutes prior to freezing, so that assessment of live and heat-inactivated virus responses could be assessed later on.

VP4 AND VP6:

Rotavirus VP4 and VP6 proteins were kindly provided by Medical Biochemistry, Department of molecular and cell biology, University of Cape Town (Courtesy of Inga Hitzeroth and Ayesha Mohammed). VP4, together with VP7, make up the viral spike

of Rotavirus. Since VP4 and VP7 are the outer most part of the virus capsid, these are very important immunogenic antigens for the immune system (Kindler, Trojnar, Heckel, Otto, & Johne, 2013). VP6 makes up the second layer of the Rotavirus capsid and is known to be highly antigenic (Beards et al., 1984). These properties make VP4 and VP6 good antigens for detection of Rotavirus responses. The proteins were derived in E.coli, potentially resulting in high levels of lipopolysaccharide (LPS) contamination. LPS is an endotoxin expressed by most gram-negative bacteria and can trigger Toll like Receptor 4 receptors on numerous cell types, thus eliciting non-specific cytokine release. For this reason, it was considered important to remove LPS from the VP4 and VP6 protein preparations (Petsch & Anspach, 2000). For removal, we used the Pierce high capacity endotoxin removal resin (Thermo Scientific, Cat#88274). The Peirce Litmulus Amebocyte Lysate chromogenic endotoxin quantitation kit (Thermo Scientific, Cat#8828) was then used to quantify LPS levels present in the protein, to verify that purification was successful. Both kits were used as per manufactures instructions.

BORDETELLA PERTUSSIS (BP) ANTIGEN

Difco™ FA Bordetella pertussis antigen from BD Bioscience USA, was used to test acellular Pertussis (aP) vaccine specific T-cell responses. The antigen was stored in 500ul aliquots at 4^{o}C. In the 12-hour whole blood assay, 2.5ul of the antigen was used to stimulate 250 ul of blood

WHOLE BLOOD ASSAY

CONTROLS AND CO-STIMULATORY MOLECULES

Phytohemagglutinin (PHA) (Remel UK), was used as a positive control at a final concentration of 5µg/ml. Negative controls included RPMI in place of antigens. Anti-CD28 (clone L293, BD Bioscience, USA) and anti-CD49 (clone L25, BD Bioscience, USA) co-stimulatory antibodies were used in all tubes, including negative and positive controls, at a final concentration of 0.25µg/ml to enhance stimulation.

STIMULATION

Up to 24 hours prior to incubation with blood, 2ml polypropylene tubes were prepared to contain anti-CD49, anti-CD28 and either RPMI, BP antigen, Rotavirus DS-1, VP4

and VP6 or PHA. Tubes were kept at 4^{o}C either in the fridge or in a cooler box with ice during transportation to the clinic. All tubes were brought to room temperature (RT) immediately before addition of 250µl blood. Undiluted, sodium heparinized blood was added in a dropwise manner to each tube within 1 hour of blood draw on site by the clinic staff. The tubes were immediately incubated at 37^{o}C in a portable incubator and transported to the laboratory. Upon arrival at the lab, all tubes were immediately transferred to a 37^{0}C water bath for the remainder of the incubation period. At 5 hours of incubation, 10µg/ml of Brefeldin A (BFA)(Sigma Aldrich, Germany) was added to each tube. For birth and 36-week-old infants, 100µl of plasma was removed prior to addition of BFA. BFA is a protein transport inhibitor, which traps newly formed cytokines at the golgi complex or endoplasmic reticulum of a cell. BFA-containing tubes were placed back into 37^{o}C water baths, which were pre-set to automatically switch of upon completion of the 12-hour incubation. Incubations usually ended in the middle of the night; therefore samples remind in a cooling water bath (37^{0}C-18^{0}C) up to 10 hours post cessation of stimulation before being harvested.

HARVESTING OF LEUKOCYTES

For harvesting, blood was transferred to 15ml falcon tubes and incubated with 3ml of 1X red blood cell (RBC) lysis buffer (BioLegend cat#420301) for 15 minutes. RBC lysis buffer lyses red blood cells, while leaving white blood cells intact. The blood was then centrifuged at 1500rpm for 10 minutes. The pelleted cells were washed with 10ml phosphate buffer saline (PBS, WhiteSci, South Africa) and re-centrifuged. A 1:40 pre-diluted solution of ViVid PacBlue (Life Technologies) viability marker was made and 4µl was added to each resuspended pellet. After 20 minutes incubation at RT in the dark, cells were washed with 10ml PBS. After the wash, the cells were incubated with 3ml of 1xFACS lysing solution for 10 minutes at RT in the dark. The cells were washed once again, then cryopreserved.

CRYOPRESERVATION AND STORAGE

For cryopreservation, whole blood cells were suspended in 1ml of RT fetal calf serum (FSC). Then 1ml of 4^{0}C freezing solution (20% DMSO in fetal calf serum) was added to the cells in a dropwise manner, with continuous mixing between each drop. The dropwise addition of freezing solution after the addition of FSC prevents the cells

from going into thermal shock. In addition, DMSO is toxic to the cells at temperatures above -80^{0}C, therefore the 1ml of FCS serves to dilute the DMSO as it makes contact with the cells prevent cell death. The cryopreserved cells were transferred to 2ml cryotubes and placed in a 1^{o}C Mr Frosty. Mr Frosties were immediately transferred to -80^{o}C freezers and incubated overnight. The next morning, the frozen cells were transferred to liquid nitrogen, where they were stored until use.

PERIPHERAL BLOOD MONONUCLEAR CELL ISOLATION

Prior to the start of isolation, 15ml Leucosep tubes were filled with 3ml of ficoll and centrifuged for 1 minute at 1500rpm. Centrifugation moves ficoll below the porous barrier of the leucosep tube. Sodium heperanised blood was centrifuged in order to remove plasma. After plasma removal, the blood was diluted in a 1:1 ratio with PBS. Between 4-6ml of diluted blood was then layered on the porous barrier of the 15ml leucosep tube. Tubes were then centrifuged at 2500rmp for 10 minutes without brakes. Centrifugation allowed for separation of the different blood components with PBMC forming a layer of cells above the porous disc (Fig 2.2.1). The PBMC layer of the blood was pipetted into a clean 15ml tube and washed with 10ml PBS. Cells were centrifuged at 1500rpm. Figure 2.2.1 below illustrates this procedure. PBMC's were re-suspended in 10ml of PBS, 10ul of which was stained in a 1:1 ratio with trypan blue (Thermo Fisher Scientific) for counting under a microscope. The rest of the cells was washed for a second time, supernantant was discarded and PBMC pellet was re-suspended and used in the Rotavirus stimulation assays.

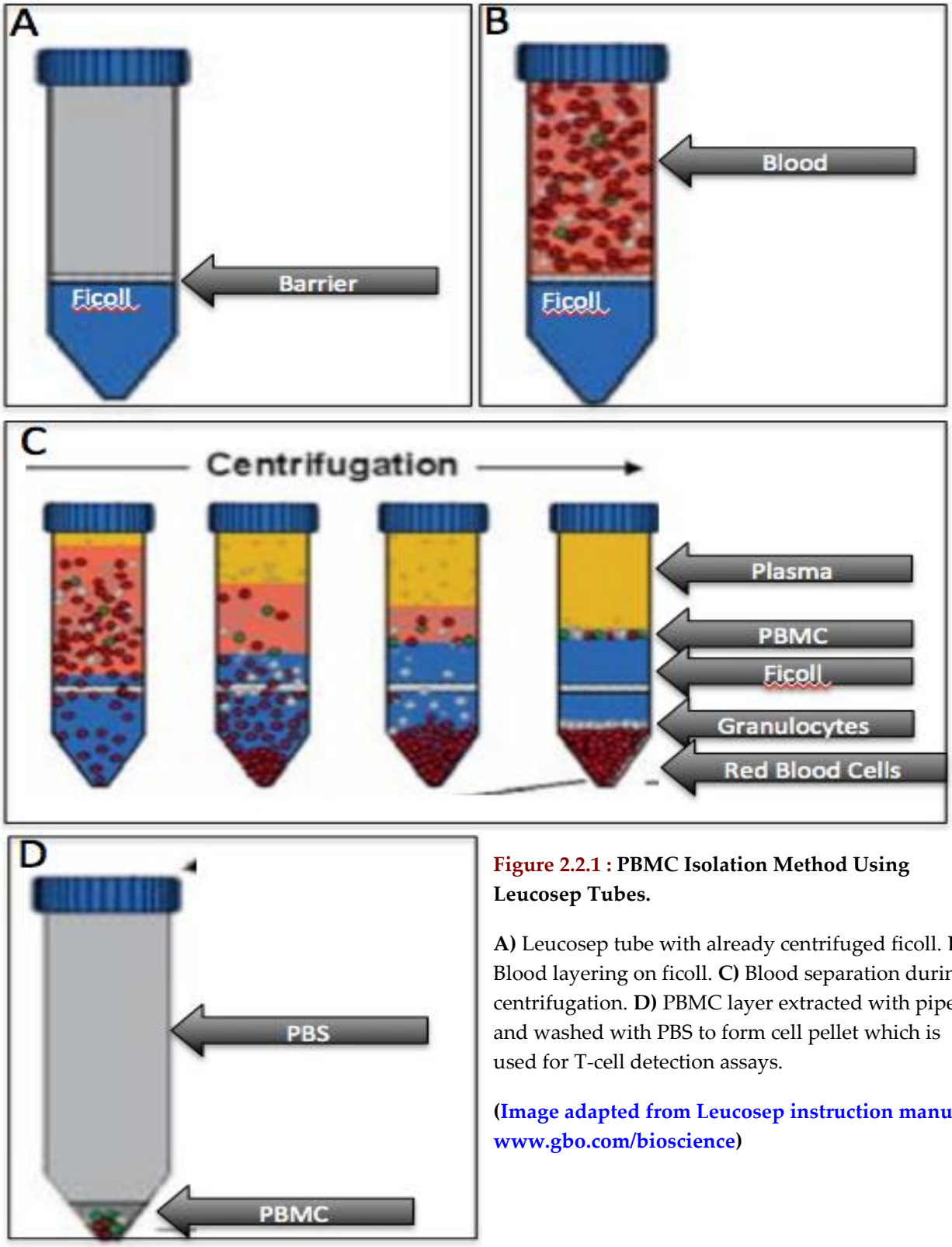


Figure 2.2.1 : PBMC Isolation Method Using Leucosep Tubes.

A) Leucosep tube with already centrifuged ficoll. **B)** Blood layering on ficoll. **C)** Blood separation during centrifugation. **D)** PBMC layer extracted with pipette and washed with PBS to form cell pellet which is used for T-cell detection assays.

(Image adapted from Leucosep instruction manual-www.gbo.com/bioscience)

FLOW CYTOMETRY

Cells were acquired on an LSRII (BD). Up to 500000 events or all the cells in the tube were acquired, whichever came first. After acquisition, events were analyzed using FlowJo (TreeStar). Forward and side scatter was used to identify singlets and lymphocytes, as well as to exclude any doublets and debris (Fig 2.2.2). Other artifacts that may have been present during acquisition were excluded using time gates. Dead cells were also excluded based on Vivid staining. Within the live CD3+ populations, CD4+ and CD8+ lymphocytes were identified and gated on. Several gates were completed on both sets of lymphocytes. CD45RA and CD27 were used to identify memory populations within the CD4+ and CD8+ cells. HLA-DR and Ki67 was then used to identify activated and proliferating CD4+ and CD8+ T-cells. In addition, IFNg, IL-2 and TNFa experessing T-cell were also identified within the CD4+ and CD8+ T-cell populations.

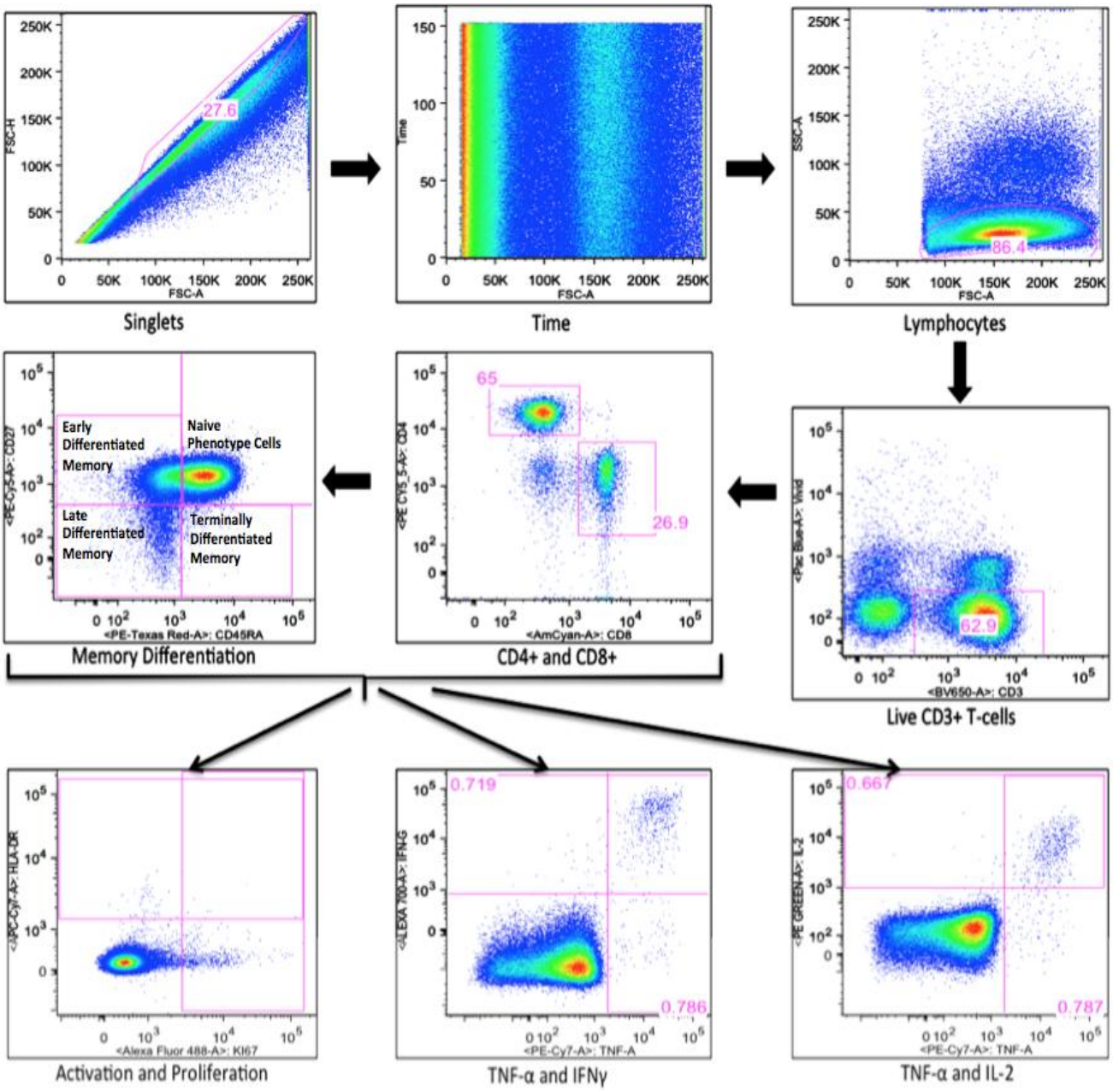


Figure 2.2.2: Flow cytometry Gating Strategy

STAINING FOR FLOW CYTOMETERY

Cryopreserved white blood cells were thawed in a 37^{o}C water bath. The thawed cells were transferred to FACS tubes using pasture pipettes and centrifuged at 1500 RPM for 10 minutes. The supernatant was discarded and the cells were washed with 2ml of 1xBD Permwash at 1500 RPM for 10 minutes. After washing, the supernatant was discarded and cells were transferred to 96 well V-bottom plates. Plates are centrifuged at 1500 RPM for 10 minutes. Any excess supernatant was discarded and 100ul of Permwash was added to each well. Plates were incubated for 15 minutes at room temperature and then centrifuged as before, to permiabilise the cells

for intracellular staining. Supernatant was discarded again and 50ul of a pre-prepared antibody mastermix was added to each well. Antibody concentrations were determined via titration (table 2.2.2). After a 45-minute incubation at 4°C, a further 100ul of Permwash was added to cells. Plates were centrifuged and the supernatant was discarded. The cells were washed again, with 150ul of Permwash before being centrifuged. Supernatant was discarded and 200ul of 1% formaldhyde was added to each well, the solutions were then transferred back to FACS tubes for flow cytometry acquisition.

Table 2.2.2: Flow Cytometry Antibodies

Antibody	Flurochromes	Clone	Manufacturer	Volume added/reaction	Function
CD3	BV650	OKT3	BioLegend	0.25 µl	T-cell Categorisation
CD4	PE Cy5.5	S3.5	Invitrogen	0.05 µl	T-cell Categorisation
CD8	AmCyan	RPA-T8	BD Bioscience	0.20 µl	T-cell Categorisation
CD27	PE Cy5	0323	eBioscience	0.50 µl	Memory Differentiation
CD45RA	Texas red	2H4LDH11LDB9	Beckman Coulter	0.50 µl	Memory Differentiation
HLA-DR	APC Cy7	L243	BioLegend	0.125µl	Activation Detection
Ki67	Alexa 488	B56	BD Bioscience	1.25 µl	Proliferation Detection
IFN-g	Alexa 700	B27	BD Bioscience	0.10 µl	Functional Detection
TNF-a	PE Cy7	MAb11	BioLegend	0.063µl	Functional Detection
IL-2	PE Green	Mq1-17H12	BD Bioscience	0.050µl	Functional Detection

COMPENSATION CONTROLS

Compensation controls are necessary for multiparameter flow cytometry, to correct for spectral overlap within the staining panel. Spectral overlap occurs when a percentage of the emitted light from one fluorochrome is detected in the detection channel of another flurochrome. This may result in the detection of false populations.

For the preparation of compensation controls, each fluorochrome-conjugated antibody in the staining panel was added to one drop of positive BD CompBeads (BD Bioscience, USA). The volume of antibody added to each compensation tube can be found in Table 2.2.3 below.

Compensation tubes containing antibody and beads were vortexed and then incubated for 5 minutes at 4^0C in the dark. After the incubation, 150µl of PBS was added to each tube. Unstained compensation controls were also made up in the same manner but no antibodies were added to the tube. The unstained control was needed for identification of negative populations. Each compensation tube was then acquired on the flow cytometer on the same day and at the same voltages used for acquisition of samples.

Table 2.2.3: Volume of Antibody in Compensation Control Tubes

Antibody conjugate	volume [µl]
CD3 BV650	4µl
CD4 PE-Cy5.5	1µl of a 1 in 20 dilution in permwash
CD8 V500	1µl of a 1 in 5 dilution in permwash
CD27 PE-Cy5	1µl of a 1 in 3 dilution in permwash
CD45RA ECD	0.5µl
HLA-DR APC-Cy7	1µl of a 1 in 8 dilution in permwash
Ki67 Alexa488	1.25µl
IFNγ Alexa700	1µl of a 1 in 10 dilution in permwash
TNFα PE-Cy7	1µl of a 1 in 24 dilution in permwash
Il-2 PE	1µl of a 1 in 20 dilution in permwash
CD3 PacBlue	1 µl

ANALYSIS

Compensation and post-acquisition analysis was done with FlowJo version 9.9 (Tree Star). Excel was used to organize output data from FlowJo and to combine multiple experiments into one file. Text files generated from excel data was used to complete background subtractions in Pestle 1.7 (Vaccine Research Center, National Institute of Allergy and Infectious Diseases) and Spice (National Institute of Allergy and Infectious Diseases). All polyfunctional cytokine analyses were completed in Spice. Spice and GraphPad Prism version 6.0 (San Diego) were used to complete all statistical analyses and graphical representations. A Shapiro-Wilk test was used to determine the distribution of the data. The parametric unpaired t test or non-parametric Mann-Whitney test was used to compare the means and medians of two unmatched groups. A kruskal-Wallis test was used to compare medians of matched groups. P-values ≤ 0.05 were considered statistically significant.

Unstimulated controls that underwent the exact treatment as antigen stimulated blood but without added antigen, were used for background subtraction. Within the population of interest, the frequencies of cells found in the unstimulated tubes were subtracted from the frequency of cells found in the antigen-stimulated tubes to yield a net response. This was only completed for comparative cytokine analyses between two groups of infants. For all other analyses, gross responses were used.

CHAPTER 3:

CELLULAR IMMUNE RESPONSES TO ROTAVIRUS VACCINATION IN INFANTS.

SECTION 3.1:

INTRODUCTION

Rotavirus is the primary cause of severe acute gastroenteritis in infants. An annual estimate of 600,000 deaths and 2 million hospitalizations in children under the age of five years occurred globally due to rotavirus infection prior to 2010 (J Chan, H Nirwati, R Triasih, M D Danchin, 2010). Transmission of the virus occurs through the fecal-oral route, via contaminated hands, objects and surfaces (Butz, Fosarelli, Dick, Cusack, & Yolken, 1993). Thus, poor hygiene and unsanitary environments allow for successful transmission (Glass et al., 2006). Primary infections caused by Rotavirus results in destruction of enterocytes, which are the cells responsible for digestion and absorption in the jejunum (H. W. Moon, 1994). A lack of functional enterocytes leads to reduced water and nutrient absorption, followed by malnutrition and dehydration and in some cases death. While symptomatic treatment is fully available for Rotavirus infections, many rural settings lack access to medical facilities, thus the number of deaths caused by Rotavirus infections in such areas are high (Pesavento, Crawford, Estes, & Prasad, 2006). With every subsequent Rotavirus infection, symptoms diminish, until protective immunity renders infections asymptomatic. Consequently, symptomatic Rotavirus infection rates are highest in children (Glass et al., 2006).

ROTAVIRUS STRUCTURE AND PROTEINS

Rotavirus is a double stranded RNA virus from the Reoviridae family. It is made up of a nonenveloped, multilayered icosahedral capsid containing 11 RNA segments (Pesavento et al., 2006). These dsRNA segments encode structural and non-structural proteins necessary for viral replication (Matthijnssens et al., 2011; Pesavento et al., 2006).

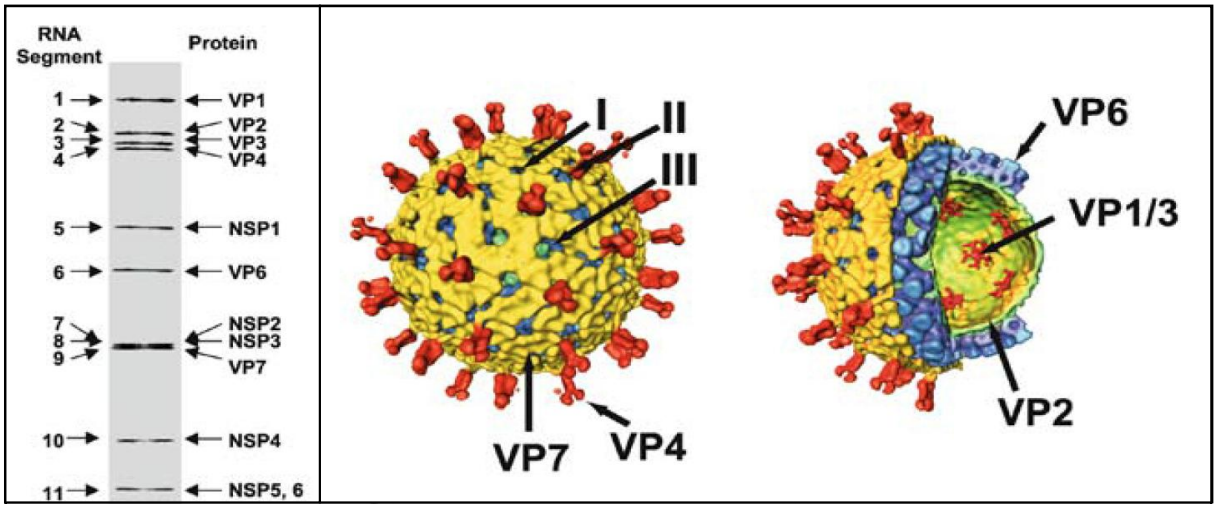


Figure 3.1.1: Structural and non-structural Rotavirus Proteins. A) dsRNA segment encoding for the different proteins. B) 3D illustration of structural proteins (Pesavento et al., 2006).

Figure 3.1.1 and table 3.1.1 (Pesavento et al., 2006) shows and describes the structure, properties and functions of Rotavirus proteins. VP4 and VP7 form the outer portion of the virus and play important roles in the development of immunity to the virus (Kindler et al., 2013). VP6 is the most antigenic part of the virus, therefore is commonly targeted in laboratory testing (Beards et al., 1984).

Table 3.1.1: Properties and Function Of Rotavirus Proteins (Pesavento et al., 2006)

Gene segment	Protein	Mass (kDa)[b]	Post-translational modification(s)	Location (no. of copies)	Functional properties
1	VP1	125	–	SLP (12)	RNA-dependent RNA polymerase, RNA binding, interacts with VP2 and VP3
2	VP2	95	Cleaved	SLP (120)	RNA binding, interacts with VP1
3	VP3	88	–	SLP (12)	Guanylyl and methyl transferase, ssRNA binding, interacts with VP1
4	VP4 (VP5* + VP8*)	85 (58+27)	Cleaved	TLP (120)	Hemagglutinin, neutralization antigen, virulence, protease-enhanced infectivity, cell attachment, fusion region
5	NSP1	53	–	Nonstructural	RNA binding, antagonist of interferon response
6	VP6	45	–	DLP (780)	Hydrophobic trimer, group and subgroup antigen
7	NSP3	34	–	Nonstructural	Important for viral mRNA translation, PABP homologue, RNA binding, interacts with eIF4G
8	NSP2	35	–	Nonstructural	Important for genome replication/packaging, main constituent of viroplasm, NTPase, RNA binding, interacts with NSP5
9	VP7	34	Cleaved signal sequence, high mannose glycosylation and trimming	TLP (780)	RER integral membrane glycoprotein, neutralization antigen, Ca^{++} binding
10	NSP4	20	Uncleaved signal sequence, high mannose glycosylation and trimming	Nonstructural	RER transmembrane glycoprotein, role in morphogenesis, viral enterotoxin
11	NSP5	26	Phosphorylated, *O*-glycosylated	Nonstructural	Constituent of viroplasm, interacts with NSP2, RNA binding, Protein kinase
11	NSP6	11	–	Nonstructural	Constituent of the viroplasm, interacts with NSP5

ROTAVIRUS CLASSIFICATION

Rotavirus can be classified into seven groups, A-G, based on the migration pattern of dsRNA in polyacrylamide gel electrophoresis. Group A to F have also been identified using clustering patterns of NSP4 amino acids in dendrograms (Matthijnssens et al., 2008). Of all the groups, group A is the most significant cause of gastroenteritis in infants worldwide (Jiang et al., 2003). Annually, this group is associated with 453,000 childhood deaths in African and Asian countries (Nyaga et al., 2014). Group A rotavirus has been further classified based on whole-genome sequencing, giving rise to the two main human rotavirus strains, which are the WA and DS-1 strain of Rotavirus (Nyaga et al., 2014). Group A rotavirus are further subdivided into P and G types. This subdivision is based on classification of two outer viral proteins, VP7 which is a glycoprotein giving rise to the "G" type virus and VP4 which is protease sensitive, giving rise to the "P" type virus (Nyaga et al., 2014).

ROTAVIRUS VACCINES

There are currently two approved oral, live-attenuated Rotavirus vaccines, Rotarix and RotaTeq, which are part of the national immunization programs in over 150 countries (Gray, 2011). Rotarix is a monovalent vaccine derived from the G1P[8] human rotavirus strain. It relies on heterotypic protection from antigenically similar viruses of different serotypes/genotypes. RotaTeq is a pentavalent human-bovine reassorted vaccine containing G1, G2, G3, G4 and P[8] human surface rotavirus proteins (Gray, 2011). This vaccine exhibits homotypic protection as it contains antigenic proteins from a range of the most common rotavirus strains. Both of these are live vaccines, which confers an advantage, in that both vaccines mimic natural infection by allowing replication of the virus (Gray, 2011). Their oral administration allows the vaccines to stimulate immune responses where it is required, the gut, which is the point of entry for rotaviral infections (Gray, 2011). In South Africa, infants are given Rotarix as part of the expanded program of immunization (EPI) (Ngcobo, 2010).

Vaccination with Rotarix and RotaTeq has shown an 80-95% reduction of severe rotavirus disease in high and middle-income countries, but are less efficacious in protection of infants in developing countries (Qadri, Bhuiyan, Sack, & Svennerholm,

2013). The reasons for reduced efficacy of Rotavirus vaccines in developing countries are not yet clearly defined but are suggested to be influenced by multiple factors, including; co-infections, like HIV and TB, higher levels of maternal Rotavirus antibodies, malnutrition, co-administration with OPV and feeding practices (Chan et al., 2011).

INFANT ROTAVIRUS T-CELL IMMUNITY

It has always been assumed that antibodies directed against the outer protein capsid of Rotavirus mediate protection, however no such correlate of protection has yet been demonstrated in humans (Ward, McNeal, & Sheridan, 1992). This suggests that T-cells may be necessary for Rotavirus protection. Many animal and human studies support this hypothesis and demonstrate the important role T-cells play in Rotavirus protection (Franco, Angel, & Greenberg, 2006; Johansen et al., 1999; Offit & Dudzik, 1988). Most healthy children and adults have circulating Rotavirus T-cells during the convalescent phase of Rotavirus gastroenteritis (Mäkelä, Marttila, Simell, & Ilonen, 2004). Mouse studies show that cytotoxic T-cells develop during Rotavirus infections and play a role in resolving the infection (Offit, Cunningham, & Dudzik, 1991). Additionally, T-cell immunodeficient children, such as those with congenital T-cell immunodeficiency have been shown to experience chronic Rotavirus infection, suggesting that antibodies alone are not sufficient to control infections (Wood, David, Chrystie, & Totterdell, 1988). In support of this, Mäkelä et al. showed that T-cell responses in infants were accompanied by increased antibody responses, suggesting that they may be interdependent. They found that adults had strong Rotavirus specific cellular T-cells while T-cell responses in children declined shortly after infection (Mäkelä et al., 2004). This suggests that T-cells require multiple infections to develop. Another study examining the humoral and cell-mediated responses to Rotavirus NSP4 in vaccinated adults found that NSP4 induced T-cell proliferation and production of IL-2 and IFN-γ in vitro.

In addition to the protective role T-cells play in Rotavirus clearance, assessing T-cell responses may have other advantages. Being a ubiquitous virus, most pregnant female adults have strong rotavirus specific antibodies (Kapikian et al., 1976). These antibodies may be passively transferred to infants during pregnancy and breast-feeding, thus, assessing antibody responses in infants as a measure of vaccine

response may be inaccurate (GITLIN, KUMATE, URRUSTI, & MORALES, 1964). Conversely, a limited number of T-cells cross the placenta, so assessing T-cell responses would allow for the distinguishing of maternal immune responses from active infant immune responses (Palmer et al., 2007).

T-CELL CYTOKINE RESPONSES TO ROTAVIRUS

TH1 and TH2 cytokines have been shown to play a role in the pathogenesis and clearance of Rotavirus disease in children (Jiang et al., 2003). A study comparing levels of 9 different cytokines in the sera of children with acute gastroenteritis versus healthy controls found that IL-2 and IFNγ were mostly expressed in the late phase of acute infection (Jiang et al., 2003). Both this study and that of Azim et al found that IL-10 and TNF-α and levels were higher in children with persistent Rotavirus diarrhea compared to uninfected controls (Azim et al., 1999; Jiang et al., 2003). TNF-α is a pro-inflammatory cytokine that has anti-viral activity but in the setting of Rotavirus infections has been associated with increased diarrheal episodes (Jiang et al., 2003). In contrast with TNFα, IFN-γ and IL-2 were shown to have a negative association with Rotavirus symptoms, suggesting that these cytokines may form part of the host's protective immune response (Jiang et al., 2003).

Given the seemingly important role that T-cells and their corresponding cytokines play during Rotavirus infections, there is major gap in the current literature of vaccine-induced T cell responses to the currently licensed rotaviral vaccines. Further, in HIV exposed uninfected infants, there are even fewer studies looking at Rotavirus specific T-cell responses. Given the major health risks associated with rotavirus infections in HIV exposed infants, especially in areas of poor hygiene and sanitation due to poverty, studies assessing T-cell responses to Rotavirus infections are highly relevant. Background levels of Rotavirus infection in such populations are high, causing multiple deaths and hospitalizations in children every year. Improved understanding of the immune response to Rotavirus infections would inform vaccine interventions and treatment options.

Thus, the main aim of this chapter, as mentioned in chapter 1 was to develop an in vitro assay to measure Rotavirus-specific T-cell responses in infants. To accomplish this, various rotavirus antigens including; DS-1 whole virus, VP4 and VP6 surface

proteins, were evaluated as antigens for the detection of Rotavirus specific CD4+ and CD8+ T-cell cytokine responses in an in vitro whole blood assay or PBMC stimulation assay. For mathematical calculations and a detailed outline of each experiment described in this chapter refer to appendix 1.

SECTION 3.2:

RESULTS

DS-1 ROTAVIRUS CYTOKINE RESPONSES

The first step of optimization was to culture DS-1 Rotavirus in Vero cells (Methods page 49). Half the virus-cell suspension was heat inactivated at 57°C for 45 minutes so that comparisons between live and heat inactivated virus responses could be conducted. Both the heat-inactivated and live virus-cell mixtures were then serially diluted (Appendix 1) and used to stimulate adult blood in a 12-hour whole blood assay (Methods, page 50). Serial dilutions of Live and heat inactivated virus was conducted as shown below:

15µl DS-1 + 15µl RMPI → 15µl dilution 1 + 15µl RMPI → 15µl dilution 2 + 15µl RMPI → 15µl dilution 3 + 15µl RMPI → 15µl dilution 4 + 15µl RMPI → 15µl dilution 5 + 15µl RMPI

The experiment was set up in duplicate and 250µl of blood was used for each stimulation condition. For the titrations, 5µl of stock virus or 5µl of the appropriate diluted stock was added to 250µl of blood in a 2ml tubes. A negative, unstimulated control and positive PHA stimulated control were included (Appendix 1). Blood from a single individual was used for this experiment. Adult blood was used due to the large volume of blood required for the experiment (4ml). The aim of the experiment was to select optimal titrations, which stimulated responses in adults to stimulate infant blood, so as to minimize the volume of blood required from infants.

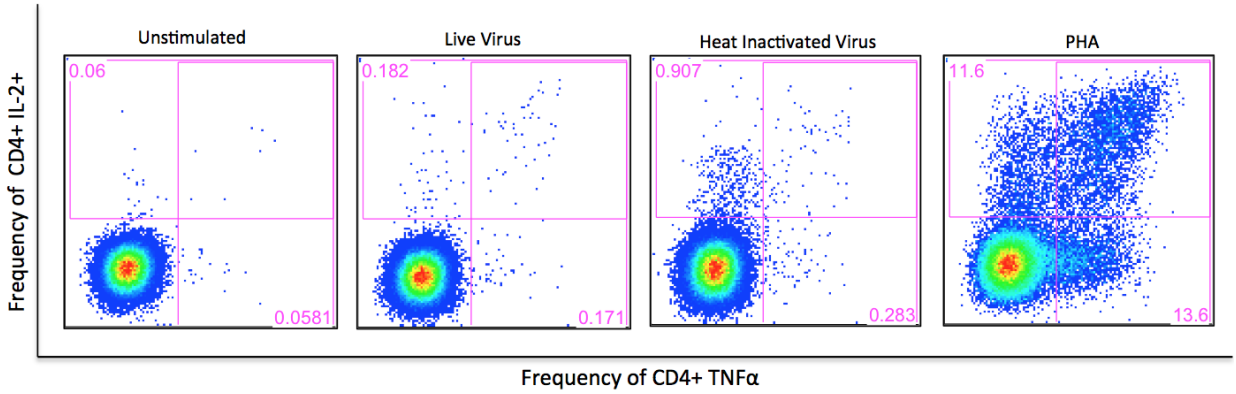


Figure 3.2.1: Representative plots of adult CD4+ IL-2 and TNFα responses in unstimulated blood or 5µl live DS-1 Rotavirus, 5ul heat-inactivated DS-1 Rotavirus and PHA blood stimulated blood in a 12 hour whole blood assay.

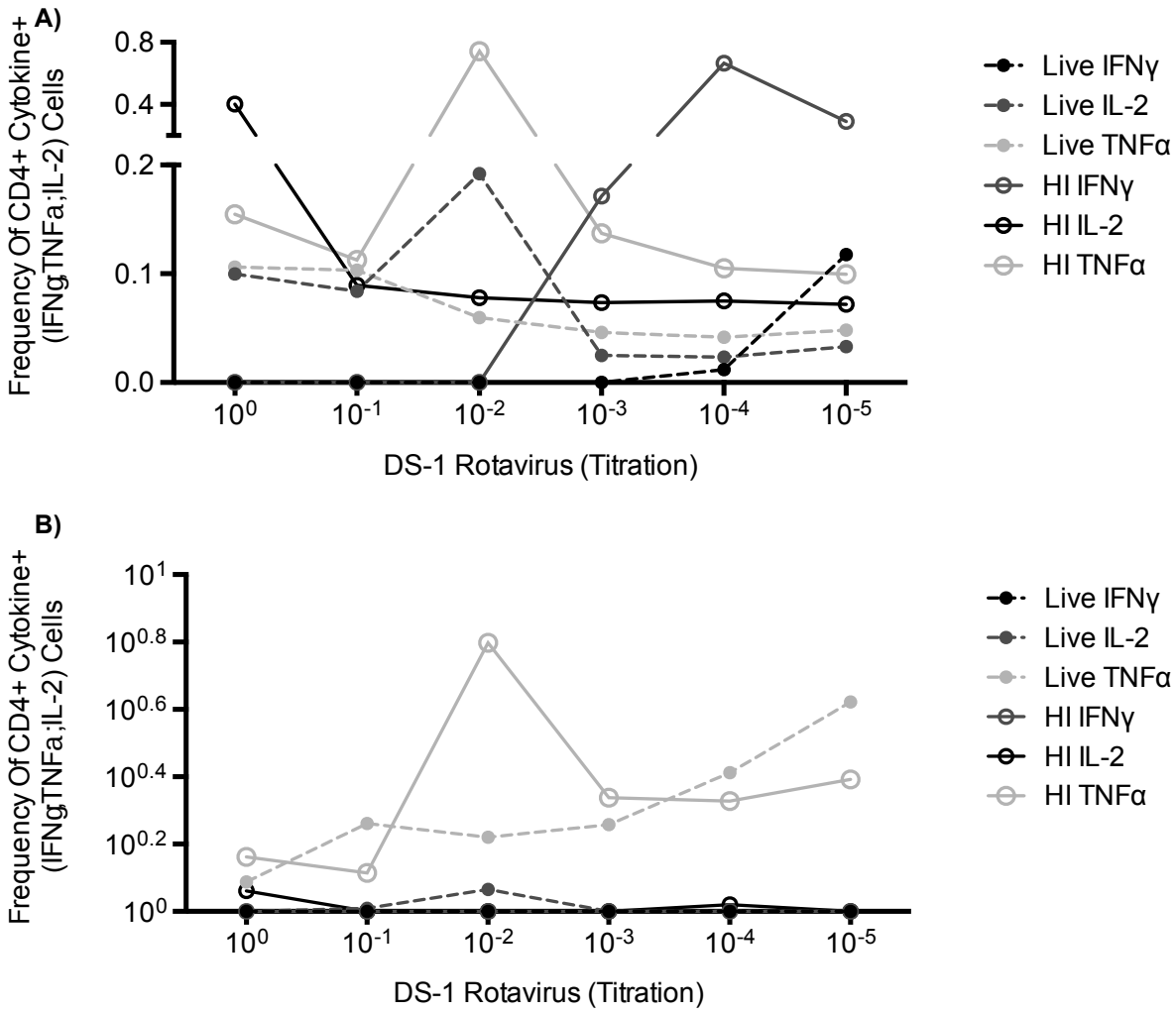


Figure 3.2.2: Net CD4+ and CD8+ Rotavirus specific adult cytokine responses. Panel A and B represent net CD4+ and CD8+ responses respectively from a single adult healthy volunteer after 12hours of stimulation with either live or heat-inactivated DS-1 Rotavirus. Dashed lines with solid symbols represent live virus stimulated responses while solid lines with open symbolsrepresent heat inactivated (HI) virus responses. The volumes of DS-1 virus-cell mixture added to each condition decreases from left to right on the x-axis. The frequency of cytokine expressing cells is on the y-axis.

A good representative of the differences in responses between live and heat-inactivated virus of the same volumes is seen in figure 3.2.1. It is clear in this figure that heat-inactivated virus stimulates more cytokine production then live virus. No definite volume of DS-1 Rotavirus induced distinctly better cytokine responses than the other volumes used for stimulation in the whole blood assay (Figure 3.2.2). However, it was found that heat-inactivated virus elicited better T-cell cytokine responses compared to live virus when used at the same volumes. This can also be seen in figure 3.2.2, where solid lines representing heat-inactivated virus responses are higher at most volumes of stimulation compared to live virus responses. Additionally, both heat-inactivated and live virus stimulation induced higher cytokine

expression then is found in unstimulated blood (figure 3.2.1). Therefore, a second titration was completed in the same manner as the first titration but using only heat-inactivated virus for stimulation. This titration was set up with blood from two different adult individuals and the average total responses of both individuals were plotted in figure 3.2.4.

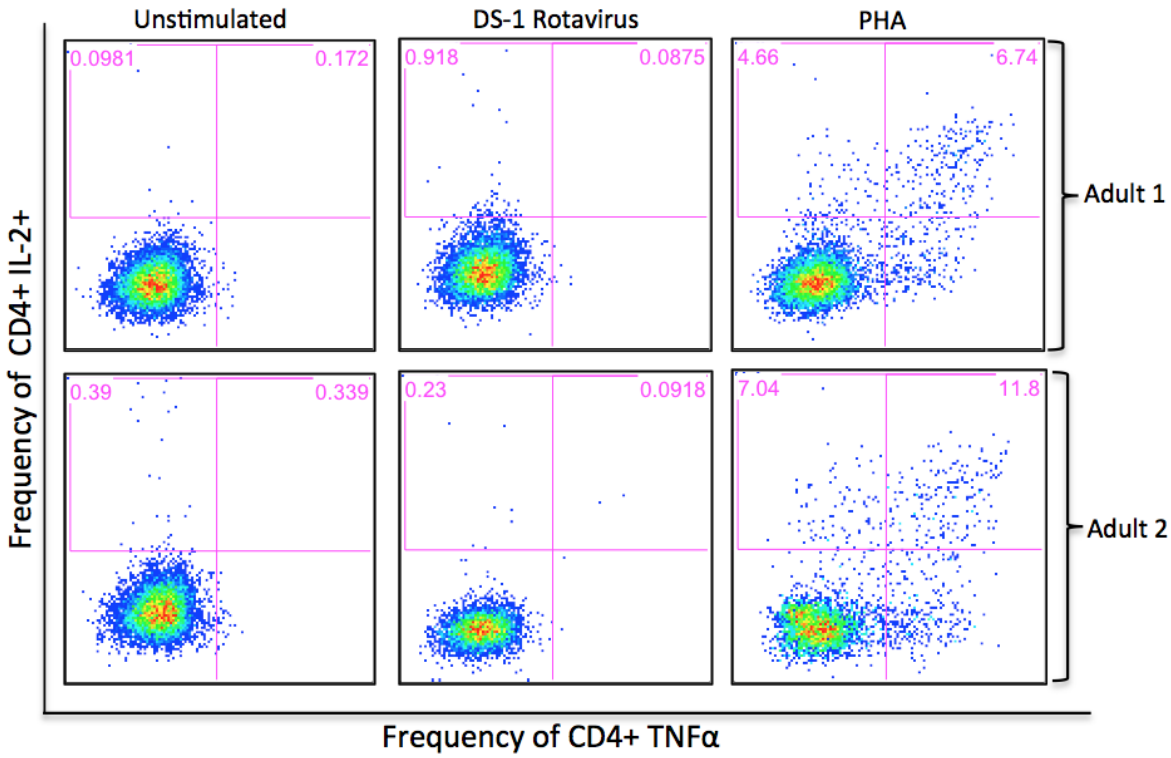


Figure 3.2.3: Representative plots of CD4+ IL-2 and TNFα expression in unstimulated, heat-inactivated DS-1 and PHA stimulated blood in 2 different adults. The top row represents adult 1 responses and the bottom row represents adult 2 responses. Columns 1 ,2 and 3 represent unstimulated, DS-1 Rotavirus and PHA stimulated responses.

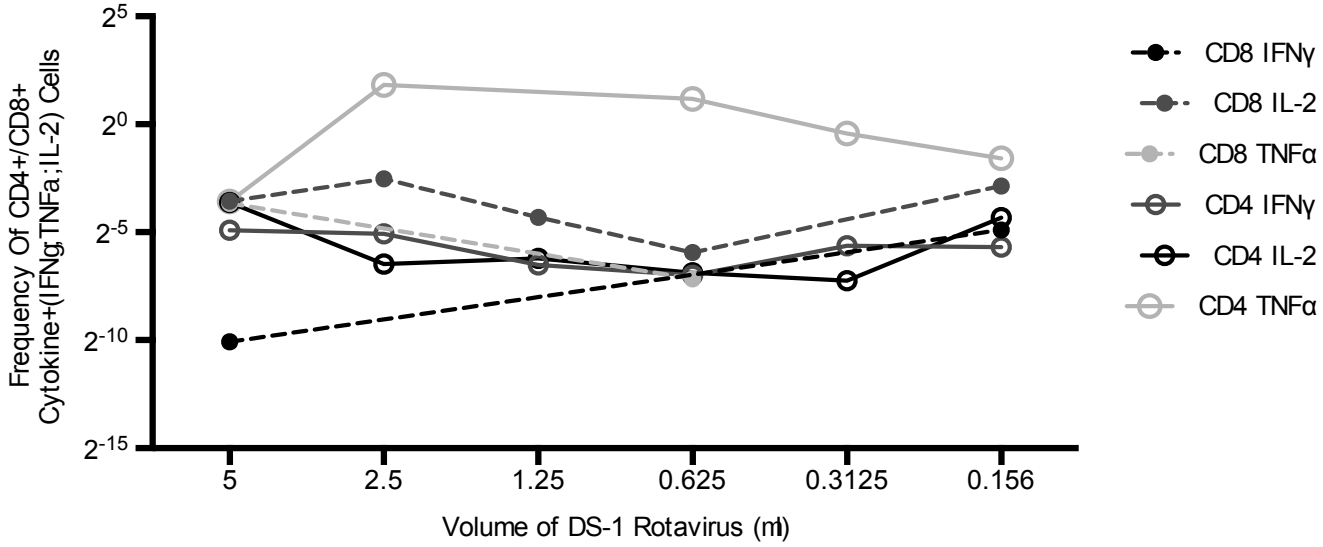


Figure 3.2.4: Net CD4+ and CD8+ adult cytokine responses to heat inactivated DS-1 Rotavirus. Solid lines with open symbols represent net average CD4+ responses and dashed lines with solid symbols represent net average CD8+ responses of 2 adults.

As seen in figure 3.2.3, DS-1 induced responses were very similar to that of unstimulate blood and indicate that these adults possessed no re-call responses to the RV antigens. This demonstrates that cytokine expression induced by DS-1 varies in different individuals. Additionally, no optimal volume of DS-1 was found for stimulation in this experiment, possibly due to the strong neutralizing antibodies found in adults, which may clear the virus prior to T-cell engagement. Figure 3.2.4 demonstrates that all volumes of virus induced similar levels of cytokine. As there appeared to be variability in adult responses to DS-1 virus, it was difficult to determine the optimal volume of virus for testing in infants. A more robust approach was then considered, which consisted of using RV capsid proteins VP4 and VP6.

SECTION 3.3:

VP4 AND VP6 ROTAVIRUS CYTOKINE RESPONSES IN ADULTS

E.coli. derived Rotavirus VP4 and VP6 proteins were obtained from the Bio-pharming research lab at Molecular and Cell Biology, UCT (Courtesy of Inga Hitzeroth, PhD). Three different batches of proteins were received, all with differing concentrations.

Initially, 10-fold dilutions of each protein from the first batch were used in a titration experiment, ranging from 0.4ug/ml to 40ug/ml of VP4/VP6. Endotoxin was removed from the protein prior to use in the assay. However, the purification did not remove all LPS and thus the protein still had some LPS contamination. The concentration of LPS found in the 40µg/ml (highest concentration) stimulation for both VP4 and VP6 was calculated. The 40µg/ml VP4 stimulation was found to have the highest level of LPS contamination, at a final concentration of 0.26EU/ml. (For detailed calculations refer to appendix 1). This experiment was conducted using blood from three different adults and the average responses. Flow cytometry plots of CD8+ IL-2 and TNFα responses for all three adults used in this experiment are depicted in figure 3.3.1. After subtraction of background, responses are plotted in figure 3.3.2.

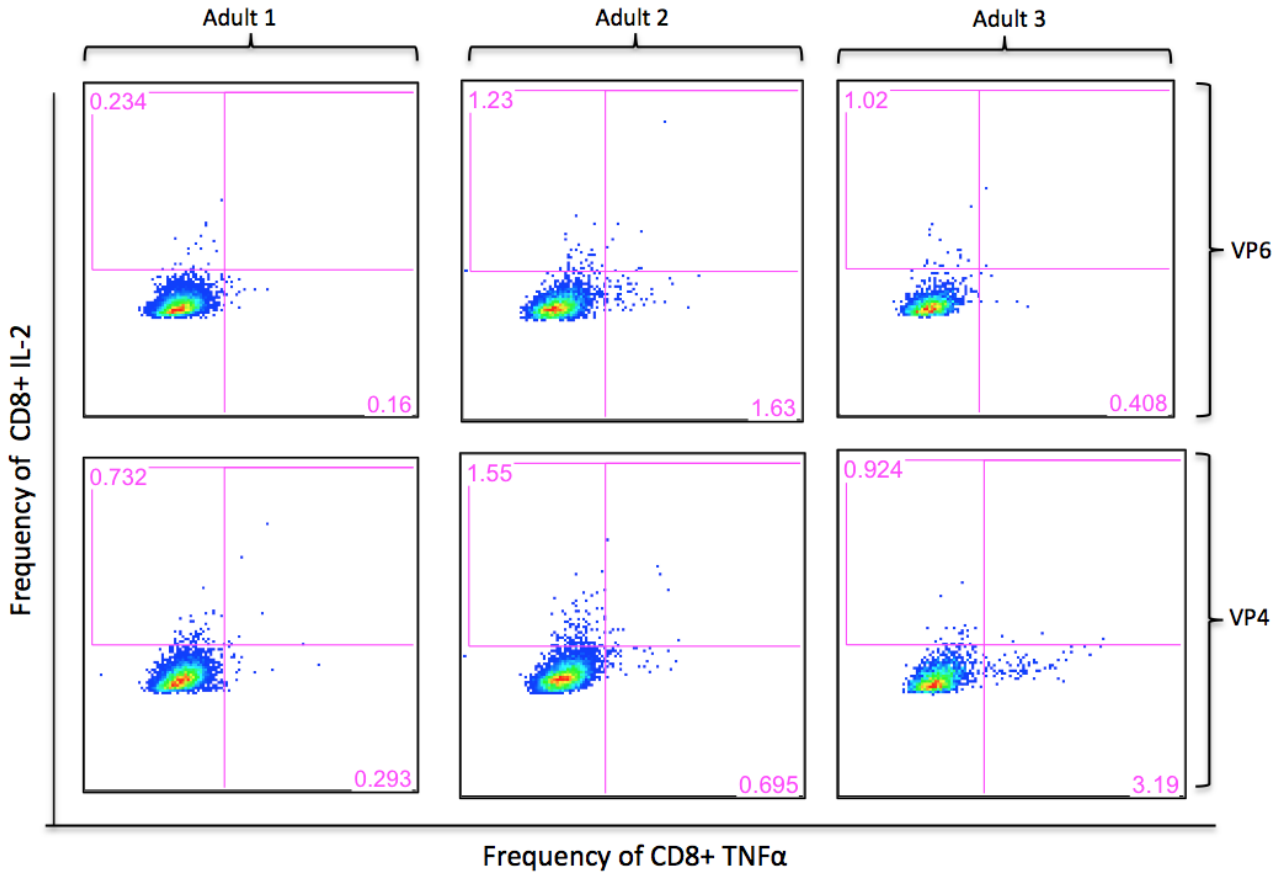


Figure 3.3.1: Adult CD8+ IL-2 and TNFα responses to 40µg/ml VP4 and 40µg/ml VP6 stimulation in a 12 hour whole blood assay. The top and bottom rows show adult responses to VP6 and VP4 respectively. The first, second and third column represent adult 1, adult 2 and adult 3 responses respectively.

VP4 stimulated responses were best detected at a concentration of 40µg/ml (Figure 3.3.2 A). At lower concentrations, only CD8+ IL-2 and CD8+ TNFα responses were detectable at levels above background. CD8+ IL-2 and CD8+ TNFα responses were the only two cytokine responses detected above background levels in VP6 stimulated blood (Figure 3.3.2 B).

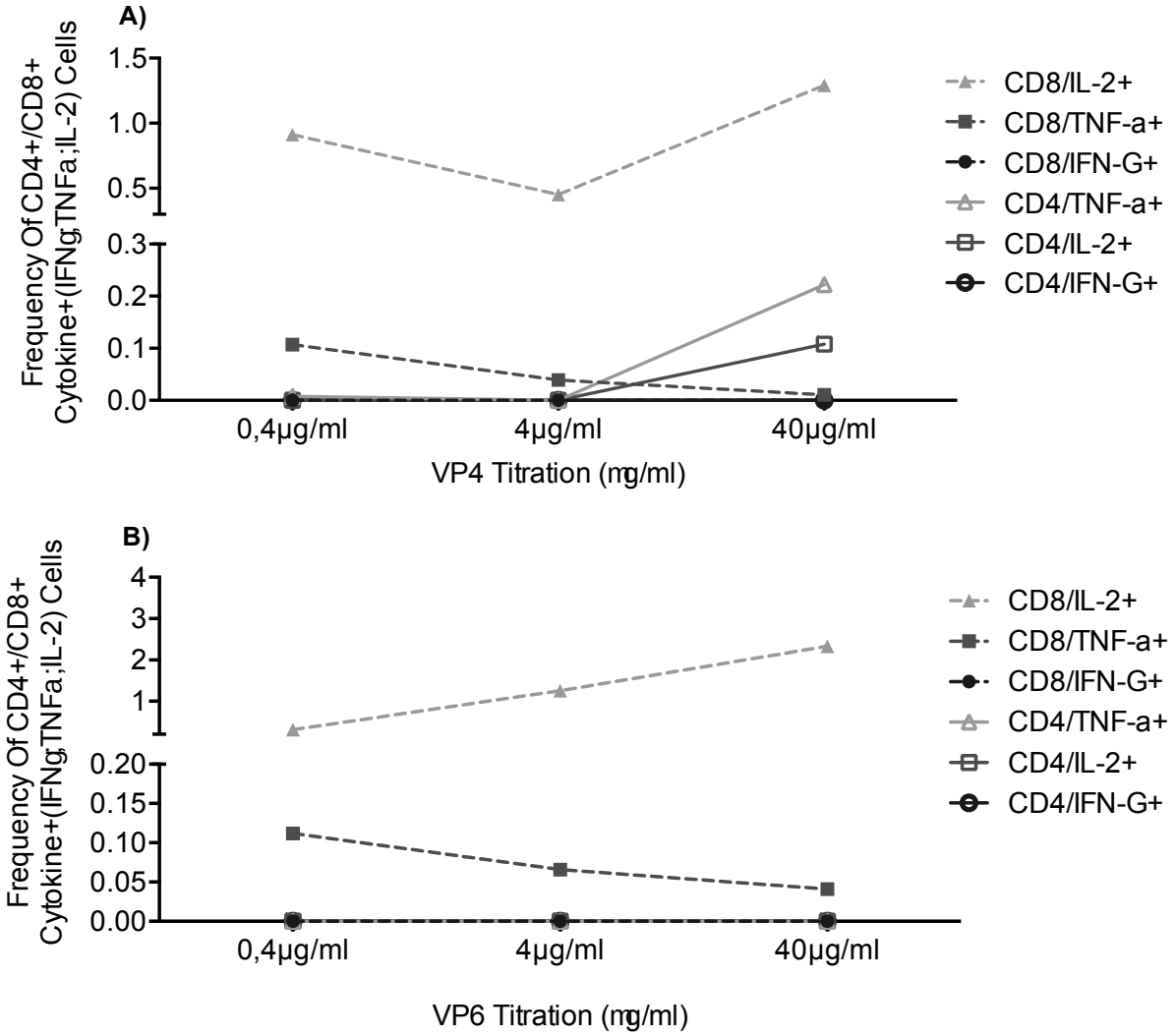


Figure 3.3.2: Net CD4+ and CD8+ adult cytokine responses to VP4 and VP6 Rotavirus Proteins. Solid lines with open symbols represent net CD4+ responses and dashed lines with solid symbols represent net CD8+ responses. Panel A and B represent VP4 stimulated and VP6 stimulated responses respectively. The experiment was conducted in 3 different adults and mean responses were plotted.

Since 40µg/ml VP4 yielded detectable amounts of CD4+ and CD8+ IL-2 and TNFa, and 40µg/ml of VP6 stimulated the highest CD8+ IL-2 response, a narrow range titration of proteins around 40µg/ml was subsequently conducted. The second titration was completed using blood from 'adult 3', who had the overall best responses in the 10-fold titration experiment. The same batch of proteins was used in both experiments. The highest concentration of protein used for stimulation was 320µg/ml, equating to an LPS concentration of 2.26EU/ml (Appendix 1). Representative CD4+ flow plots are plotted in figure 3.3.3 and background subtracted responses are plotted in figure 3.3.4.

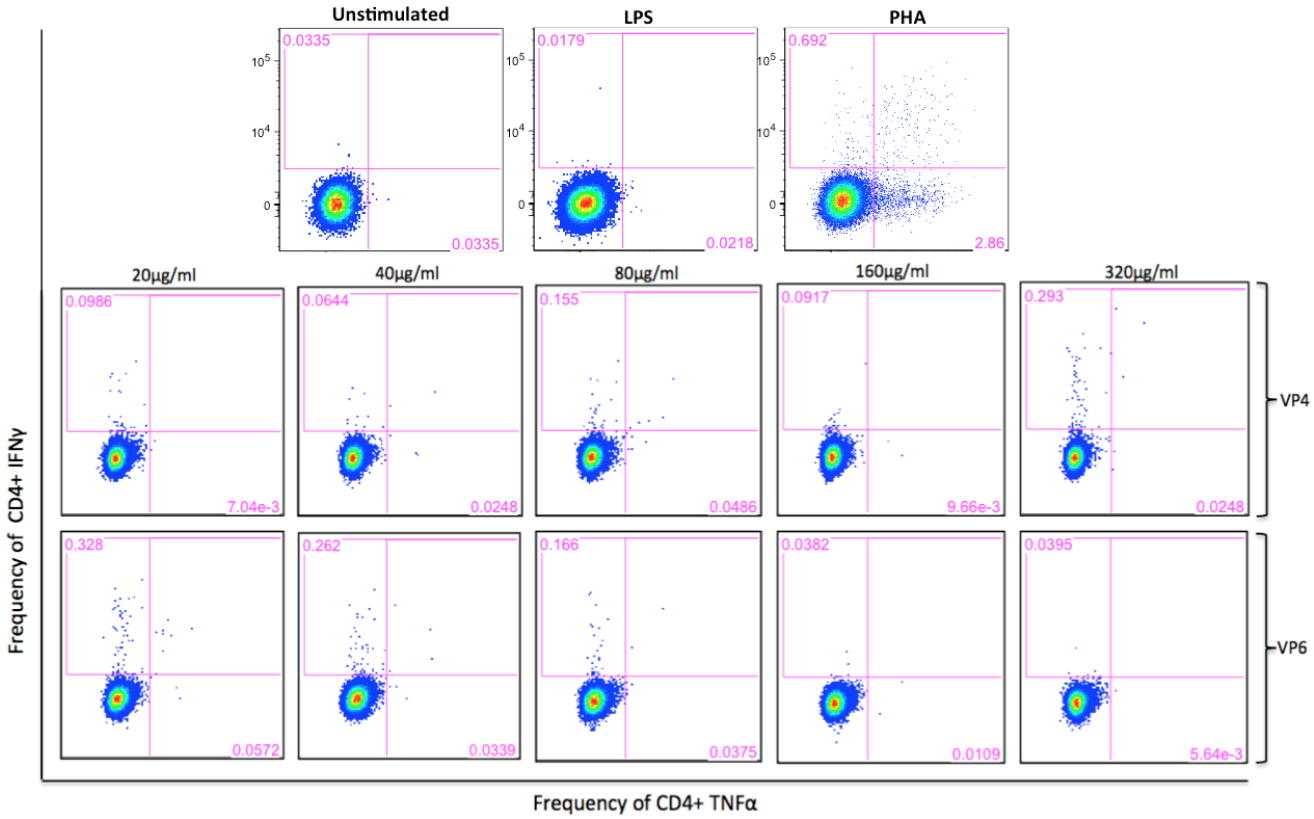


Figure 3.3.3: Adult CD4+ IL-2 and IFNγ responses to varying concentrations of VP4 and VP6 stimulation in a 12 hour whole blood assay. The top row shows unstimulated, LPS and PHA stimulated controls. The second row shows VP4 responses and the bottom row shows VP6 responses. Columns 1 to 5 show 2-fold increasing concentrations of proteins.

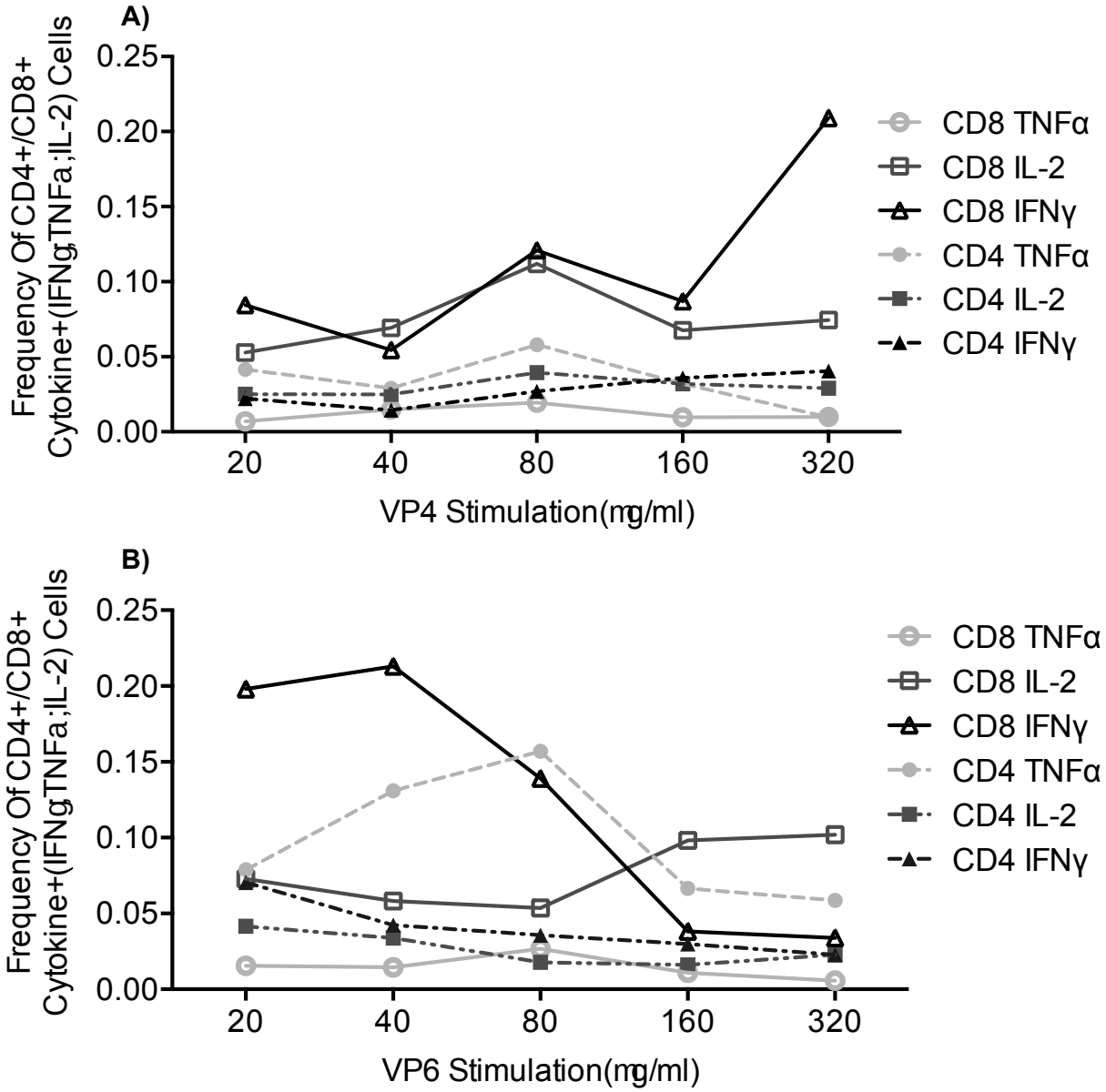


Figure 3.3.4: Net CD4+ and CD8+ adult cytokine responses to VP4 and VP6 Rotavirus Proteins. Solid lines with open symbols represent net CD4+ responses and dashed lines with solid symbols represent net CD8+ responses. Panel A and B represent VP4 stimulated and VP6 stimulated responses respectively.

According to figure 3.3.4 A, a concentration of 40µg/ml VP4 induces the highest overall expression of cytokines. For VP6 (Figure 3.3.4 B), both 20µg/ml and 40µg/ml induced similar levels of cytokine expression. Therefore, these optimal adult concentrations were then tried in infant blood. Additionally, infant responses to different combinations of VP4 and VP6 were also assessed.

SECTION 3.4:

VP4 AND VP6 TITRATION CYTOKINE RESPONSES IN INFANTS

The 12-hour whole blood assay was used to determine optimal protein concentrations needed for detection of rotavirus specific T-cell cytokine responses in infants. In South Africa, Rotarix is given at six and 14 weeks of age, thus blood from five 15-week-old infants, who have completed the Rotarix vaccine schedule, were used for optimization. Figure 3.4.1 shows representative flow plots from one of the five infants that were used for this titration, and Fig.3.4.2 depicts the net median responses of all five infants.

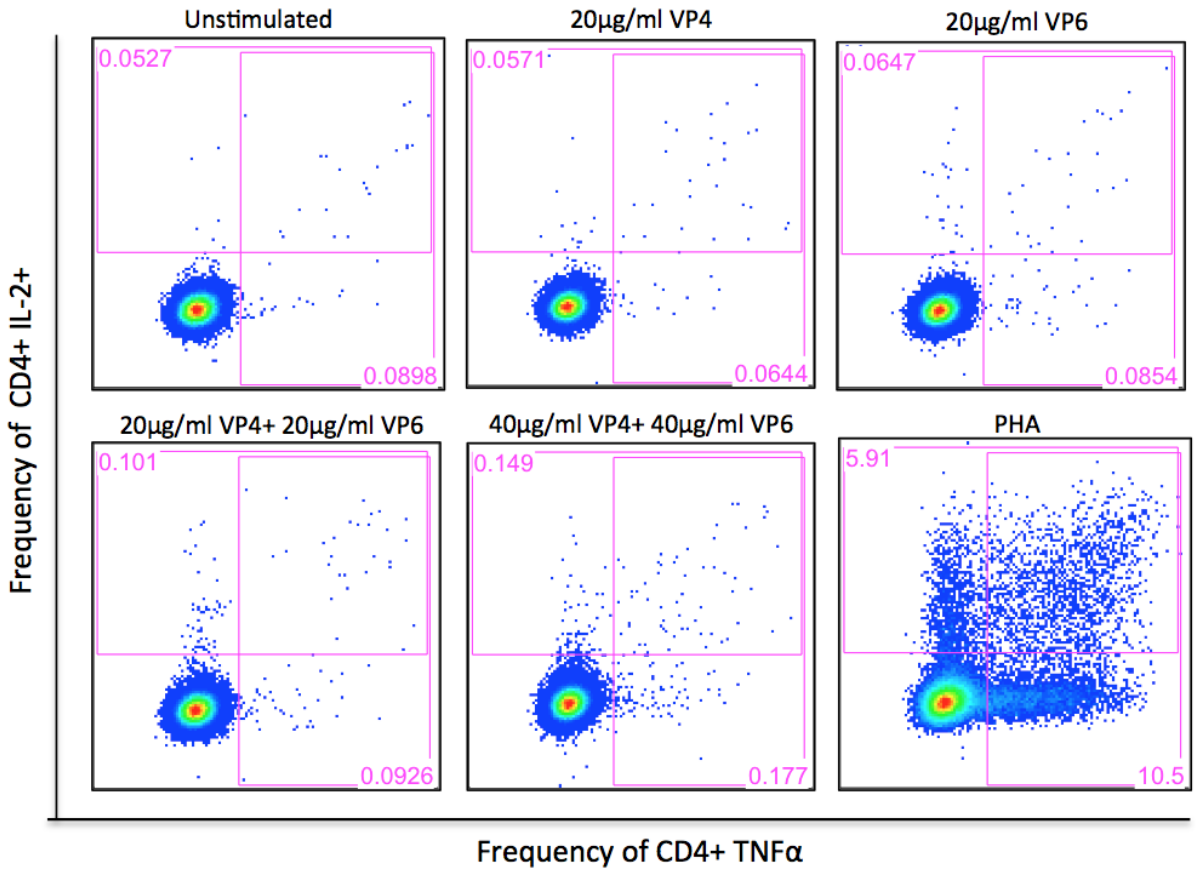


Figure 3.4.1: Flow plots of one week 15 infant, showing CD4+ TNFα and IL-2 responses to different combinations of VP4 and VP6 stimulation done in a 12 hour whole blood assay.

Stimulation with 20µg/ml of VP4, VP6 or a combination of both proteins at 20µg/ml each yielded responses similar to that of unstimulated blood. However, as evident in

figure 3.4.1 a combination of 40µg/ml VP4 and VP6 induced responses above background levels.

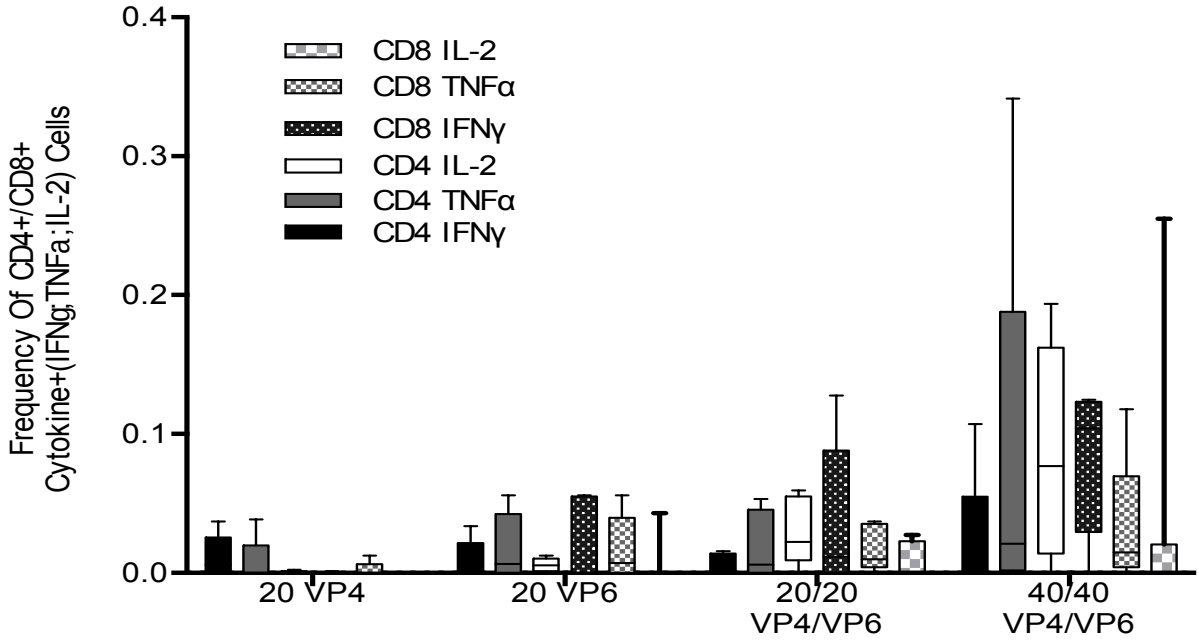


Figure 3.4.2: Net CD4+ and CD8+ infant cytokine responses to VP4 and VP6 Rotavirus Proteins. Solid box and whiskers represent CD4 responses; patterned box and whiskers represent CD8 responses.

Upon analysis of all responses (n=5), it was found that a combination of 40ug/ml VP4 and 40ug/ml VP6 rotavirus proteins was the best concentration of proteins for the detection of CD4+ and CD8+ cytokine responses. This concentration of proteins was implemented in further whole blood assays.

SECTION 3.5:

VP4 AND VP6 ROTAVIRUS CYTOKINE RESPONSES IN PRE-VACCINATED AND POST-VACCINATED INFANTS

As previously mentioned, the VP4 and VP6 proteins used in the whole blood assays were produced in E.coli. This resulted in high levels of lipopolysaccharide endotoxin (LPS) contamination. A comparison of responses induced by LPS contaminated protein and LPS free protein in a 12-hour WBA was assessed in 3 different infants. The second batch of Rotavirus VP4 and VP6 proteins were utilized in this experiment.

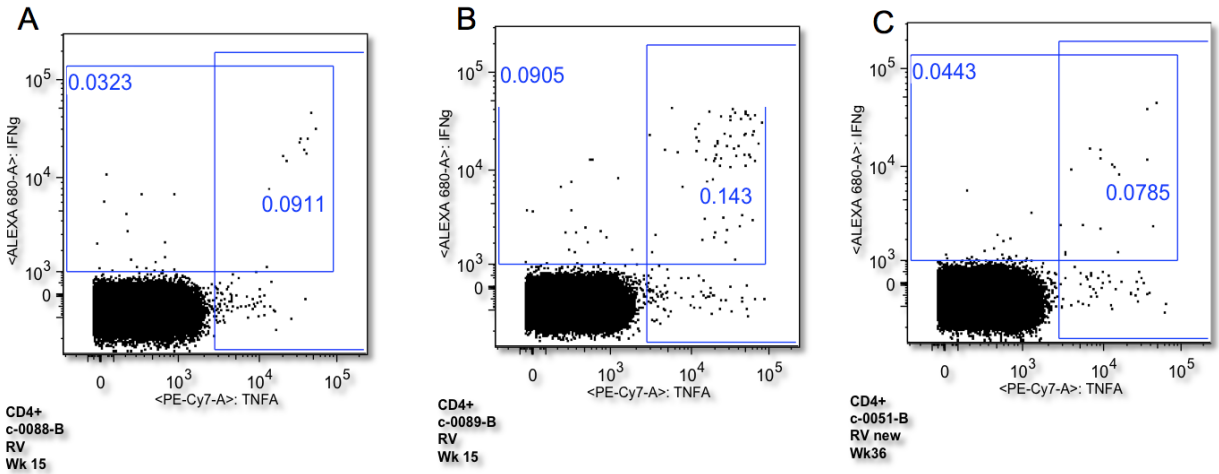


Figure 3.5.1: CD4+ Rotavirus IFNγ and TNFα Responses. Panel A and B show responses to LPS contaminated proteins while panel C shows responses to LPS free VP4 and VP6.

Figure 3.5.1 shows representative flow plots of CD4 IFNγ and TNFα responses to unpurifed batch 2 proteins (A and B), or partially purified batch 2 protein (C). From the figure it seems like all three infants respond in a similar manner, dispite A and B having a much higher concentration of contaminating LPS. This suggests that LPS may not drastically alter T-cell responses.

Therefore, a final experiment was conducted in which pre-vaccinated infant responses were compared to post vaccination infant responses. Blood from four 4-7 day old infants, three week 15, and four week 36 infants was analyzed in this experiment. The volumes of protein used in this experiment were based on the assumption that the endotoxin removal kits were able to remove LPS and did not deplete protein concentrations. Batch 2 proteins were used in this experiment after being purified once. Flow plots of the CD4 IL-2 and TNFα responses from this

experiment may be found in figure 3.5.2 below. Quantification of the protein was only completed after the experiment was the endotoxin removal kit had not only reduced the LPS concentration, but also severely depleted the protein concentration. Consequently, the volume of protein initially assumed to be 40µg/ml, was actually 0.048µg/ml VP4 and 0.0041µg/ml VP6. In addition, the LPS concentration in the protein was still relatively high, resulting in a final LPS concentration of 2.6832EU/ml in each vial.

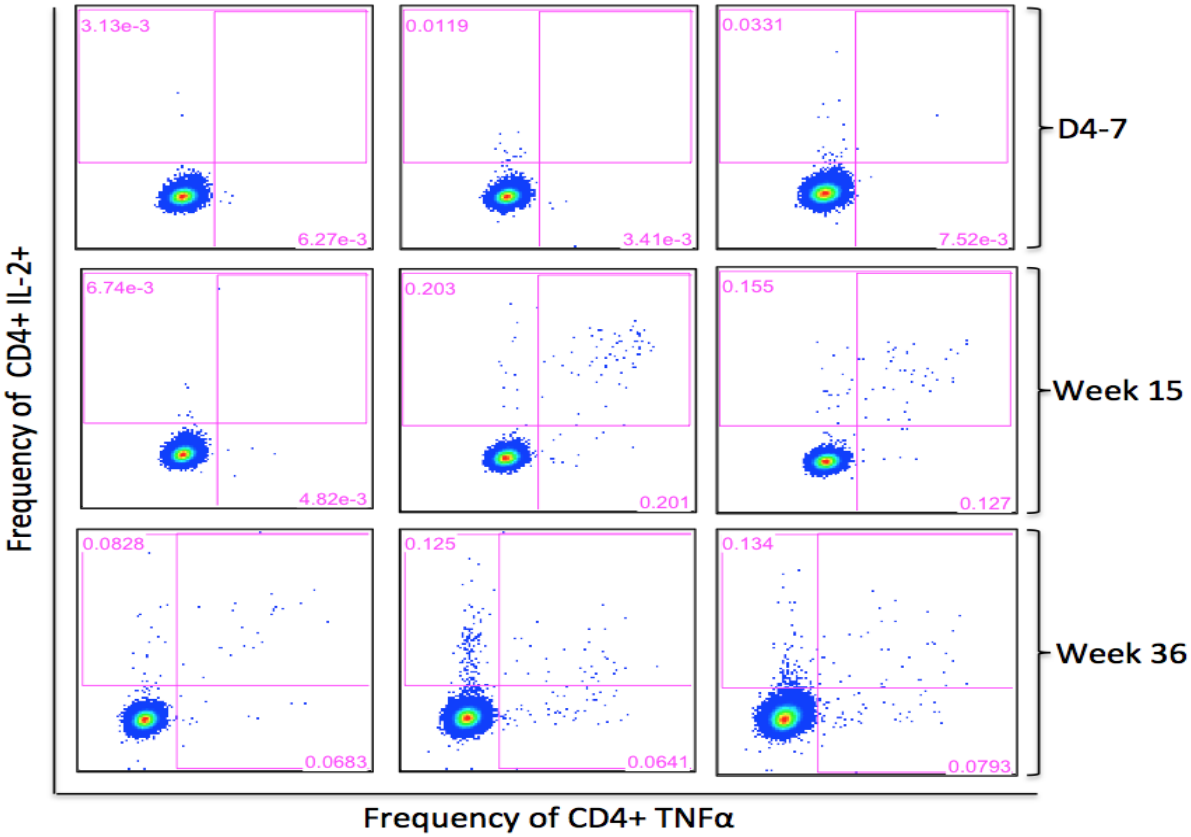


Figure 3.5.2: Representative flow plots of the CD4+ IL-2 and TNFα responses to VP4 and VP6 in different infants at different time points. Row 1 represents responses of 3 different D4-7 infants, row 2 represents responses of 3 different week 15 infants and the third row represents responses of 3 week 36 infants to VP4 and VP6 stimulation in a whole blood assay.

The results of the experiment indicated that overall cytokine responses at week 36, which is 22 weeks post completion of the vaccine schedule, was higher than D4-7 responses which are pre-priming (Fig 3.5.3). Additionally CD4+ IL-2 expression was significantly higher at week 36 compared to both week 15 and D4-7.

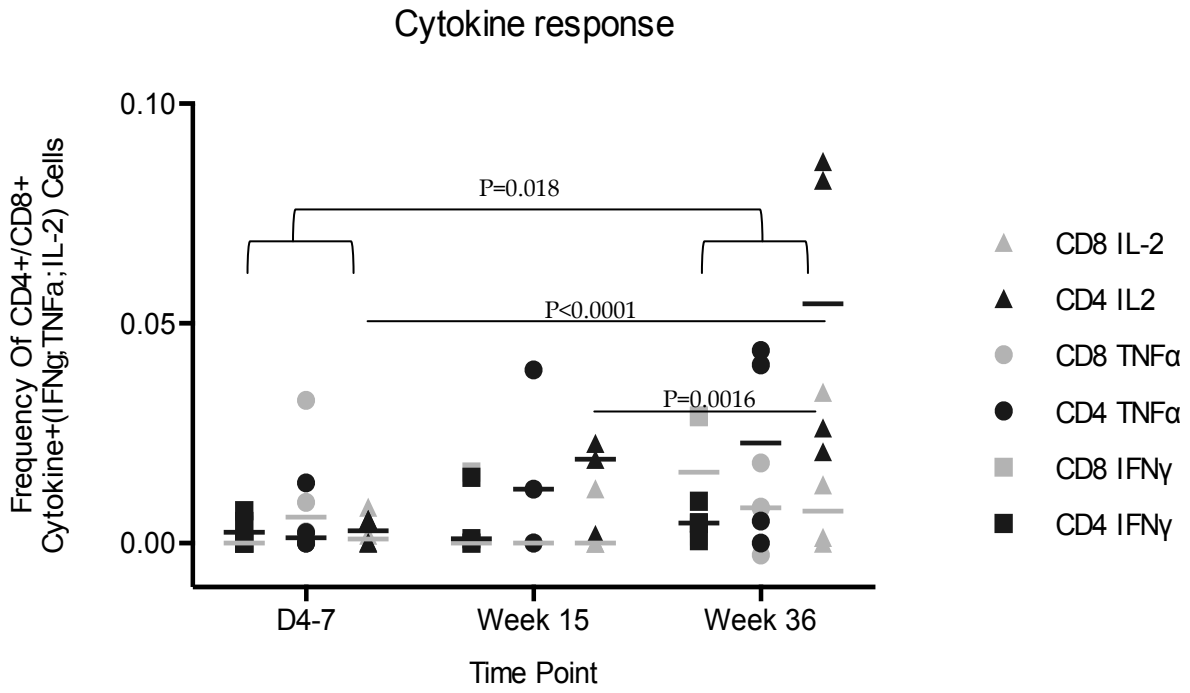


Figure 3.5.3: CD4+ and CD8+ infant cytokine responses to VP4 and VP6 Rotavirus Proteins. Grey symbols and bars represent net CD8 responses and medians respectively. Black symbols and bars represents net CD4 responses and medians respectively. P-value was generated using a 2-way ANOVA analysis and adjusted with the Turkey multiple comparison test

It is evident that post-vaccinated infants respond better to VP4 and VP6 stimulation than pre-vaccinated infants (figure 3.5.2 and 3.5.3). This suggests that Rotavirus T-cell responses are present in Rotarix vaccinated infants and that this assay is able to detect some rotavirus specific responses. However, it would be necessary to determine how much of the responses detected are due to stimulation with LPS.

SECTION 3.6:

LIPOPOLYSACCHARIDE REMOVAL FROM ROTAVIRUS VP4 AND VP6 PROTEINS

LPS is a major component of gram-negative bacteria. It binds to LPS-binding protein (LBP) and toll like receptor 4 (TLR4) on monocytes and myeloid cells (Akira, Takeda, & Kaisho, 2001). Once bound to monocytes, such as dendritic cells (DC), it causes the DC to mature. Mature DC express proinflammatory cytokines and upregulated costimulatory molecules. In addition, mature DC also have increased antigen-presenting abilities. All of these functions allow DC to stimulate T-lymphocytes (Akira et al., 2001). Since the aim of this study was to assess Rotavirus specific responses, having LPS contamination could make it difficult to distinguish between LPS and Rotavirus vaccine responses (Petsch & Anspach, 2000). Therefore, LPS was removed from the RV protein preparation prior to use in subsequent experiments (Methods page 49).

The original batch of proteins used were at concentrations of 700µg/ml VP4 and 2840µg/ml VP6. The LPS concentrations after purification were 4.44EU/ml in VP4 and 7.53EU/ml in VP6. Only the LPS concentrations found in the proteins were measured after purification, the proteins concentrations itself was not. Therefore, the final concentrations of proteins utilized in experiments 1 to 6 remained unknown. Since the original batch of proteins were utilised in experiments 1 to 6, a new batch had to be used for subsequent experiments. The second batch of proteins received were far more concentrated at 1200µg/ml VP4 and 7800µg/ml VP6. However, the second batch of protein also had a very high concentration of LPS, at 430EU/ml in VP4 and 700EU/ml in VP6. The proteins were purified yielding concentrations of 60EU/ml in VP4 and 120EU/ml in VP6. Since 60EU/ml and 120EU/ml LPS were still too concentrated to work with a second purification was done resulting in LPS concentrations of >1EU/ml in both proteins. Nevertheless, as evident in figure 3.6.1,a large amount of protein depletion occurred during subsequent purifications. After the first purification the proteins concentrations dropped from 1200µg/ml VP4 to 1.4µg/ml and VP6 decreased from 7800µg/ml to 0.8µg/ml. After the second purification, the proteins were undetectable on the western blot.

Proteins were quantified by constructing a BSA standard curve ranging from 12.5µg to 0.78µg on a SDS-PAGE gel. Varying amounts of protein ranging from 1µl - 20µl in PBS plus protein loading dye were loaded on gel. SynGene GeneTools software was used to quantify the amount of VP4 and VP6 (Red rectangles on figure 3.6.1) in relation to the BSA standards (Purple rectangles on figure 3.6.1). As depicted in the figure, protein concentrations found in batch 2 were measured after the first and second purification. A third batch of protein was also obtained and purified. The protein concentration in this batch was measured after purification and found to be undetectable prior to running any experiment. Since the protein was undetectable, no experiments were run with this batch of proteins.

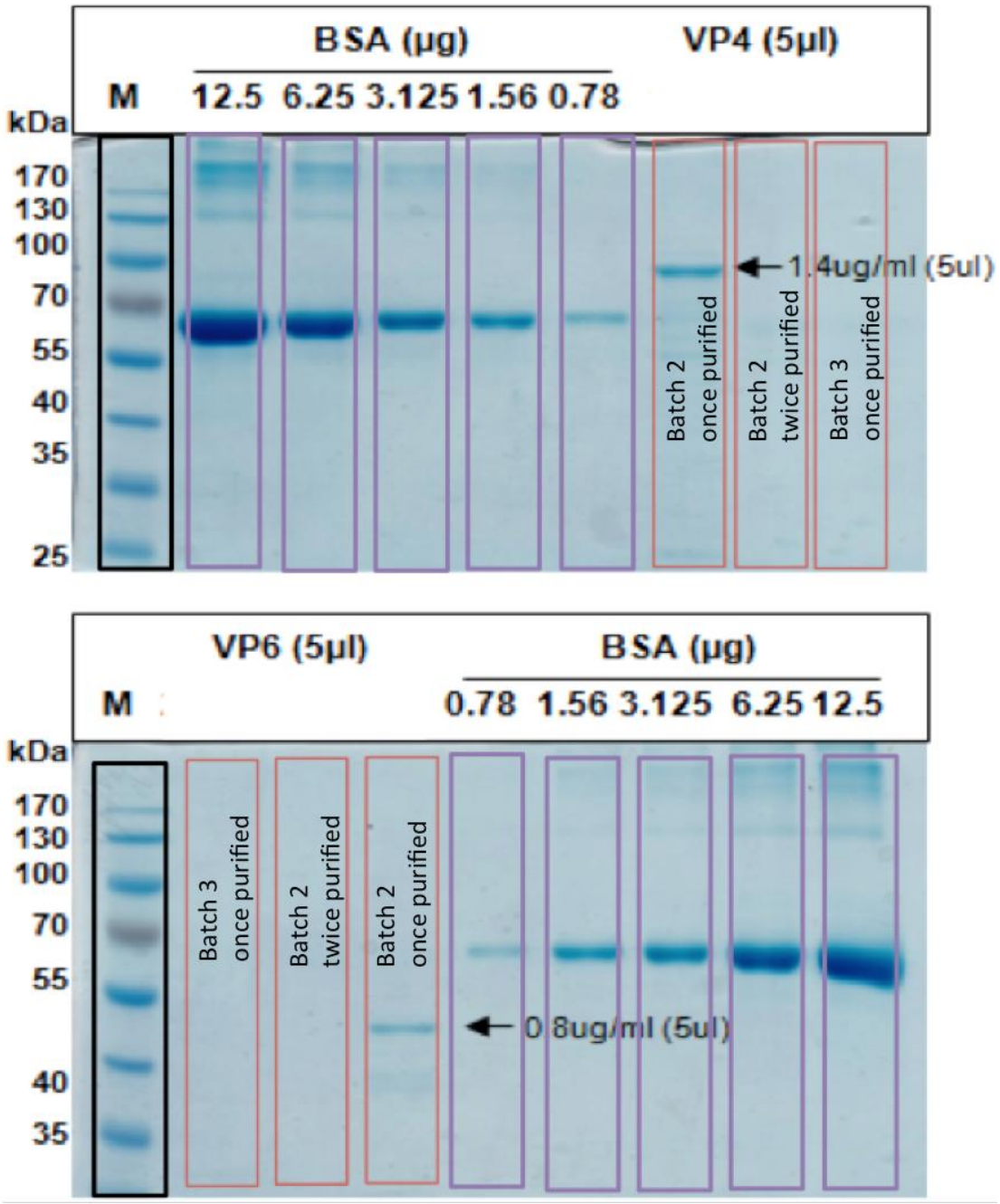


Figure 3.6.1: BSA Quantification Of Endotoxin Free Rotavirus VP4 and VP6 e.coli Derived Protiens. M (Black), which is the bands in the first column of each gel is a DNA ladder, containing DNA fragments of known sizes, that can be used as a reference, to measure other bands on the gel. In the top gel, columns 2-6 (Purple) represent BSA standards and columns 7-9 (Red) represent different batches of protein. In the bottom gel, column 2-4 (Red) represent different batches of protein and column 5-9 (Purple) represent the BSA standards. Each batch of protein has a red rectangle around it. The arrow indicates the extraoplated concentrations of the detected proteins.

Given that LPS acts throught monocytes and myeloid cells and do not directly activated T-cells, it was not clear if LPS at the concentrations in the contaminated protein solution, once diluted in vitro would have any effect on T-cell responses in the 12 hour incubation time. Thus, due to the inefficency in endotoxin removal from proteins, a series of experiments were conducted to determine if any effects that LPS contamination had on the detected responses could be quantified.

A titration experiment was initially conducted to determine if it was possible to find a concentration at which protein-stimulated responses were above LPS-stimulated responses (Appendix 1). Three dilutions were used for this experiment, ranging from 0.1µg/ml to 0.001µg/ml. Additionally; an LPS control was added with the equivalent amount of LPS to that of the most contaminated protein stimulation condition. This experiment was done using blood from a 15-week-old infant. Figure 3.6.2 below depicts CD4+ IFNγ and TNFα responses at the three concentrations of protein tested in the experiment, alongside the LPS stimulated responses.

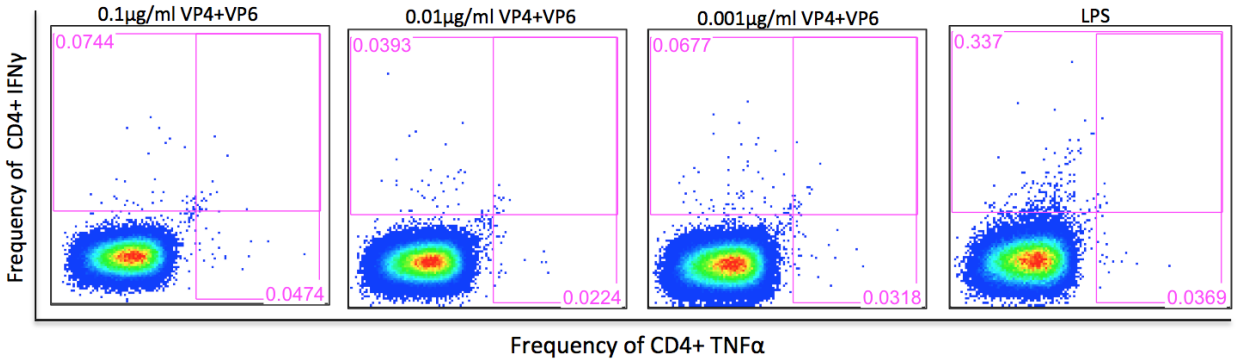


Figure 3.6.2: Infant CD4+ IFNγ and TNFα responses to LPS and varying concentrations of VP4 and VP6

As apparent in figure 3.6.2, LPS stimulated CD4+ TNFα responses were similar for protein stimulated and LPS stimulated blood. This can also be seen in figure 3.6.4 A and B below. CD4 and CD8 IL-2 responses were also similar in protein and LPS stimulated blood. However, IFNγ responses were higher in LPS stimulated blood.

A second experiment was conducted in the same manner as the first but with a wider range of dilutions, ranging from 0.001µg/ml to 1µg/ml. Again, blood from a week 15 infant was used in this experiment. Figure 3.6.3 shows representative plots of CD4+ IFNγ and TNFα responses at the different concentrations of protein used in this experiment, as well as LPS responses.

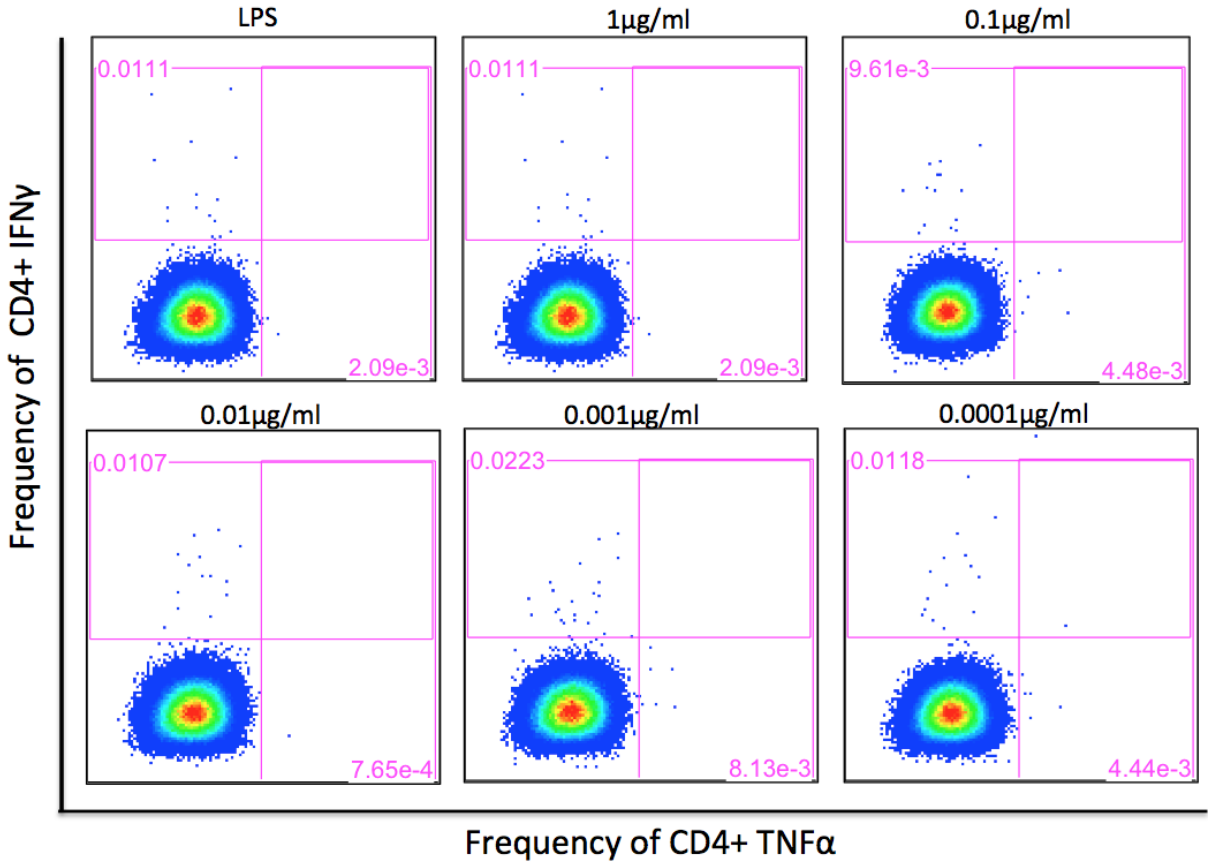


Figure 3.6.3: Infant CD4+ IFNγ and TNFα responses to LPS and varying concentrations of VP4 and VP6.

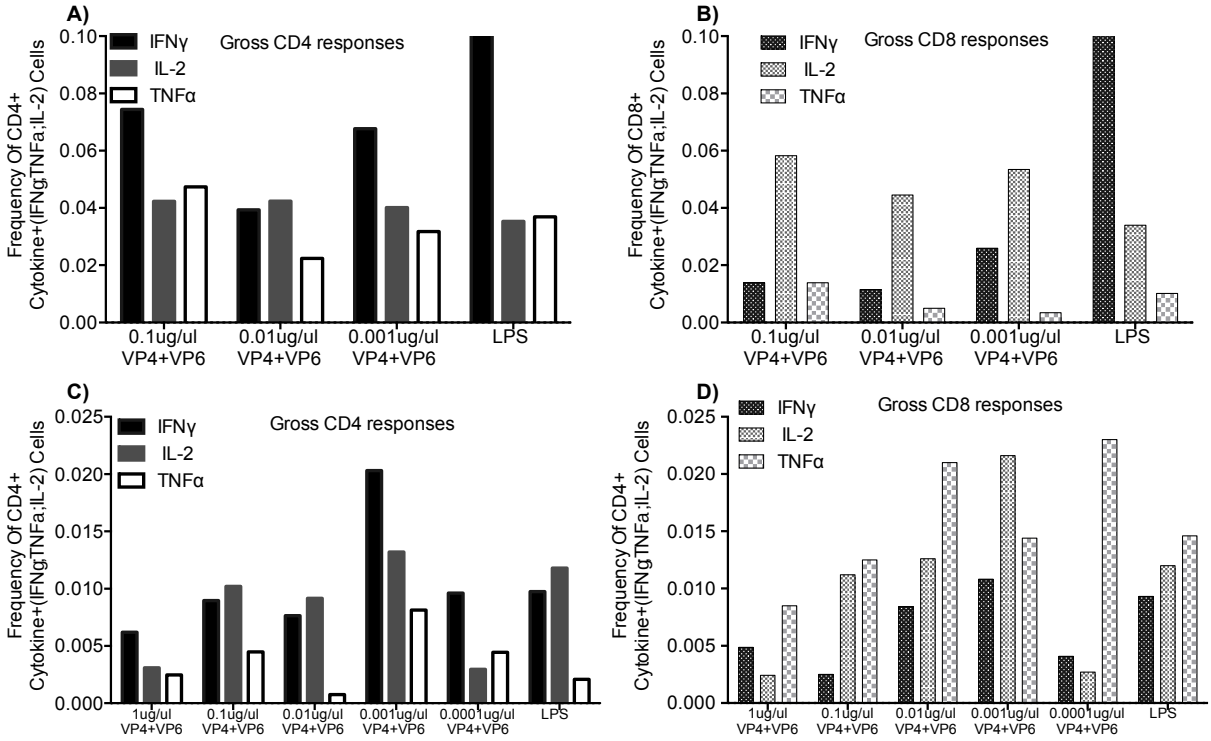


Figure 3.6.4: Effects of LPS Contamination On Outcome Of VP4 And VP6 Cytokine Responses. Panel A and C show total CD4 responses while B and D represent total CD8 responses. Additionally, responses to LPS at a concentration 2x that in the most contaminated protein stimulation tube are displayed. Panel A and B [LPS] = 0.0688EU/ml and in panel C and D [LPS] = 0.7EU/ml.

As evident in figure 3.6.4 C and D, at a concentration of 0.001µg/ml VP4 and 0.001µg/mlVP6, majority of responses were slightly higher in protein-stimulated blood compared to LPS stimulated blood. At other concentrations, most protein responses were of similar levels to that of the LPS stimulated responses. This suggests that all T-cell responses, which were detected after stimulation with LPS contaminated VP4 and VP6, were at least in part, due to the LPS contamination.

SECTION 3.8:

DISCUSSION

The main aim of this chapter was to optimize an assay capable of detecting Rotavirus vaccine induced T-cell cytokine responses. We assessed three different antigens, including whole DS-1 rotavirus, as well as Rotavirus surface proteins VP4 and VP6 for this purpose. However, we were unable to optimize a consistent assay. We encountered multiple challenges in developing a rotaviral T cell assay.

Firstly, using DS-1 rotavirus in a mixture with Vero-cells resulted in inconsistent responses. This was possibly due to a change in the ratio of virus to cells in our stimulation mixture. This would result in different concentrations of virus being present within the same volume of mixture. Additionally, virus was prepared by freeze-thaw cycles of virus-infected Vero cells; we did not purify viral particles from cellular constituents. Vero-cells are non-human cells and foreign antigens, therefore are also capable of eliciting an immune response in human cells. This may have made it difficult to distinguish if the responses we detected were in fact Rotavirus-specific responses or allogeneic responses. One way around this issue would be to use Vero-cells alone as a control. Any responses detected over and above that caused by Vero-cell stimulation could then be attributed to DS-1. Another alternative would be to separate the virus from the Vero cell mixture, possibly using ultracentrifugation. This would be advantageous in eliminating the issue of distinguishing which antigen is eliciting our detected responses.

With VP4 and VP6 proteins derived from E.coli, the main issue was endotoxin contamination. Endotoxins are an integral part of gram-negative bacterial cell membranes (Vaara & Nikaido, 1984). Bacteria, such as E.coli, release endotoxins during cell growth and division. This consequentially leads to high concentrations of LPS contamination in products derived from bioprocessing within the bacteria (Raetz, 1990). This poses a problem for biological assays, as even minute levels of LPS have been shown to have major biological impacts on immune cells (Petsch & Anspach, 2000). Endotoxins activate the immune system, particularly macrophages and monocytes, through TLR4 engagement. In response, these cells release TNF, interleukins and other immune mediators, which may stimulate T-cell responses

(Rietschel et al., 1994). A paper assessing the kinetics of a T-cell response to LPS stimulation showed that T-cells only start proliferating after 4 days in culture and no T-cell responses were detected prior to 4days of incubation (Ulmer, Flad, Rietschel, & Mattern, 2000). Based on the finding of this article, it may be assumed that 12 hours is insufficient time for LPS to stimulate T-cells. However, despite this indirect method of T-cell activation, and the short incubation time, the data in this study suggests that LPS contamination plays a large role in masking vaccine specific responses.

The threshold of acceptable endotoxin contamination in in vitro assays is generally 1EU/ml (Berthold & Walter, 1994). Due to the stability of endotoxins, meeting this threshold of 1EU/ml is usually challenging. In comparison to proteins, the biologically active parts of endotoxins are able to survive extreme temperatures and pH (Sharma, 1986). There is a variety of products available for endotoxin removal. Each product is tailored to work with specific biological agents (Petsch & Anspach, 2000). We chose to use the Pierce High Capacity Endotoxin Removal Resin, due to its reported high capacity to recover proteins. A major issue experienced with this kit was that it severely depleted our proteins. One explanation for this may be that our target proteins aggregated with the endotoxin. This would result in removal of the target proteins with the removal of endotoxins. In addition, some batches of protein had higher initial levels of LPS contamination and required multiple purification cycles to complete endotoxin removal. Mild protein depletion occurring with every cycle may have attributed to the major depletion that eventually resulted. In future, different endotoxin removal methods could be employed in an effort to reduce protein depletion.

Furthermore, rotavirus is often asymptomatic in most adults, and thus it was difficult to select adult participants who may have been recently exposed to rotavirus. This meant that even blood from the same individual might yield different responses on different days, depending on when the adult was last exposed. Also, adults generally have very strong antibody responses to rotavirus, which may neutralize the virus without stimulating an in vitro cellular response.

Another issue with optimizing this assay is that Rotavirus T-cell cytokine responses are reportedly found in very low frequencies, making them difficult to detect (Parra et al., 2014). Studies done by M Parra et al and M C Mesa et al (Mesa, Gutiérrez, Duarte-Rey, Angel, & Franco, 2010; Parra et al., 2014) showed the level of circulating rotavirus T-cells normally found in healthy adults and Rotavirus infected children, compared with those of tetanus toxoid (TT) and Flu. They found that adults had CD4+ and CD8+ T-cell cytokine responses to rotavirus were only marginally higher than PBS stimulated responses. In comparison to tetanus and influenza T-cells, rotavirus-specific T-cells were also found in lower quantities. In children, the majority of the CD4+ T-cell responses were below the limit of detection. This suggests that even if Rotavirus specific responses were been detected, the large effect of LPS contamination would mask the miniscule responses normally found to Rotavirus. Therefore, the first step of completing optimization would be to find an appropriate LPS removal kit.

Given the important role T-cells seem to play in Rotavirus infection, it is necessary that an assay capable of assessing Rotavirus specific T-cell responses be developed. Such an assay would provide imperative information that may help inform vaccine and treatment approaches in future.

Chapter 4

CELLULAR IMMUNE RESPONSES TO BORDETELLA PERTUSSIS VACCINATION IN INFANTS

SECTION 4.1

INTRODUCTION

Bordetella Pertussis is the causative agent of Pertussis or whooping cough, a highly contagious respiratory disease. It is a gram-negative aerobic coccobacillus (Domenech de Celles, Magpantay, King, & Rohani, 2016), characterized by rigorous coughing spasm, which frequently lead to vomiting. In infants, the disease causes further complications such as; encephalopathy, pneumonia, apnea, and in some cases, death (Edwards, 2005).

Like many severe diseases, pertussis is vaccine preventable. Whole cell Pertussis (wP) vaccines have been available since the 1940s (Domenech de Celles et al., 2016). In developing countries, a 90% reduction in pertussis cases were reported after the implementation of wP vaccines in routine paediatric immunization programs (Edwards, 2005). About 10 years ago, wP vaccines were replaced with acellular pertussis (aP) vaccines in most countries due to the high rate of reactogenicity caused by wP vaccination (Higgs, Higgins, Ross, & Mills, 2012). In spite of the availability of a vaccine, there is still an alarming rate of Pertussis disease, 90% of which is found in developing countries. In young infants, pertussis still causes approximately 400,000 fatalities a year.

Most immunocompetent hosts are able to clear pertussis infections but the immune response is not sustained long-term (Fedele, Cassone, & Ausiello, 2015). Immunity conferred from natural pertussis infections may last anything from 3.5 to 30 years. Vaccine-induced immunity lasts for approximately 5-14 years with wP vaccination and 4-7 years with aP vaccination. This waning of immunity leaves adolescents and adults vulnerable to infections (Kilgore et al., 2016), which in turn leave infants more susceptible to infections due to increased exposure.

Up until now, there is no known correlate of protection against Bordetella Pertussis. Serum IgG and mucosal IgA both play an important role in anti-B. Pertussis immune mechanisms but are insufficient for complete protection. In addition to this, neutralizing antibody levels rapidly decline post vaccination (Fedele et al., 2015). Contrastingly, T-cell responses to pertussis vaccines last beyond the decline of antibody responses. These T-cell responses were first assessed during the safety

and efficacy trials conducted on the aP vaccines (Podda et al., 1991). It has been demonstrated that TLR-4 and CD4+ TH1 cells are of particular importance in protective immunity generated with wP vaccination; that is, TH1 cells alone are able to protect against secondary pertussis infections (Domenech de Celles et al., 2016).

Additionally, studies of pertussis infection in children found that, induction of IFN-g secreting TH1 cells was associated with recovery from infection (Mascart et al., 2003; Ryan et al., 1997). Experimental studies in mice demonstrated that IFN-g is required for control of B. pertussis infections and mice that had targeted disruptions in their IFNγ receptor genes, developed a fatal infection (Barbic, Leef, Burns, & Shahin, 1997; Mahon, Sheahan, Griffin, Murphy, & Mills, 1997). Other mice experiments involving adoptive transfer or cell depletion also convincingly showed that TH1 cells play a role in the clearance of primary infection after wP vaccination (P. Byrne, McGuirk, Todryk, & Mills, 2004; Mills, Barnard, Watkins, & Redhead, 1993).

Moreover, in infants, IL-2 and TNFα also play a role in protecting against systemic pertussis dissemination by enhancing macrophage phagocytosis (Kilgore et al., 2016). During natural infection, T-cells secreting IFN-g and IL-2 can be found in infants recovering from Pertussis. These results as well as those from other studies suggest that natural B. pertussis infection in children stimulates TH1 responses similar to that found in wP immunized children. Interestingly, a fraction of 'naïve' children and adults with no history of natural infection or previous vaccination have detectable B. pertussis specific TH1 responses. The authors suggest that this is due to subclinical exposure to natural Bordetella Pertussis, which result in the acquisition of antigen-specific cell mediated immunity (Ausiello, Lande, la Sala, Urbani, & Cassone, 1998).

In contrast with wP vaccination, aP immunization induces TH2-type or mixed TH1/TH2 responses (Warfel & Edwards, 2015). This response does not seem to be as effective as TH1 clearance of B. pertussis infections, even though aP promotes humoral responses in addition to T-cell responses. Clinical studies conducted in children support this finding. These studies have demonstrated that children immunized with wP have strong TH1 cell responses and children immunized with aP have a dominant TH2 cell response with strong antibodies (Higgs et al., 2012). It is thus clear that T-cells play an important role in prevention of pertussis disease after

vaccination. This chapter focuses on the kinetics of CD4+ and CD8+ T-cell responses in newborn children after receiving acellular pertussis vaccination. By so doing, aims 3 and 4 of the dissertation are addressed, that is to assess the impact of HIV exposure and maternal feeding practice on T cell responses to aP vaccination.

More specifically, the first section (Section 4.2) describes the immune system of infants, with specific focus on memory ontogeny, activation and proliferation of CD4+ and CD8+ T-cells across time. Unstimulated and BP stimulated T-cells were assessed to determine if vaccine specific responses could be distinguished within the bulk CD4 and CD8 T-cell populations. The second section (Section 4.3) is concentrated on the ontogeny of BP-specific CD4 and CD8 T-cell cytokine and memory responses. The third section (Section 4.4) pays particular attention to the differences between HEU infants and HU infants and the last section (Section 4.5) assesses the differences between breast-fed and formula-fed infants.

SECTION 4.2

THE INFANT IMMUNE SYSTEM

As described in Chapter 2 (Methods, page 50), a 12-hour whole blood assay was conducted and 3 different groups of infants were examined; HIV unexposed breast fed (U BF), HIV exposed breast fed (E BF), and HIV exposed formula fed (E FF) infants. The assay was completed on infants of 4 different ages, at 4-7 days (n=24) and at 7 (n=47), 15 (n=37) and 36 weeks (n=38). All the infants were vaccinated with aP vaccines. Table 4.1.1 below demonstrates the characteristics of the cohort. There were no significant differences in characteristics among the three groups of infants at any time point. In some groups, there were a disproportionately higher number of males compared to females. Acellular Pertussis vaccines were given at roughly the same median age for all groups of infants, ranging from 42-49days of age for the first dose, 71-76 days for the second dose and 99-108 days for the last dose. The numbers of exclusively breast-fed infants were in general very low, and lower in HIV unexposed (HU) infants compared to HEU.

Throughout this section, immunological changes across time were measured separately within each group of infants, that is, no direct comparisons between HEU and HU, or breast-fed and formula-fed infants were completed.

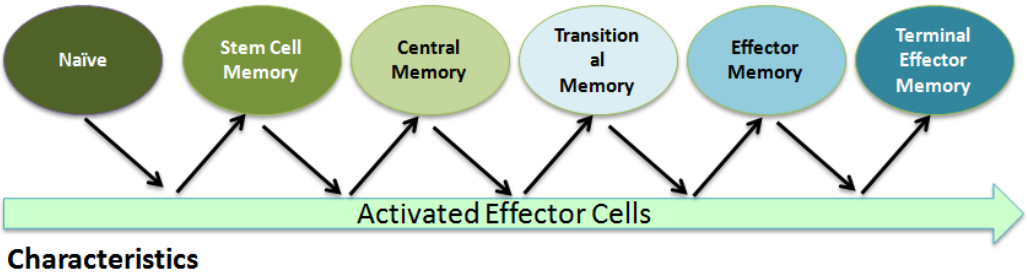
TABLE 4.1.1: COHORT CHARACTERISTICS

Time Point	Groups	Total Number of Participants	Median gestational age in weeks (IQR)	Median gestational weight (kg) (IQR)	Median weight (Kg) (IQR)	Number of females (%)	Median age of first BP vaccine (Days) (IQR)	Median age of second BP vaccine (Days) (IQR)	Median age of third BP vaccine (Days) (IQR)	Exclusively breast-fed (%)
D4-7	U BF	7	39 (38-39)	3.20 (3.04-3.37)	3.46 (3.08-3.52)	28,57	44 (40-49)	75.5 (70-82.5)	102.5 (97.5-110.5)	71,4
	E BF	10	39 (37-39)	2.91 (2.70-3.39)	2.91 (2.70-3.39)	60	45 (41.5-229.5)	76.5 (72.25-264.5)	108 (106-483)	100
	E FF	7	40 (39-40)	3.42 (2.95-3.77)	3.46 (3.06-4.14)	57,14	49 (45-57)	76 (70-78)	103.5 (101-106)	na
Week 7	U BF	18	39 (38-39)	3.06 (2.89-3.42)	4.90 (4.43-5.72)	55,5	44 (42-48.5)	72.5 (69.75-79.25)	103 (98-108)	55,5
	E BF	22	39 (38-40)	3.26 (2.86-3.47)	4.89 (4.59-5.38)	59,09	43 (42-49.5)	71.5 (70-79)	101 (98-106.5)	86,4
	E FF	7	39 (37-40)	3.19 (2.83-3.42)	5.48 (4.52-5.70)	71,43	46 (43-50)	71 (71-77)	103 (99.25-114.3)	na
Week 15	U BF	10	38.5 (37-39.5)	2.90 (2.81-3.30)	6.27 (5.92-7.80)	50	44 (41-44)	72 (70-83)	101 (98-113)	40
	E BF	17	39 (38-40)	3.27 (3.04-3.43)	6.06 (5.95-6.79)	70,58	42 (79.5-119.5)	71 (70-79.5)	99 (98-119.5)	58,8
	E FF	10	39 (37.75-40)	2.99 (2.91-3.55)	6.60 (6.11-7.57)	40	43.5 (42-48.5)	71 (70.5-74.5)	100 (99.25-101)	na
Week 36	U BF	10	39 (37-40)	2.93 (2.78-2.99)	8.50 (7.49-9.19)	60	43 (42.5-44.25)	72 (71-77)	107 (101-119.5)	10
	E BF	18	39.5 (38-40)	3.02 (2.84-3.40)	8.06 (7.46-8.97)	55,55	45 (42-47.5)	73.5 (70-78.25)	102 (99-105.5)	11,1
	E FF	10	38 (37-39.25)	3.04 (2.96-3.45)	8.91 (8.47-9.28)	50	43 (41.75-44.25)	71 (70-72)	100 (98.5-105.5)	na

LONGITUDINAL ASSESSMENTS OF CD4+ AND CD8+ MEMORY T-CELL DIFFERENTIATION

Antigen recognition by the immune system causes a number of changes in lymphocytes that allow for the neutralization or elimination of infectious antigens (Mahnke, Brodie, Sallusto, Roederer, & Lugli, 2013) and the development of immunological memory. Formation of immunological memory is one of the most important processes of cellular immune mediated protection. After negative and positive selection in the thymus, naïve T-cells are released into the circulation (Zhang & Bevan, 2011). Upon cognate antigen exposure, naïve T-cells proliferate and differentiate in effector cells. Effector cells migrate to the site of infection/vaccination and assist in clearing infection. The majority of effector cells die following clearance of an infection and a small proportion of the effector T-cells develop into long-lived memory T-cells (Hammarlund et al., 2003)

The spectrum of memory and effector populations which develop in response to antigen and survive as long-lived cells after the clearance of infection are heterogeneous in phenotypic characteristics, function and anatomical location (Marzo, Yagita, & Lefrancois, 2007; Masopust, Vezys, Marzo, & Lefrancois, 2001; Sallusto, Lenig, Förster, Lipp, & Lanzavecchia, 1999). In comparison to naïve cells, memory T-cells not only respond more efficiently to antigen encounters, they also require fewer signals to become activated and have the ability to produce a more extensive range of cytokines (J. A. Byrne, Butler, & Cooper, 1988; Merkenschlager, Terry, Edwards, & Beverley, 1988; Picker et al., 1995; Sanders, Makgoba, June, Young, & Shaw, 1989). In addition, memory T-cells also express several cell surface markers that differ from that of naïve cells, these markers can be used to define different memory populations of cells using flow cytometry (Akbar, Terry, Timms, Beverley, & Janossy, 1988; Sanders et al., 1988).



Phenotypic Characteristics

Classification	Naïve like		Early Differentiated	Early/late differentiated	Late Differentiated	Terminally differentiated
CD45RA	+	+	–	–	–	+
CD27	+	+	+	+/-	–	–

Functional Abilities

HLA-Dr	–	–	-/+	-/+	+	–
Ki67	–	–	-/+	-/+	-/+	–

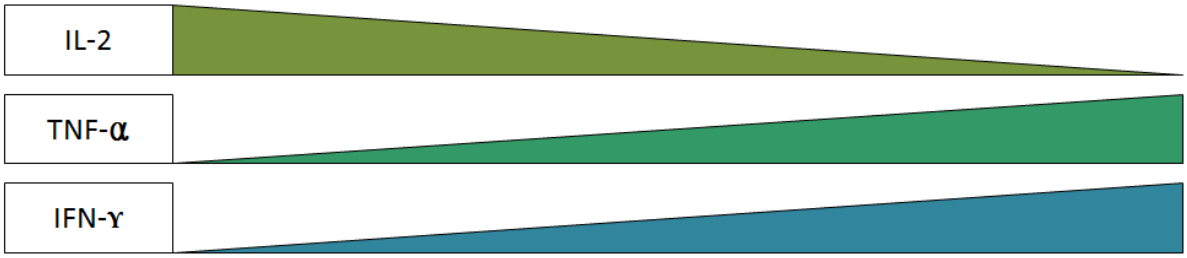


Figure 4.2.1: Heterogeneity of memory CD8+ T-cells. A) Linear progression of CD8 T-cell memory differentiation (through activation) from naïve to stem cell, central, transitional, effector and culminating in terminally differentiated memory. Antigen encounters by quiescent T-cells results in the formation of activated effector cells that assist in antigen clearance. After clearance a minor population of the activated effector cells survive forming memory cell. B) The combination of positively and negatively expressed CD45RA and CD27 markers used to identify four different subtypes of T-cell memory subsets. C) The changes in functional capacity associated with memory differentiation (Hamann et al., 1997; Mahnke et al., 2013).

Figure 4.2.1 demonstrates the different types of CD8+ memory T-cells that may be generated in response to antigen exposure, and shows selected functional differences between these populations. In this section, permutations of CD45RA and CD27 surface expression were used to differentiate between 4 stages of cell maturation (Figure 4.2.1): naïve like (naïve), early differentiated (ED), late differentiated (LD) and terminally differentiated (TD) memory cells. Naive like cells contain two subgroups of T-cells, the first group is the pure naïve group, which are cells that have not encountered antigen. The second subgroup of cells within the naïve-like group is a rare population known as memory stem cells (Gattinoni et al., 2011). Memory stem cells have a naïve cell phenotype but are functionally similar to memory cells. Early differentiated CD8 memory subgroup contains the central

memory cells, which are cells that secret large volumes of cytokines, especially IFNγ and IL-2 but lack immediate activity and can proliferate rapidly in response to antigen (Hamann et al., 1997). The ED group may also contain transitional memory cells. Transitional memory cells have characteristics of both central memory and effector memory cells. The effector memory cells, together with some transitional memory cells are classified as late differentiated memory according the CD45RA and CD27 classification used in this chapter. Effector memory cells produce a lot of IFNγ and TNFα but little IL-2 (Hamann et al., 1997). In addition, these cells are capable of inducing immediate cytotoxic effects on infected cells ex vivo. Over and above cytokine production, early and late differentiated memory cells also express high levels of the activation marker HLA-DR and have increased proliferative abilities compared to other naïve and terminally differentiated memory cells (Mahnke et al., 2013). While T-cells can be classified into different subgroups depending on surface markers and functional abilities, it is important to note that memory subsets are not mutually exclusive but rather, they exist in a continuum with overlapping characteristics in many cases (Mahnke et al., 2013). In addition, CD4+ T-cell memory differentiation is slightly different from CD8 T-cell memory, with CD4+ T-cells lacking subpopulations such as transitional memory cells.

TOTAL CD4+ T-CELLS MEMORY DIFFERENTIATION OVER TIME

As different subsets of T-cells have different effector functions, classification of vaccine specific T-cells may provide better insight on functional immune response generated against vaccines. Thus, changes in CD4+ and CD8+ naïve-like (naïve), early-differentiated (ED), late-differentiated (LD) and terminally differentiated (TD) memory T-cells over time was assessed. This analysis was conducted on total CD4+ and CD8+ T-cells after in vitro stimulation with vaccine antigens. Initial analyses included total cell populations to determine if infant T-cell phenotype distribution was similar to that of adults. The analysis was conducted independently for each infant group, that is U BF, E BF and E FF infant memory ontogeny was analysed separately. Figure 4.2.2. shows the gating strategy applied to CD4+ and CD8+ T-cell populations to classify memory subtypes. Also displayed are representative flow plots of CD4+ memory T-cell subsets found in unstimulated and BP stimulated blood of a 36-week-old infant.

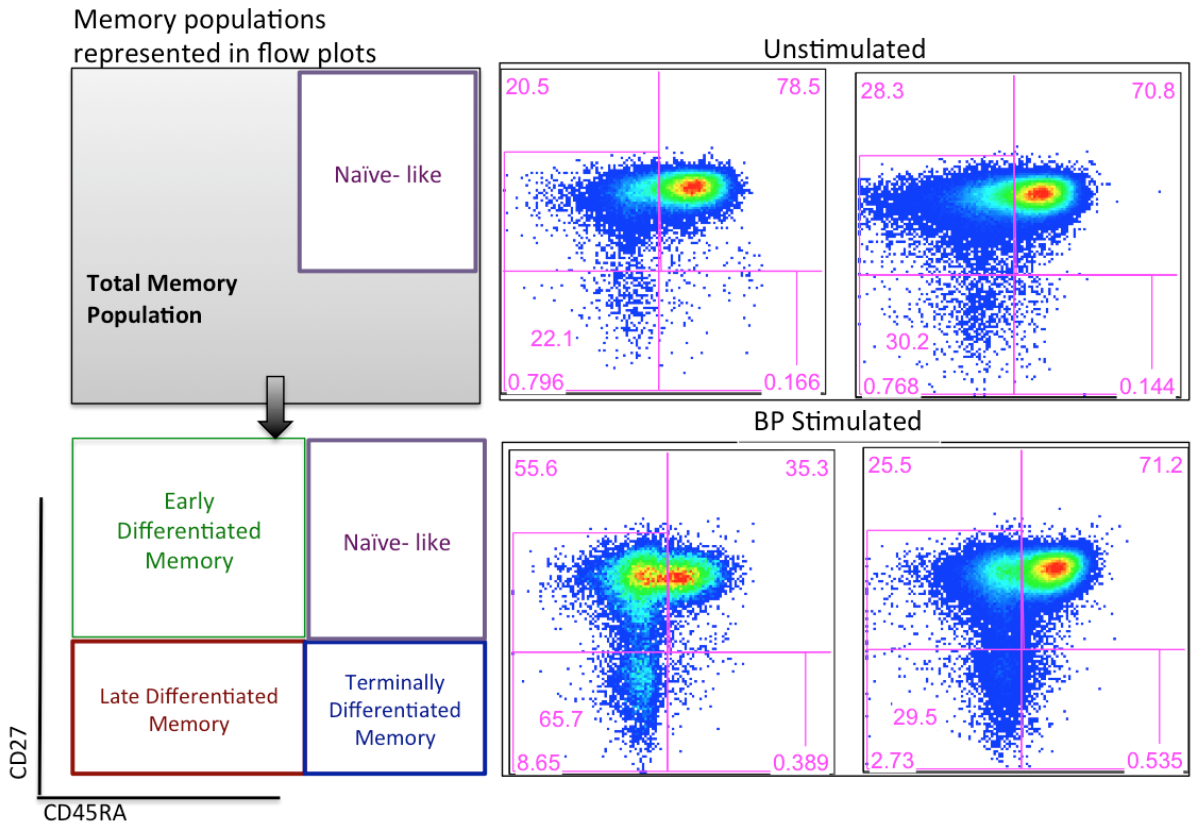


Figure 4.2.2: Representative flow plots of CD4+ memory T-cell differentiation in unstimulated and BP stimulated blood at week 36. A) Demonstrates the naïve and total memory populations. B) Represents flow plots from unstimulated blood. C) Shows the subsets of memory T-cells which are gated on in flow plots. D) Exhibits flow plots of BP stimulated blood.

Figure 4.2.3 below shows the changing memory ontogeny of CD4+ memory cells in U BF infants. In unstimulated blood, there was a decrease in naïve-like cell frequencies from day 4-7(D4-7) of life to week 7 and a corresponding increase in ED and LD memory cells from D4-7 to week 7. ED memory cells remain elevated while LD memory cells seem to decrease in frequency by week 15. This is likely due to the vast number of vaccines infants receive at week 14, which causes naïve cells to form ED memory cells. By week 36 the cells have had sufficient time to differentiate further to form LD and TD memory cells, as shown by the peak in these populations at week 36. In addition to unstimulated memory T-cells the figure also exhibits BP antigen stimulated CD4 memory T-cell ontogeny across time. Similarly to unstimulated blood, naïve-like BP stimulated cells were significantly lower at week 7 compared to D4-7. Unlike unstimulated blood however, BP stimulated ED CD4 cells did not change with time and LD and TD memory populations peaked at week 15. The peak in LD and TD memory populations at week 15 suggests that these are aP

vaccine specific CD4+ cells, thus their frequencies increase with BP antigen stimulation. However, to be certain that these are vaccine specific memory cells, cytokine expression would need to be assessed.

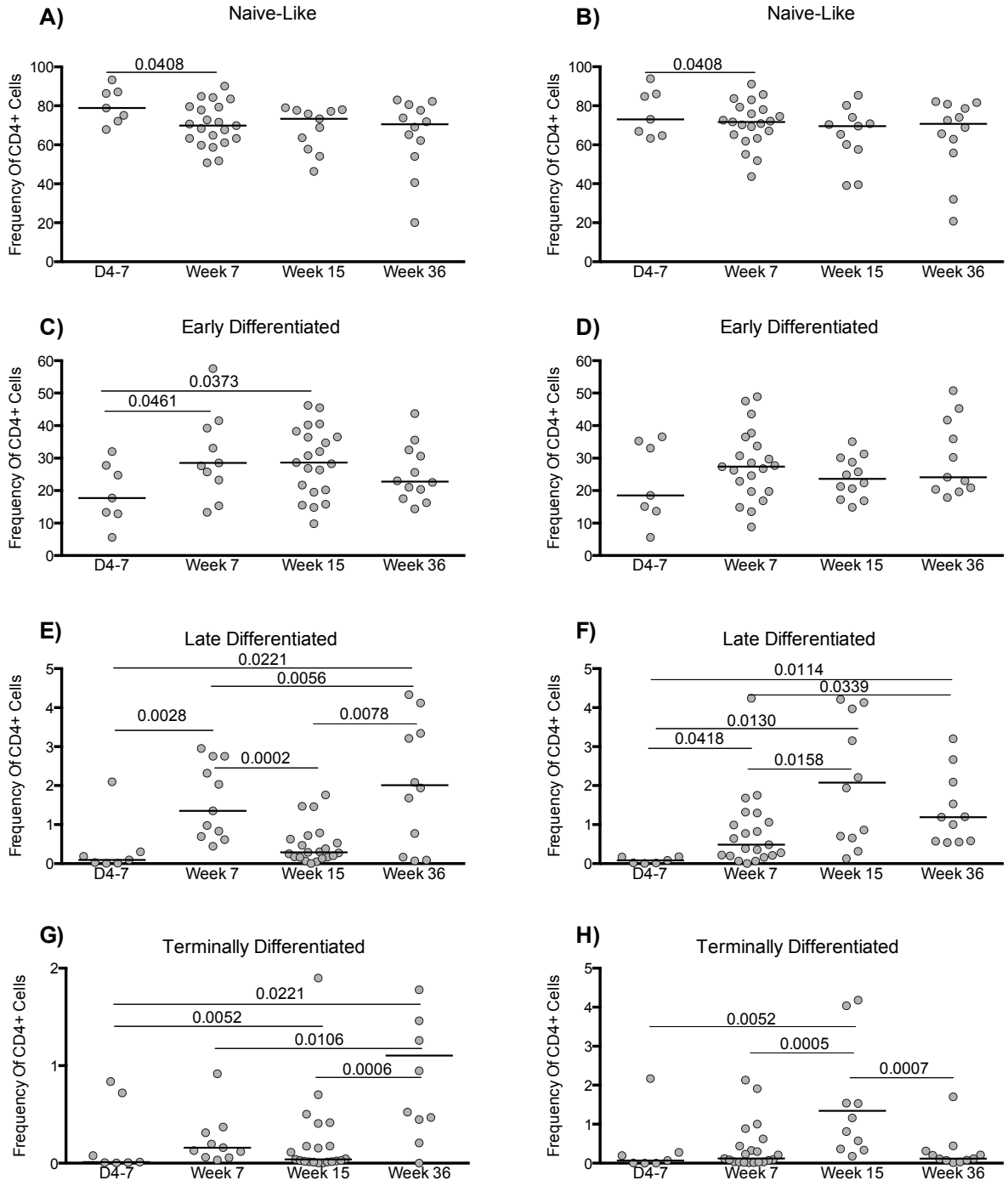


Figure 4.2.3: Changes in total CD4+ Naïve, Early Differentiated, Late Differentiated and Terminally Differentiated Memory T-Cells in Unstimulated and BP stimulated Blood at D4-7, Week 7, Week 15 and Week 36 In Unexposed Breast Fed Infants. Panels A, C, E and G represent unstimulated CD4 T-cells while panels B, D, F and H represent BP stimulated CD4 T-cells. Horizontal bars depict medians. P values were generated using the Kruskal-Wallis test and corrected for multiple comparisons with the Dunn's test.

The same analysis was conducted in E BF infants (Fig 4.2.4) and E FF infants (Fig 4.2.3). In both groups of infants, stimulation with BP antigen seemed to have minimal effects on overall memory populations, suggesting that the detected BP stimulated responses in these groups reflect the overall immune status and not vaccine specific memory responses.

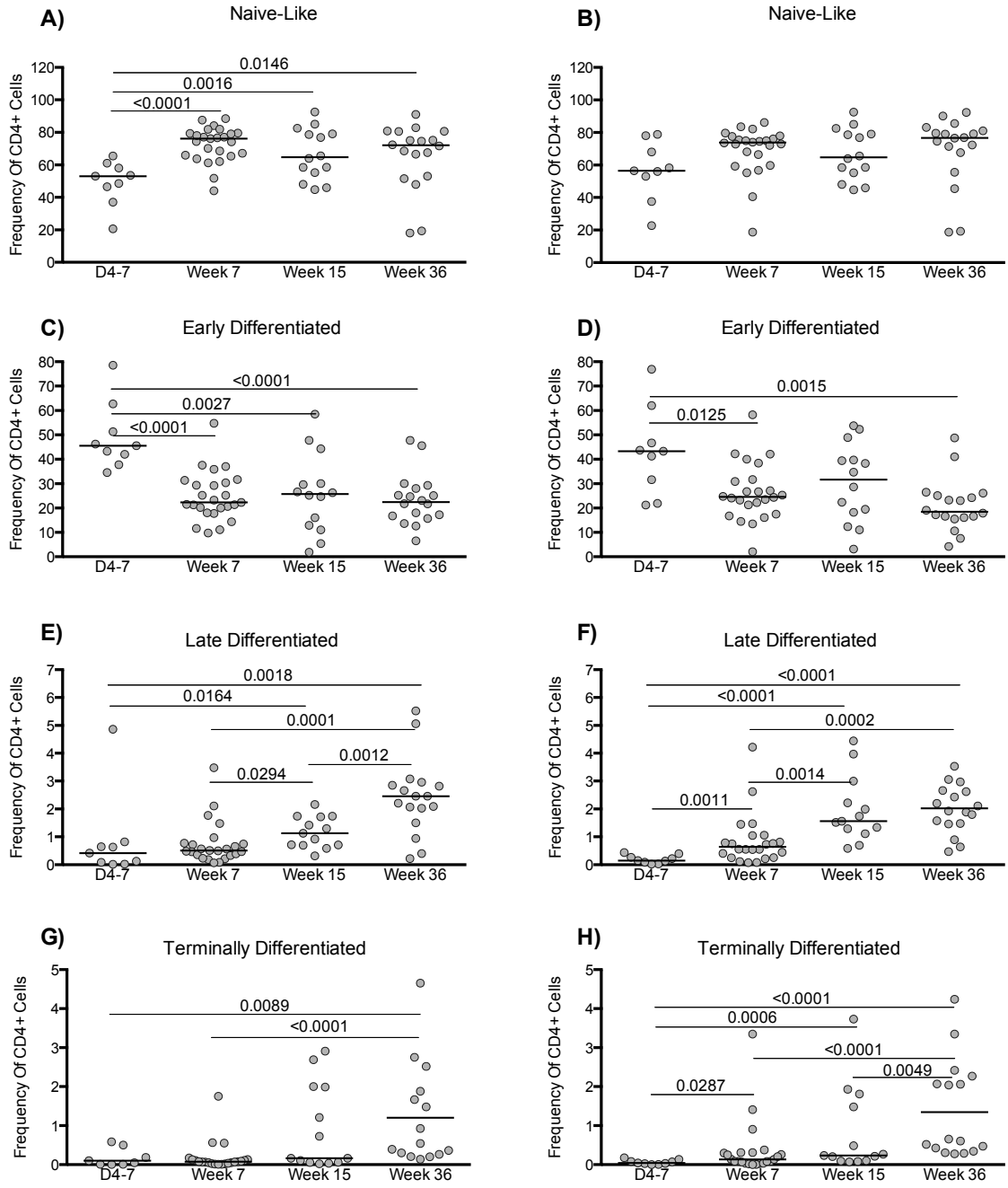


Figure 4.2.4: Changes in CD4+ Naïve, Early Differentiated, Late Differentiated and Terminally Differentiated Memory T-Cells in Unstimulated and BP stimulated Blood at D4-7, Week 7, Week 15 and Week 36 In HIV Exposed Breast Fed Infants. Panels A, C, E and G represent unstimulated responses, while panels B, D, F and H represent BP stimulated responses. Horizontal bars depict medians. P-values were generated using the Krukal-Wallis test and corrected for multiple comparisons with the Dunn's test.

E BF infants had an overall increase in unstimulated and BP stimulated LD and TD memory cell frequencies from D4-7 to week 36 (Fig 4.2.4). This is expected as memory differentiation increases with exposure to antigens and the older infants get the more antigens they are exposed to. However, a finding that is not typically seen in healthy infants, is an elevated frequency of ED memory cells as early as D4-7 of life. Also, in contrast to the expected decrease in naïve cells with age, E BF infants seem to have the lowest frequency of naïve like cells at D4-7 with increased frequencies at later time points. These results indicate that exposure to HIV causes differentiation of memory cells.

In the E FF infant group (Fig 4.2.5), naïve-like cell frequencies seem to be at the highest at week 36, while ED memory cell frequencies are lowest at week 36. It maybe possible that exposure to antigens within formula causes infants to generate memory responses to these antigens, hence they have lower levels of naïve-like cells at early time points. However, with frequent and continous exposure to formula milk, these infants eventually develop a tolerance to the antigens in formula. Whether this is related to the observed increase in the frequency of naïve-like cells observed at later time points remains to be explored. Late differentiated memory cells peak at week 15, likely due to the multitude of vaccines received at week 14. TD memory cells peak at week 36 but are also elevated at D4-7. As with E BF infants, the elevation seen at D4-7 is likely due to exposure to HIV. However in formula fed infants it seems that the D4-7 memory cells are differentiated much further than in E BF infants.

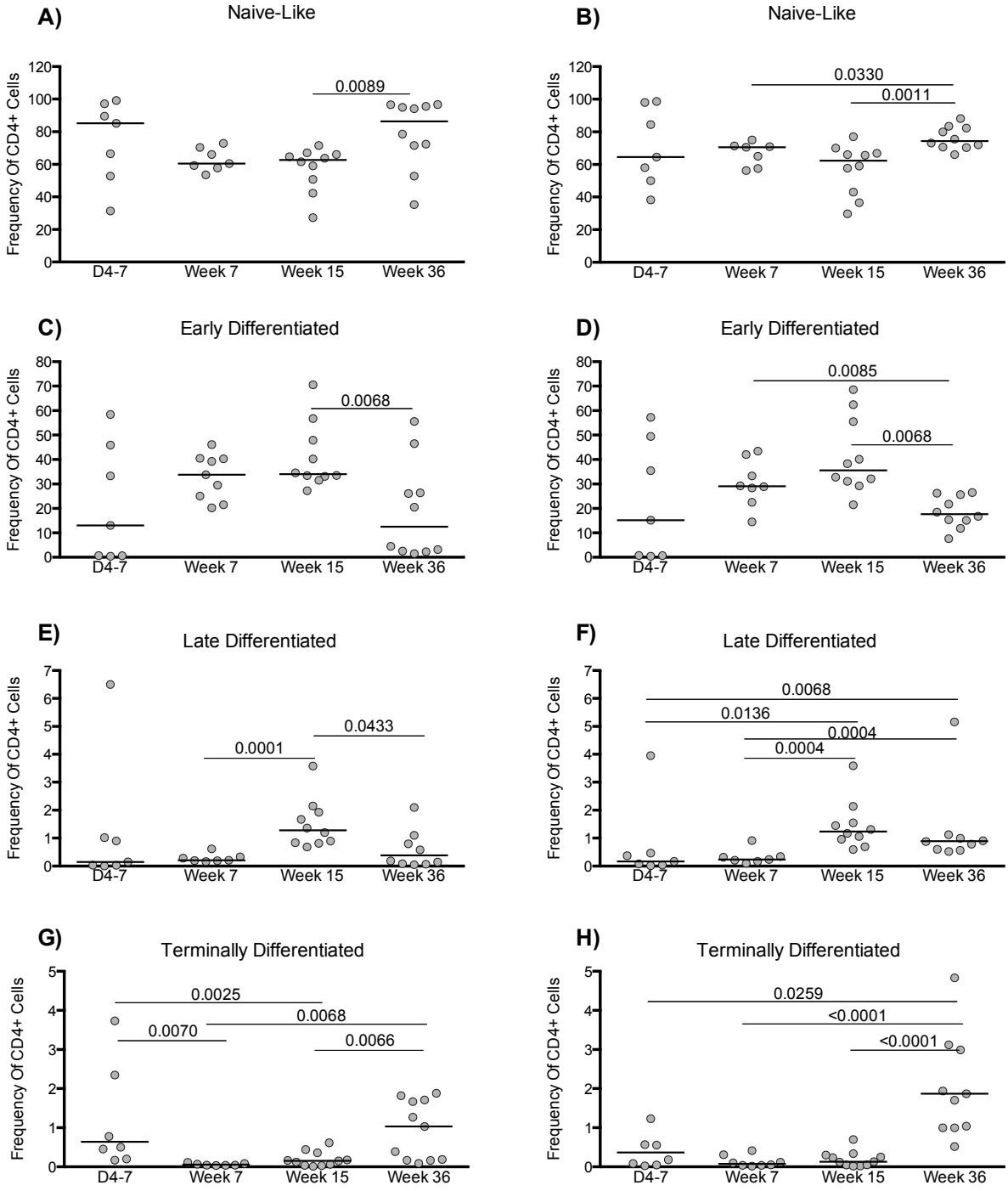


Figure 4.2.5: Changes in CD4+ Naïve, Early Differentiated, Late Differentiated and Terminally Differentiated Memory T-Cells in Unstimulated and BP stimulated Blood at D4-7, Week 7, Week 15 and Week 36 In HIV Exposed Formula Fed Infants. Panels A, C, E and G represent unstimulated responses, while panels B, D, F and H represent BP stimulated responses. Horizontal bars depict medians. P values were generated using the Kruskal-Wallis test and corrected for multiple comparisons with the Dunn's test

TOTAL CD8+ MEMORY DIFFERENTIATION OVER TIME

CD8 T-cell memory differentiation through time in the three infant groups were analysed next. U BF infants showed an overall decrease in naïve CD8+ T-cell frequencies from week 7 to week 36 (figure 4.2.6). ED memory populations remain stable at all time points and LD and TD memory cells increase in frequencies from D4-7 to week 36.

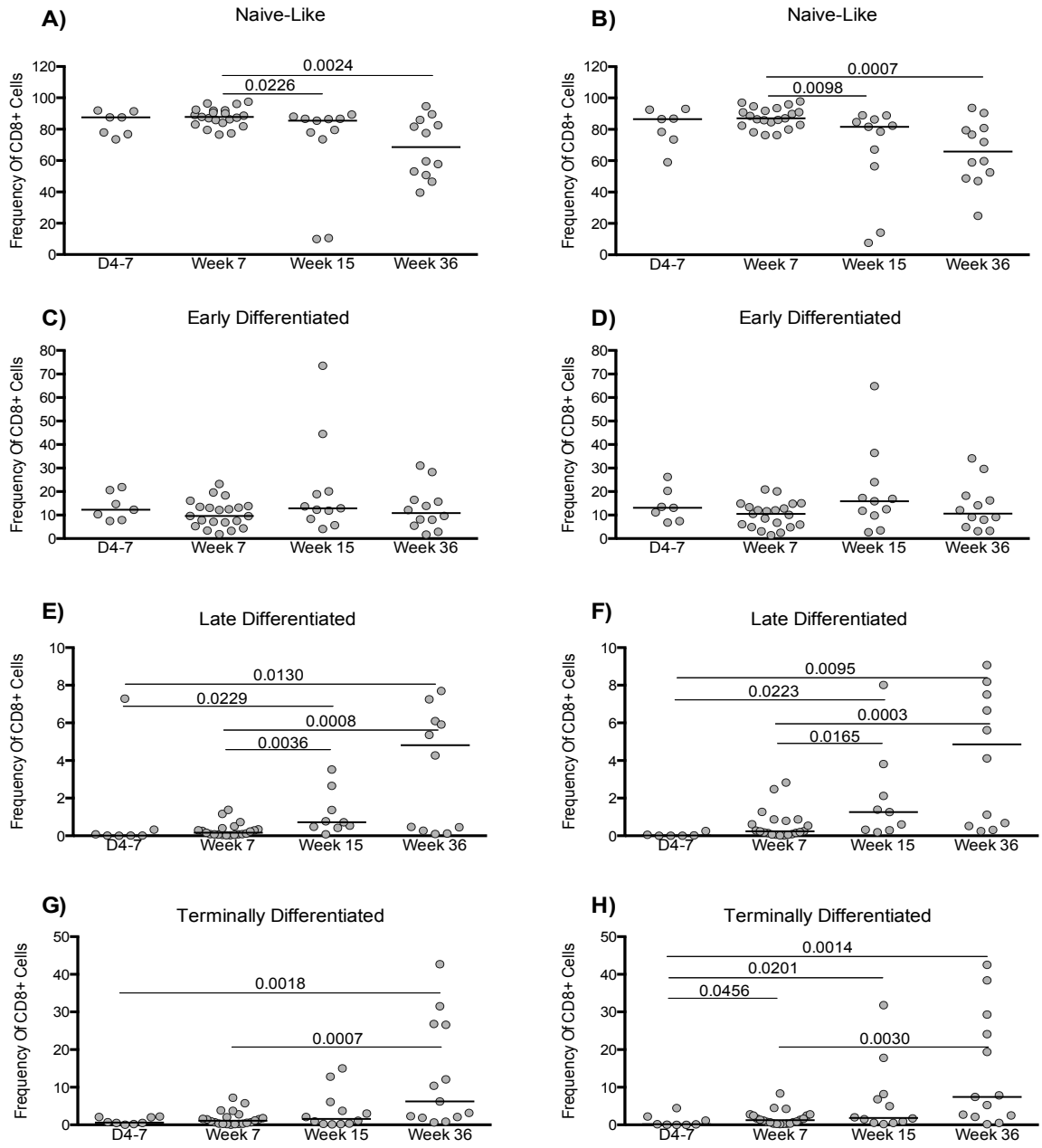


Figure 4.2.6: Changes in CD8+ Naïve, Early Differentiated, Late Differentiated and Terminally Differentiated Memory Cells in Unstimulated and BP stimulated Blood at D4-7, Week 7, Week 15 and Week 36 In Unexposed Breast Fed Infants. Panels A, C, E and G represent unstimulated responses, while panels B, D, F and H represent BP stimulated responses. Horizontal bars depict medians. P values were generated using the Krukal-Wallis test and corrected for multiple comparisons with the Dunn's test.

E BF infants show the same trend of decreasing naïve cell frequencies and increasing LD and TD memory frequencies from week 7 to week 36 (Figure 4.2.7). As with the CD4 memory cells, CD8 memory T-cells were highly differentiated at D4-7 and normalized by week 7, likely due to HIV exposure.

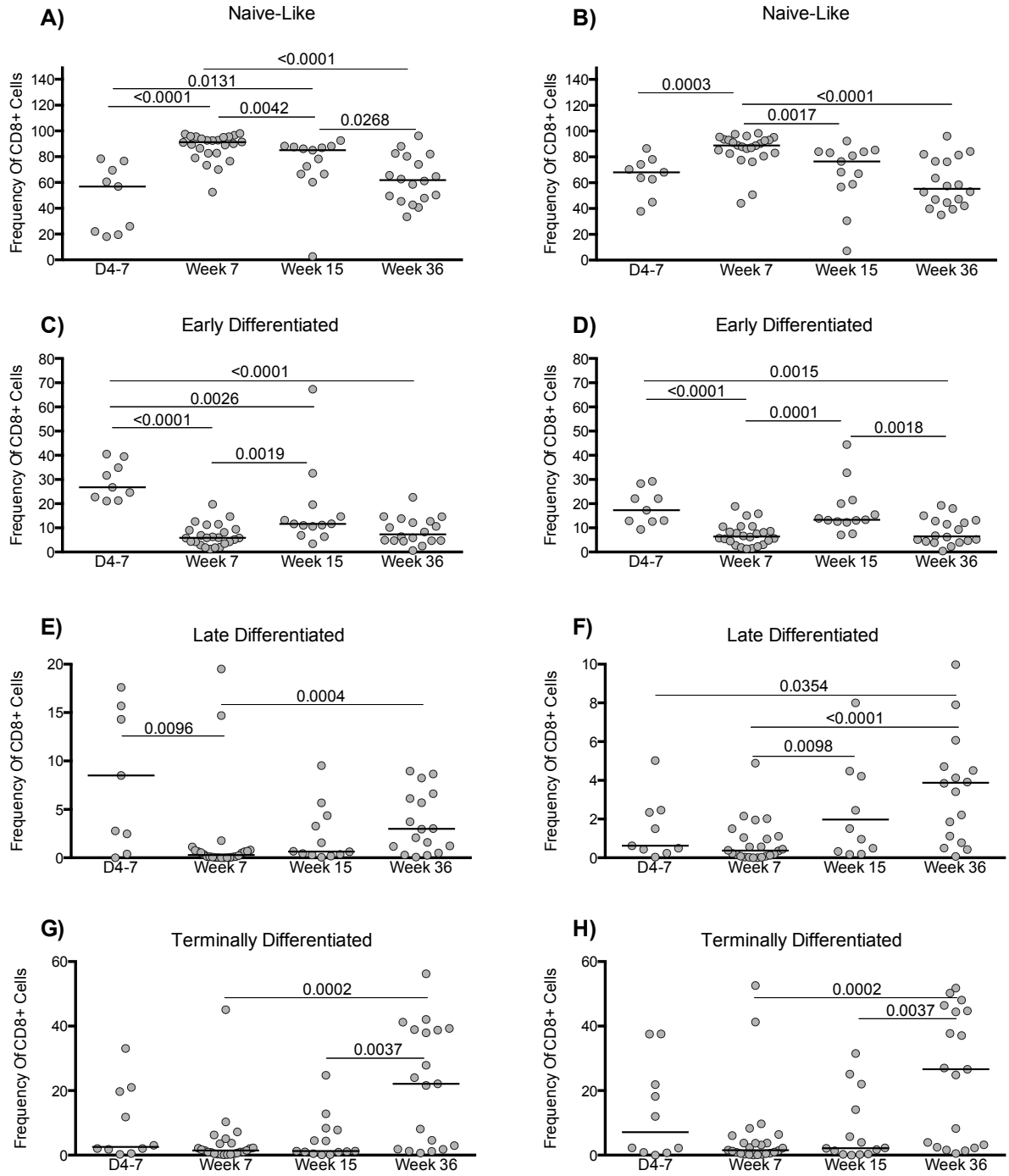


Figure 4.2.7: Changes in CD8+ Naïve, Early Differentiated, Late Differentiated and Terminally Differentiated Memory Cells in Unstimulated and BP stimulated Blood at D4-7, Week 7, Week 15 and Week 36 In HIV Exposed Breast Fed Infants. Panels A, C, E and G represent unstimulated responses, while panels B, D, F and H represent BP stimulated responses. Horizontal bars depict medians. P values were generated using the Krukal-Wallis test and corrected for multiple comparisons with the Dunn's test.

E FF infants had unchanging frequencies of unstimulated CD8 naïve-like, ED and LD memory populations. TD memory cells were elevated at week 36 and D4-7. In BP stimulated blood, naïve cell frequencies were lower at week 15 compared to D4-7. LD and TD memory cells were lowest at week 15.

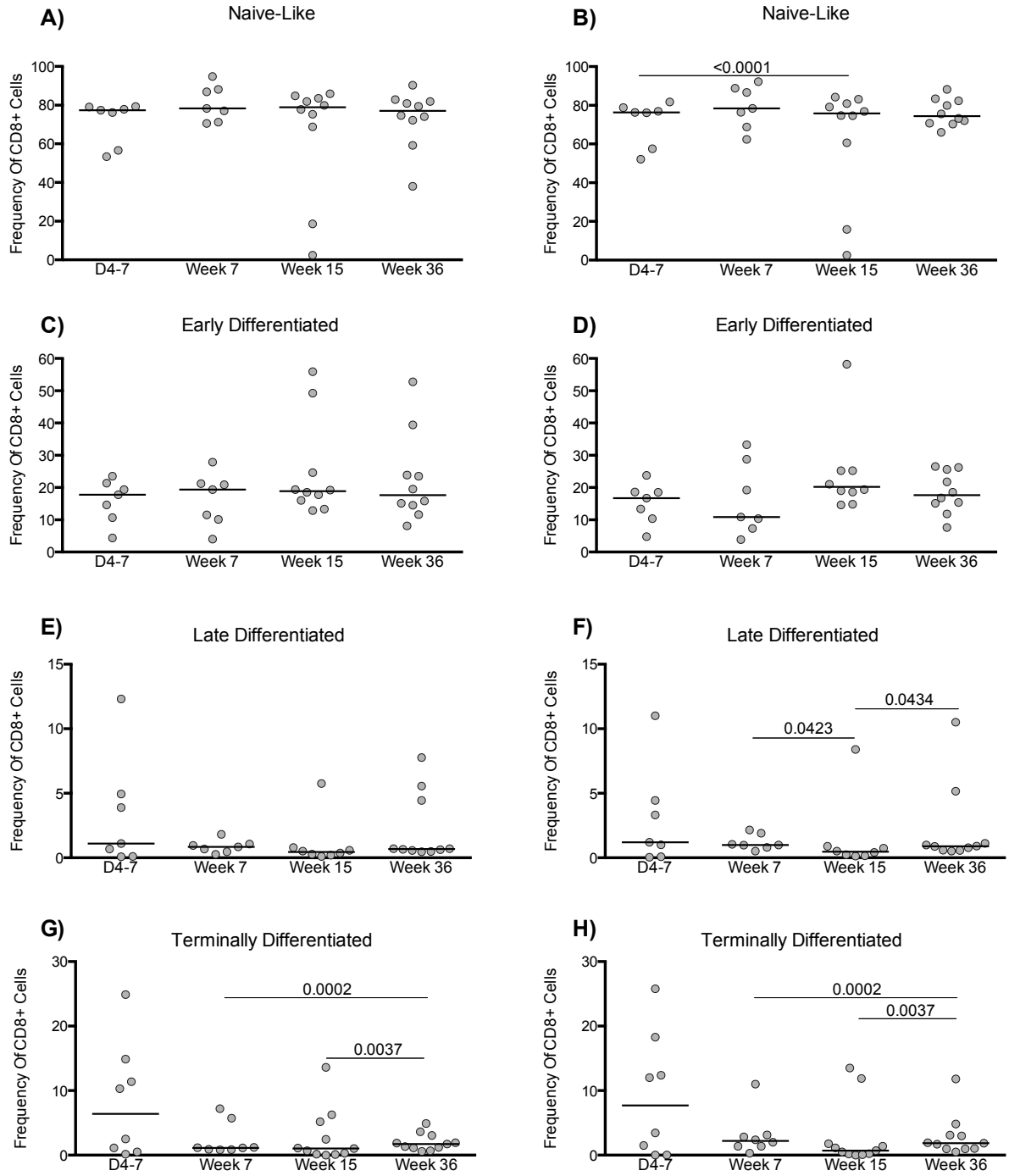


Figure 4.2.8: Changes in CD8+ Naïve, Early Differentiated, Late Differentiated and Terminally Differentiated Memory T-Cells in Unstimulated and BP stimulated Blood at D4-7, Week 7, Week 15 and Week 36 In HIV Exposed Formula Fed Infants. Horizontal bars depict medians. P values were generated using the Kruskal-Wallis test and corrected for multiple comparisons with the Dunn's test

BP STIMULATED ACTIVATION AND PROLIFERATION

After assessment of CD4 and CD8 memory differentiation other functional abilities of infant T-cells were assessed. As previously discussed, development of long-lived immunologic memory is the main purpose of vaccination. In general, a vaccine should cause naïve T-cells to differentiate into memory T-cells capable of eliminating pathogens upon recall in a rapid manner. Preceding the formation of memory cells, T-cells undergo proliferation, necessary for the development of effector cells. Therefore CD4 and CD8 T-cell proliferative responses were assessed to determine if infants have functional proliferative cells that are necessary for good vaccine responses. The marker Ki67 was used to assess proliferation. Ki67 is a nuclear antigen that is expressed by all proliferating cells in the G_1 , S and G_2 phase of the cell cycle (Scholzen & Gerdes, 2000).

HLA-DR is an activation marker found on activated T-cells 48-60 hours after antigenic challenge (Rea, McNerlan, & Alexander, 1999). An upregulation of HLA-DR has been associated with increased functional abilities such as higher expressions of TNFα and IL-2 receptor. Therefore in this study, expression of HLA-DR was used as a measure of potential immune function.

The flow plots shown in figure 4.2.9, demonstrate that CD4+ T-cell activation and proliferation responses to BP stimulation are mutually exclusive when examined at different time points after aP vaccination. This gating strategy was applied to the full cohort to determine CD4+ and CD8+ T-cell activation and proliferation statuses.

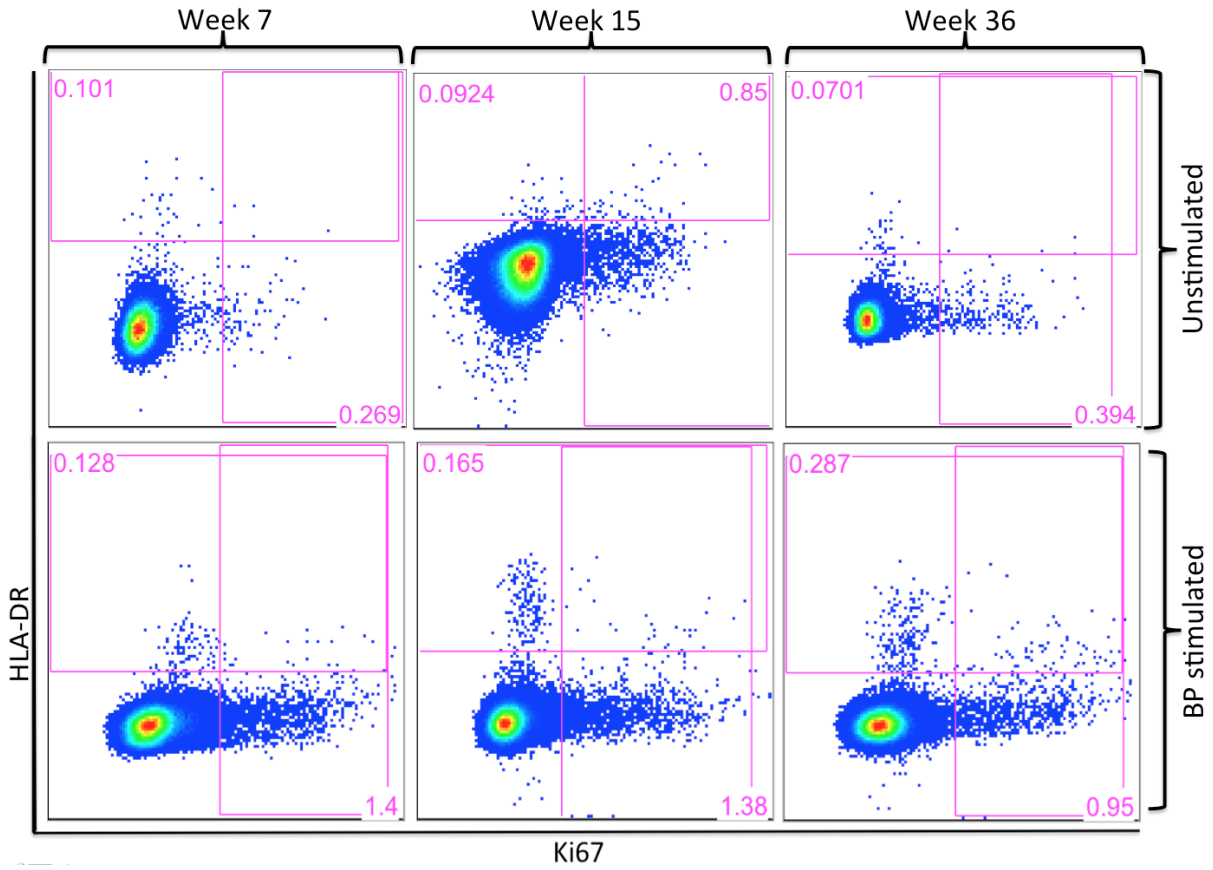


Figure 4.2.9: CD4+ T-cell Activation and Proliferation In Unstimulated and BP Stimulated Blood Of HIV Exposed Breast Fed Infants. Columns 1,2 and 3 represent week 7, 15 and 36 CD4+ HLA-dr and Ki67 responses from 3 different infants after a 12-hour whole blood assay stimulation at 37ºC. All unstimulated responses are in row one and BP stimulated responses are in row 2. HLA-DR expression is on the Y-axis and Ki67 expression is on the X-axis.

T-CELL ACTIVATION

As seen in figure 4.2.10, no significant differences were found between unstimulated and BP stimulated CD4+ T-cell activation. Peak BP stimulated activation was found at week 15 for breast fed infants regardless of HIV exposure as. In U BF infants unstimulated blood also had a significant increase in HLA-DR expression from week 7 to week 15, with a p-value of 0.0212 and a significant decrease in HLA-DR expression from week 15 to week 36, with a p-value of 0.0034. Additionally, within the E BF group unstimulated blood significantly increased from week 7 to week 15 (p-value = 0.0134). This suggests that BP stimulation had minimal effects on CD4+ T-cell activation and may instead represent in vivo activation. In addition, both HEU groups of infants had high HLA-DR expression in unstimulated blood at D4-7. This suggests that exposure to HIV, upregulates HLA-DR expression early in life, but this upregulation is not maintained, since at week 7 the HLA-DR expression is similar to that found in unexposed infants. It is also interesting to note that after BP

stimulation, HLA-DR expression decreased suggesting that the stimulation process down-regulated HLA-DR expression at D4-7.

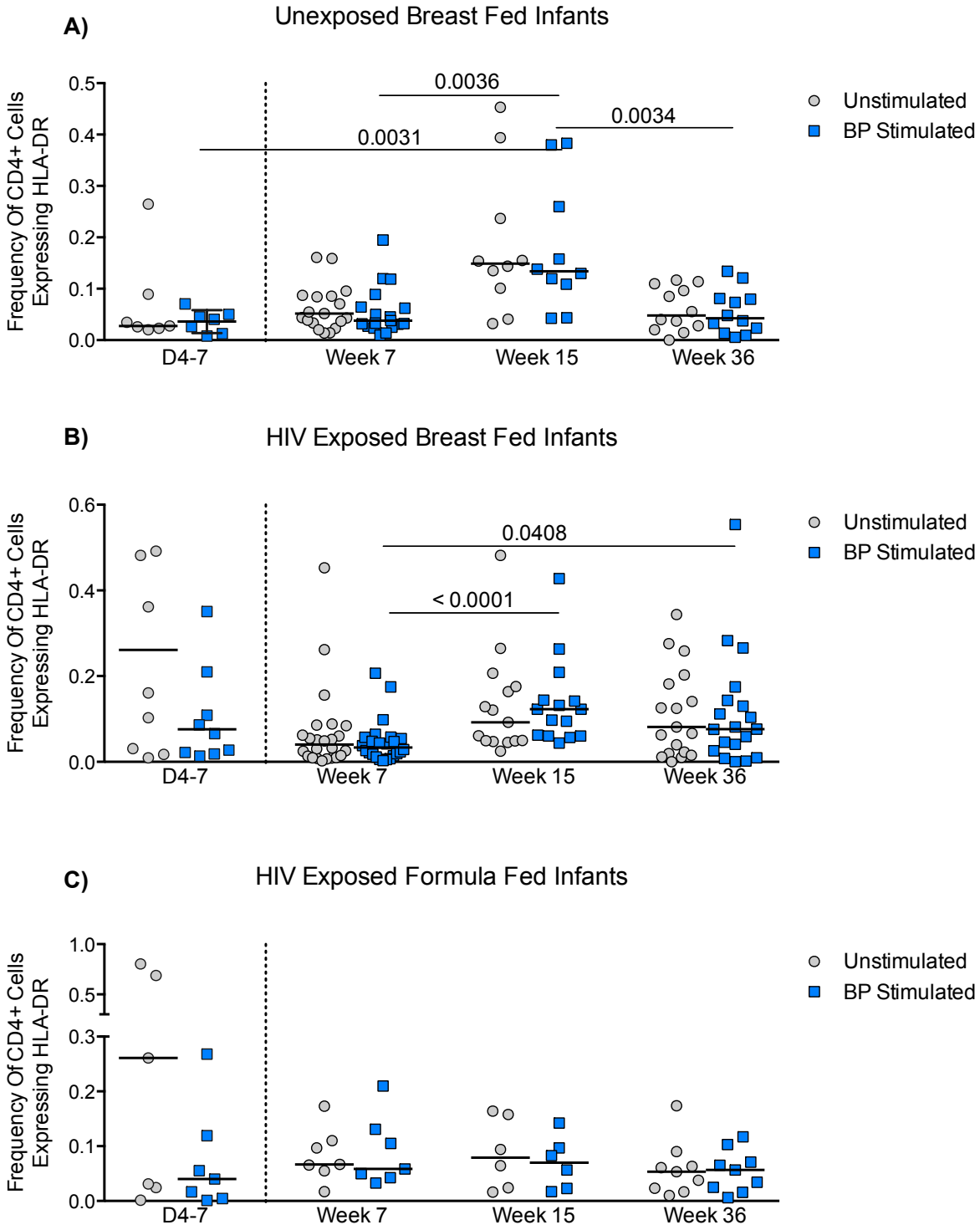


Figure 4.2.10: Changes in CD4+ T-cell Activation Over TIme. Horizontal bars depict medians. Comparison of changes over time was done with a Mann-Whitney U test, only BP changes were plotted on graph. Comparisons between unstimualted and BP stimulated blood were done with a Wilcoxon signed rank test.

As with CD4+ T-cells, the unstimulated blood demonstrated similar changes in activation compared to BP stimulated blood (Fig 4.2.11). The only difference found between unstimulated and BP stimulated blood was that E FF infants had a higher level of HLA-DR expression in BP stimulated blood at week 7. Furthermore the week 7 BP stimulated CD8+ activation is also significantly higher than BP stimulated activation at D4-7, suggesting that formula fed infants are able to develop aP specific CD8 responses earlier, at week 7 of life. Peak CD8+ T-cell activation in breast fed infants was evident at week 15. Similarly to CD4+ T-cells, E BF infants had high HLA-DR expression in unstimulated blood at D4-7, which decreased after stimulation.

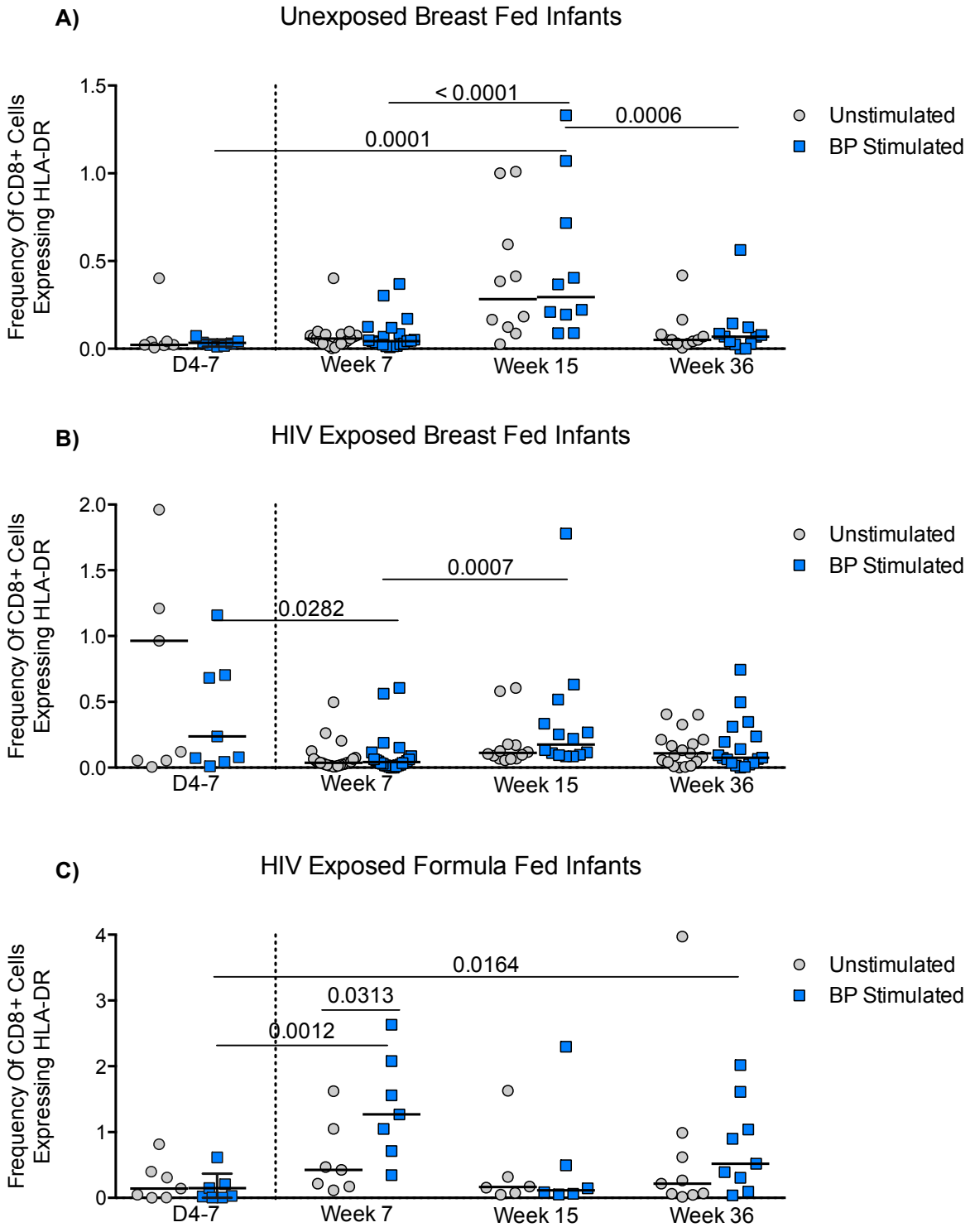


Figure 4.2.11: Changes in CD8+ T-cell Activation Over TIme. Horizontal bars depict medians. Comparison of changes over time was done with a Mann-Whitney U test, only BP changes were plotted on graph. Comparisons between unstimualted and BP stimulated blood were done with a Wilcoxon signed rank test.

T-CELL PROLIFERATION

The ability of T cells to proliferate over time after aP vaccination and in response to BP stimulation was assessed using Ki67 (Fig 4.2.12). No significant differences were found between unstimulated and BP stimulated CD4 cells, suggesting that BP stimulation has minimal effects on the proliferative ability of CD4+ T-cells. Additionally, infants express the highest level of Ki67 at D4-7. However, this proliferation is not maintained and by week 7, both U BF and E FF infants have significantly decreased Ki67 expression. Similar decreases were seen in E BF infants but not at a significant level. This level of Ki67 expression was maintained until week 36 in U BF and E BF infants. In E FF infants proliferation was not maintained as long as it is in breast fed infants, as shown by a significant decrease at week 36.

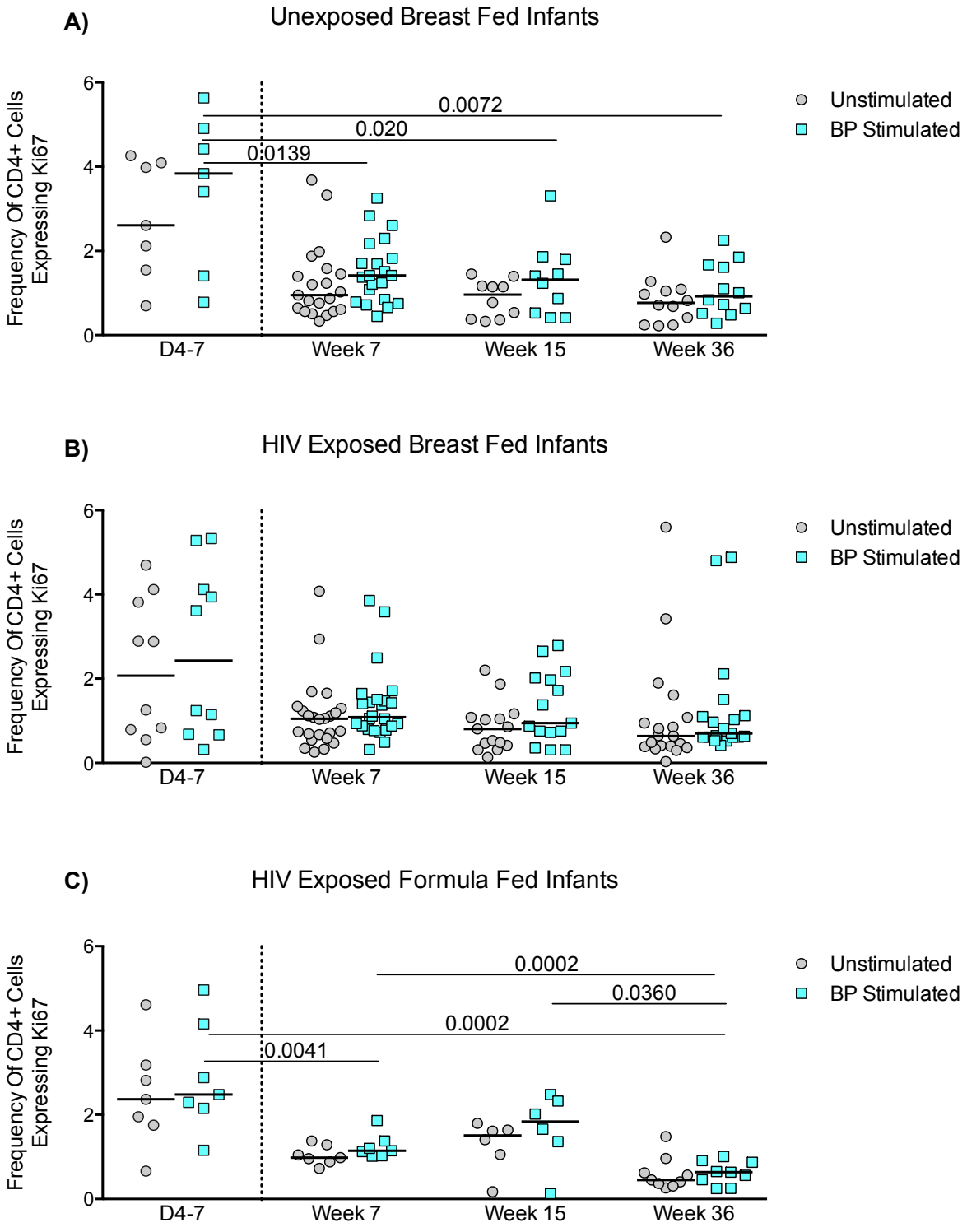


Figure 4.2.12: Changes in CD4+ T-cell Proliferation Over TIme. Horizontal bars depict medians. Comparison of changes over time was done with a Mann-Whitney U test, only BP changes were plotted on graph. Comparisons between unstimualted and BP stimulated blood were done with a Wilcoxon signed rank test.

The CD8+ Ki67 expression was very similar to CD4 Ki67 expression, with D4-7 being the highest (Fig 4.2.13). From week 7 to week 36, Ki67 expression was maintained at a constant rate in all groups. Both CD4 and CD8 Ki67 data suggests that infants have rapid T-cell proliferation within the first week of life.

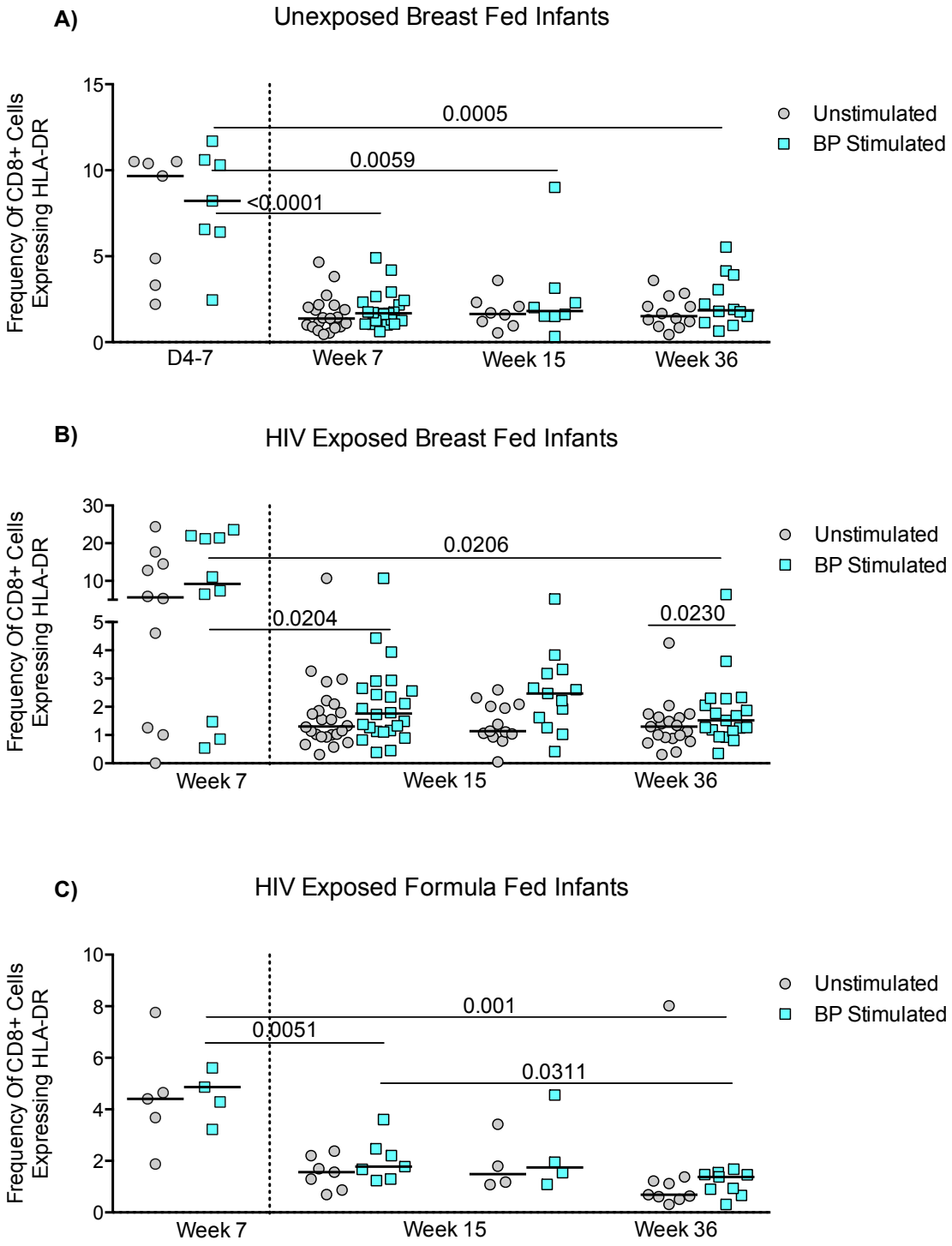


Figure 4.2.13: Changes in CD4+ T-cell Proliferation Over TIme. Horizontal bars depict medians. Comparison of changes over time was done with a Mann-Whitney U test, only BP changes were plotted on graph. Comparisons between unstimualted and BP stimulated blood were done with a Wilcoxon signed rank test.

CONCLUSION

Taken together, the data suggests that CD4+ and CD8+ T-cell responses were largely unaffected by stimulation, suggesting that the responses detected in BP stimulated blood reflect the overall immune status and does not depict responses specific to in vitro BP stimulation. These data provide valuable information on the development of the adaptive immune system in the first few weeks of life. For example, the results indicate that infants are capable of forming memory cells in the first 9 months of life, where CD4 and CD8 T-cells show different trajectories. These trajectories appear to be dependent on feeding mode and highlights the importance of understanding how infants are nursed, which have important implications for all vaccines, including aP and a potential HIV vaccine. The data also indicates that memory differentiation is a function of age and that older infants have more differentiated memory cells while younger infants have a predominant naïve-like T-cell phenotype. Whilst this may seem intuitive, this is not true for HIV exposed infants, who have an elevated level of LD and TD memory cells as early as the first week of life.

In addtion HIV exposed infants also have elevated levels of activation at D4-7 after birth. D4-7 was also the time point at which infants had the highest level of Ki67 expression. This proliferation decreased by week 7 and was maintained at a similar level thereafter. This indicates that infants have rapid T-cell turn over within the first week of life. This is likely due to the fact that infant T-cells are fundamentally different to adult cells, in that majority of T-cells found in infants are recent thymic emigrants (RTE). RTE are highly susceptible to apoptosis, and the high levels of Ki67 observed in the infants may indicate a rapid T-cell turnover to compensate for this (Marchant & Goldman, 2005).

SECTION 4.3

BORDERTELLA PERTUSSIS SPECIFIC T-CELL RESPONSES

ACELLULAR PERTUSSIS SPECIFIC CD4+ T-CELL MEMORY DIFFERENTIATION

After establishing how infants generate total memory cell populations in the CD4 and CD8 compartments, cytokine-expressing CD4+ T-cells were analysed to determine memory maturation specific to aP vaccination. This analysis was conducted in cytokine positive CD4+ T-cells after in vitro stimulation with BP antigens. This analysis was only completed for CD4+ T cells and not for CD8 T-cells as there were to few cytokine positive cells to reliably analyse.

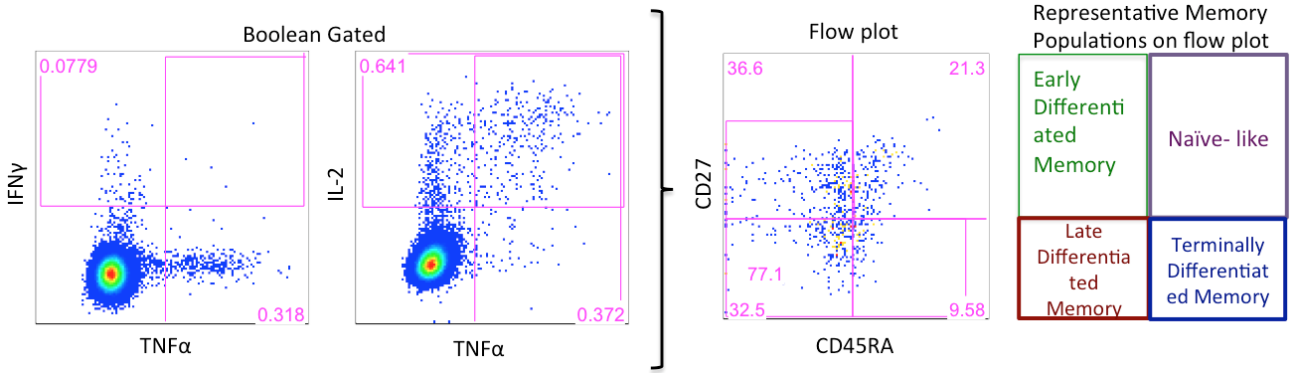


Figure 4.3.1: Gating of Bordetella Pertussis specific memory T-cells. A) Displays representative flow plots (CD4+) of the cytokine gating done within total CD4+ and CD8+ T-cell populations. Boolean gates of IFNγ+, IL-2+ and TNFα+ populations were created. B) CD45RA and CD27 was used to classify cytokine+ CD4+ and CD8+ T-cells into different memory subtypes as displayed.

Gating for the cytokine+ memory population was completed as demonstrated in figure 4.3.1, by Boolean gating on cytokine positive populations and then plotting these cytokine-positive cells using CD45RA and CD27, to define different subsets of memory cells. The analysis was completed independently for all three groups of infants.

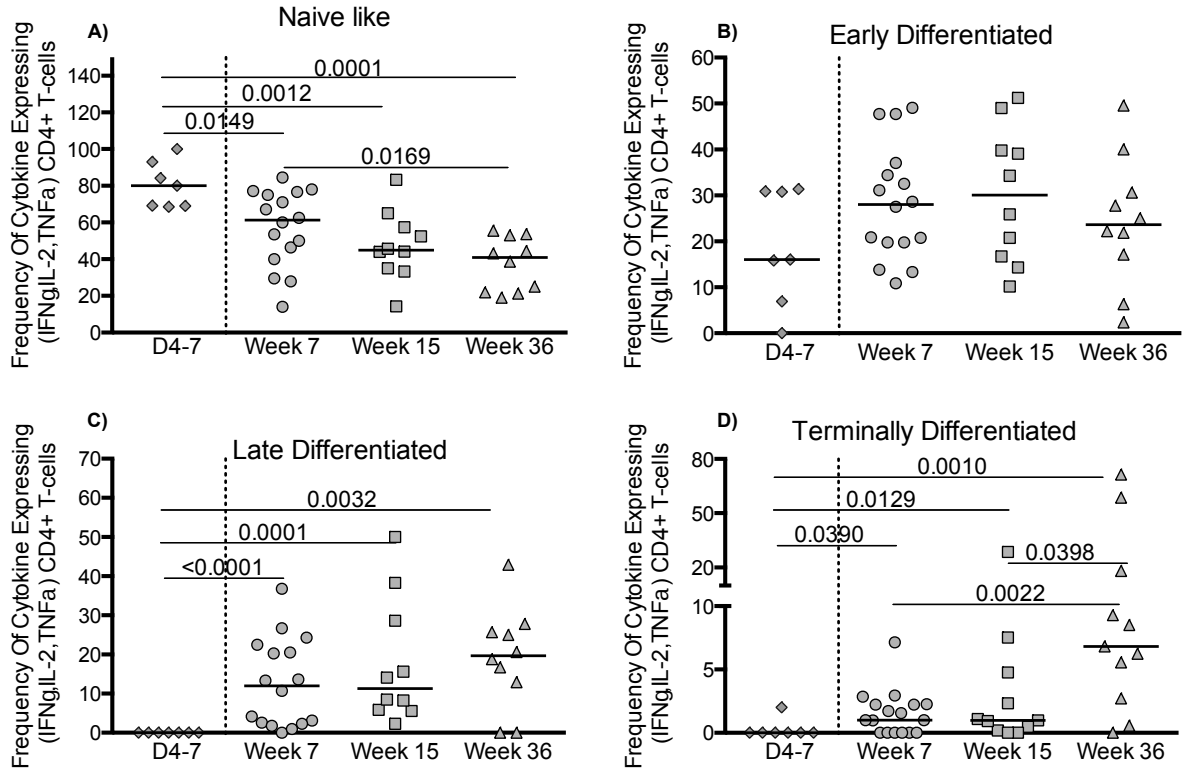


Figure 4.3.2: Changes in BP specific CD4+ Memory T-cell subtypes over time in unexposed breast fed infants. Horizontal bars depict medians. P- values were generated using a kruskal-wallis test.

As evident in figure 4.3.2, U BF infants had BP specific responses at D4-7, prior to vaccination. This is likely due to maternal factors or in utero exposure to Pertussis. Even rarer is that a substantial proportion of the cytokine positive cells at D4-7 were of a naïve-like phenotype. This is unexpected as naïve-like cells usually have poor cytokine expression. These naïve-like cell frequencies decreased with time. There was also a corresponding increase in TD memory cells from D4-7 to week 36. Additionally, there was a trend toward increase in the frequency of LD memory cells at week 36 but this was not significant. No changes were detected in the frequencies of ED memory cells over the 3 time points. As with the naïve-like cells, ED memory responses were detectable prior to vaccination.

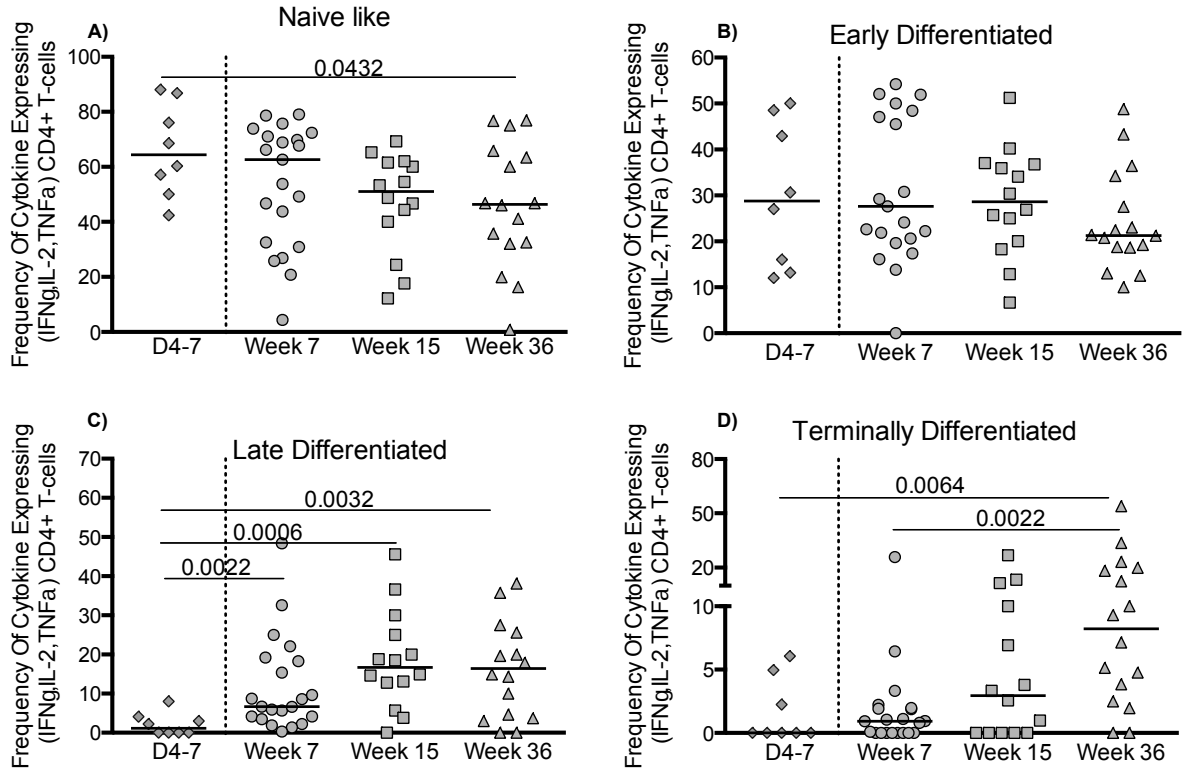


Figure 4.3.3: Changes in BP specific CD4+ Memory subtypes over time in HIV Exposed breast-fed infants. P- values were generated using a kruskal-wallis test. Horizontal bars depict medians.

E BF infants (Figure 4.3.3) had similar memory profiles to U BF infants, with increasing LD memory and decreasing naïve like memory with time, and a constant level of ED memory cells. E BF and E FF infants (Figure 4.3.4) also had BP specific responses prior to vaccination with high proportions of naïve like responding cells. Unlike U BF infants, where pre-vaccination responses were mainly of a naïve and ED memory phenotype, some HEU infants had LD and TD memory responses prior to vaccination. This was especially true for E FF infants. In addition E FF infants seem to have peak ED responses at week 15. Collectively, these data suggest that CD4+ T cell memory in HIV exposed and formula fed infants was fairly mature even after the first few days of life, suggestive that these neonates were most likely born with a mature antigen-experienced population of T cells.

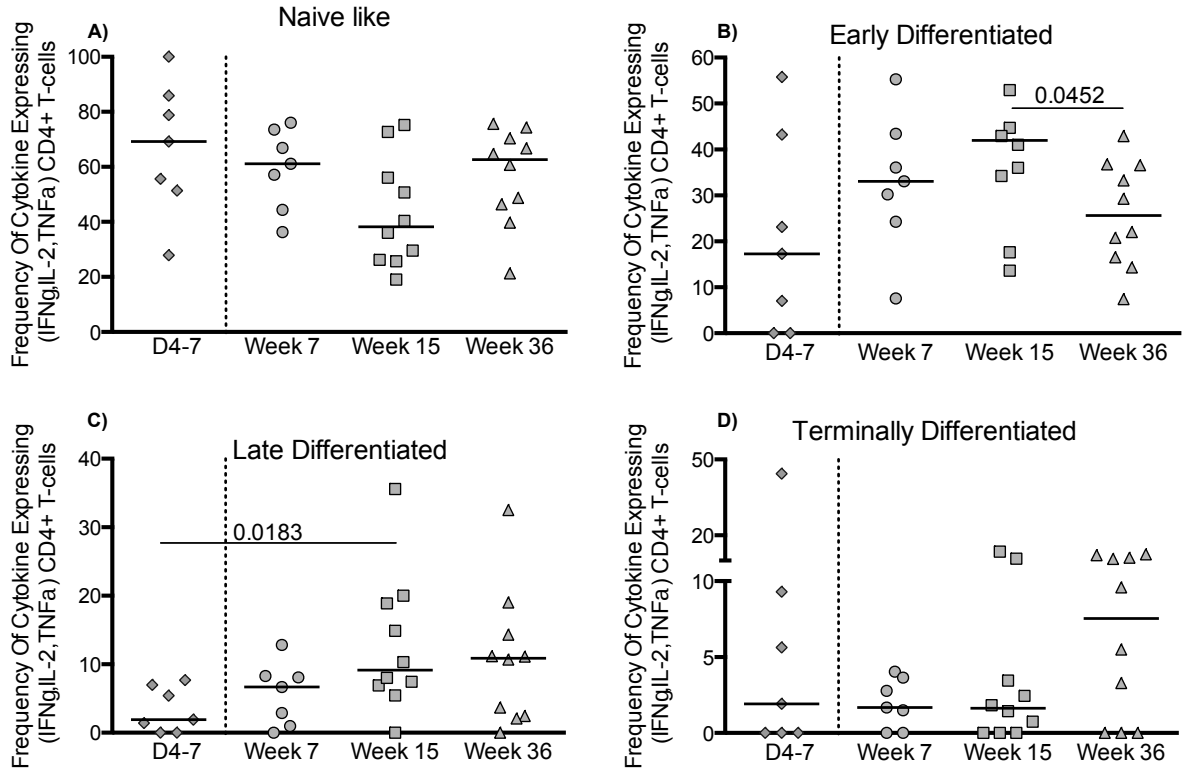


Figure 4.3.4: Changes in BP specific CD4+ Memory T-cell subtypes over time in HIV Exposed formula-fed infants. Horizontal bars depict medians. P- values were generated using a kruskal-wallis test.

BORDETELLA PERTUSSIS INDUCED CYTOKINE EXPRESSING CD4+ AND CD8+ T-CELL RESPONSES

After assessment of the memory kinetics induced by BP stimulation in aP vaccinated infants, cytokine responses were assessed. Once naïve T-cells are primed by antigen presenting dendritic cells, they will clonally expand and differentiate into various memory subsets. These memory subsets may also acquire distinct functional abilities, determined by the type of cytokine liberated. Cytokines are molecules that are essential for orchestrating a successful antigen-specific response, including those to vaccines. They shape the milieu of an immune response; control the expansion and phenotypic development of different immune cells, as well as tumorous or infected cells (Mahnke et al., 2013). Multiple cytokines can be expressed by either CD4+ and CD8 T-cells, which when co-expressed can identify the polyfunctional nature of these cells. In this section, focus was made on the ability of either CD4+ or CD8+ T cells to express IL-2, TNFα and IFNγ (Literature review, page 29).

IL-2 exerts its most prominent effects on T-cells (Gaffen & Liu, 2004) and is an early-secreted cytokine that enables expansion of antigen-specific T cells. IL-2 production is induced shortly after antigen triggering of the T-cell receptor (TCR); this is especially true for CD4+ T-cells (Mahnke et al., 2013). Upon antigen triggering of the TCR, T-cells rapidly synthesize IL-2 and express the IL-2 receptor, which allows for the selective and rapid expansion of effector T-cells (Lenardo et al., 1999). Not only does IL-2 promote T-cell proliferation in this manner, it also has effects on glycolysis and cellular metabolism that is necessary for the survival of T-cells (Rathmell, Vander Heiden, Harris, Frauwirth, & Thompson, 2000). In addition, IL-2 is required for the differentiation of CD4+ T-cells into different TH subsets and plays a role in regulation by activating T-regs (Gaffen & Liu, 2004).

Like IL-2, TNFα is also considered an early cytokine and is produced shortly after T-cell activation (Stefferl, Hopkins, Rothwell, & Luheshi, 1996). TNFα is a potent pyrogen that augments fluid drainage and activates the vascular endothelium, which results in increased cellular motility across the endothelia (Yang et al., 2005). This is important for the recruitment of immune cells to the site of inflammation/vaccination. Furthermore, as mentioned in the introduction of this chapter, IL-2 and TNFα play a direct role in protecting against dissemination of Pertussis disease by enhancing macrophage phagocytosis (Kilgore et al., 2016).

In contrast to IL-2 and TNFα, IFNγ is considered a late cytokine. Efficient IFNγ production requires T-cells to have undergone several rounds of proliferation and is thus mainly produced by late differentiated memory subtypes (Bird et al., 1998). IFNγ is a multifunctional cytokine and one of the most essential cytokines in orchestrating immune responses, specifically to viral infections. It activates macrophages by inducing amplified expression of MHC class I and II on T-cells (Fritsch et al., 2005). It stimulates the cytolytic activity of NK cells and is able to directly inhibit viral replication (Mahnke et al., 2013). In terms of Bordetella Pertussis, murine studies have suggested that IFNγ is essential for control of infection (Barbic et al., 1997; Mahon et al., 1997). Co-expression of the three measured cytokines is thus considered to delineate highly functional T cells in response to vaccination.

CHANGES IN ANY CYTOKINE EXPRESSION OVER TIME

The first analysis in this section was to determine the kinetics of multiple cytokine expression by CD4+ and CD8+ T-cells in response to in vitro BP antigen stimulation in aP vaccinated infants. IL-2, TNFα and IFNγ expression by CD4+ and CD8 T-cells were measured in infants at 4-7 days after birth, regarded as a baseline time point (before aP vaccination). All infants were vaccinated at 6, 10 and 14 weeks of age and post-vaccination measures were assessed at week 7, 15 and 36.

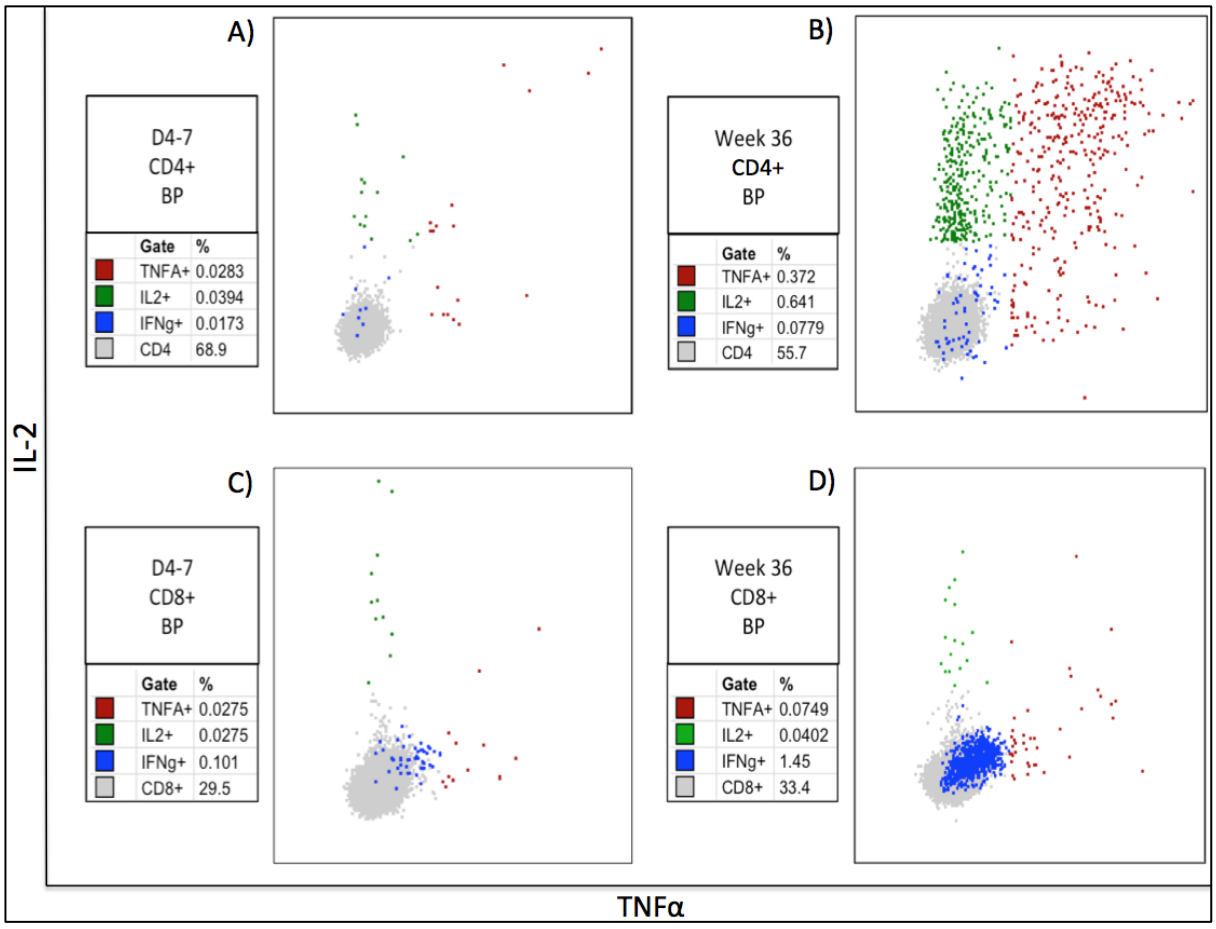


Figure 4.3.5: Representative Flow Plots of CD4+ and CD8+ T-cell Cytokine Expression At D4-7 and Week 36. Panel A and B show CD4+ T-cell responses from a 4-7 day old infant and a week 36 infant respectively. Panel C and D represents CD8+ T-cell responses from a 4-7 day old infant and a week 36 infant respectively. A discription of the infant as well as the percentage of T-cells expression IL-2, TNFα and IFNγ is found on the left of each flow plot.

Figure 4.3.5 is a representative flow plot of total CD4+ and CD8+ T-cell cytokine responses to BP in vitro stimulation at baseline (D4-7) and at week 36. This example is typical for infants in this cohort. The figure only depicts CD4+ T-cells expressing

either IL-2, TNFα or IFNγ not cells expressing different combinations of these cytokines.

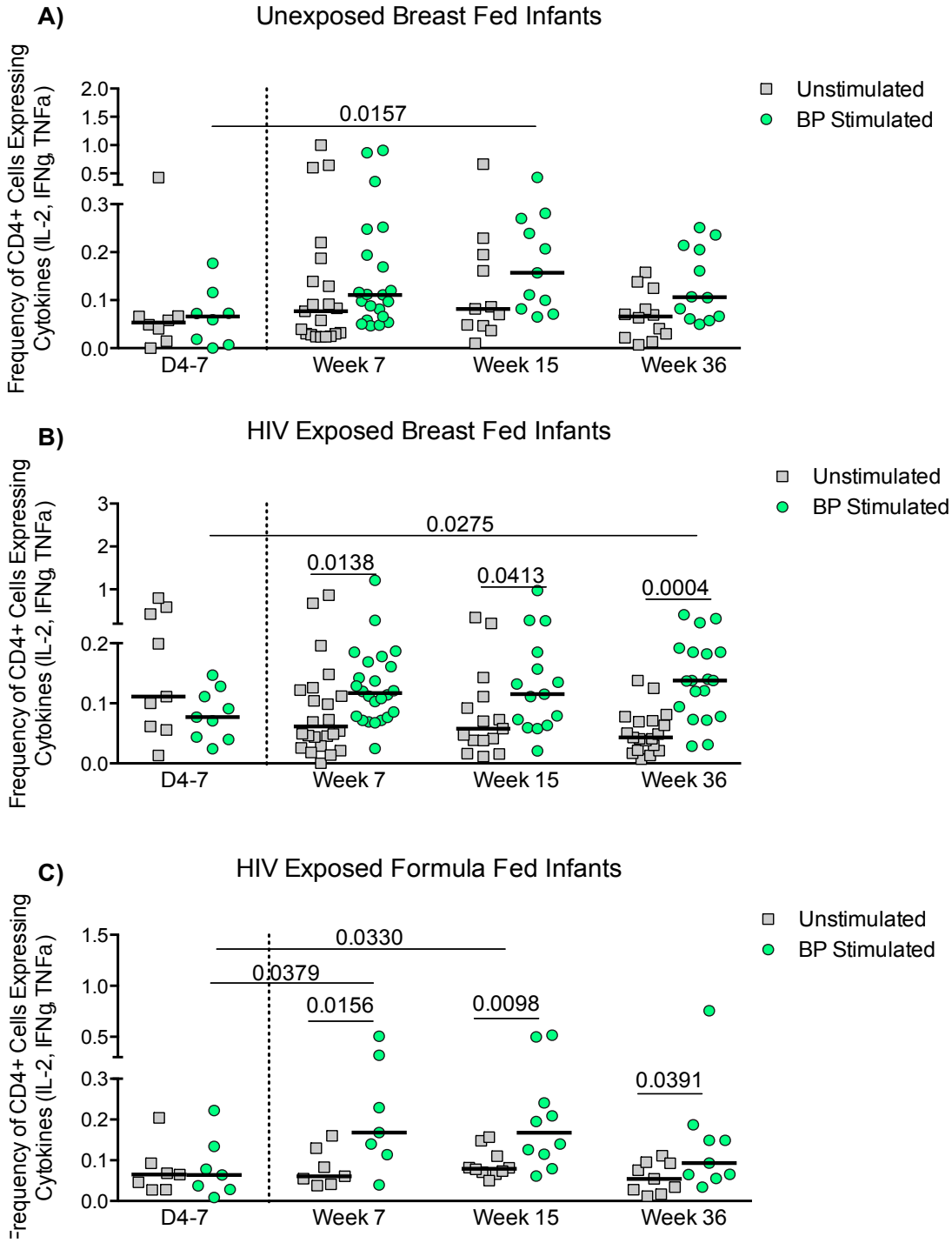


Figure 4.3.6: Comparison of changes in Unstimulated and Bordetella Pertusssis specific CD4+ T-cell any cytokine expression over time. Panel A, B and C represent different feeding groups of infants. Green circles represent BP specific cytokine responses. Gray squares represent unstimulated CD4+ T-cell cytokine responses. Horizontal bars represent medians. P-values were generated using a Wilcoxon signed rank test comparing unstimulated responses to BP specific responses. A Kruskal-wallis test comparing changes across time points within each group was also done and corrected for multiple comparisons with a Dunns test.

Analysis of BP stimulated CD4+ T-cell any cytokine expression demonstrated that infants express cytokines at a level above background at time points post-vaccination, that was not evident pre-vaccination at day 4-7 (fig 4.3.6). Of note, these responses were more evident in HIV exposed infants, and more so in breast-fed than formula fed, demonstrating that in HIV exposed babies CD4+ T-cells were responsive to BP antigens prior to vaccination. Measurement of BP responses after aP vaccination showed that both HEU feeding groups resulted in significantly elevated responses at all time points. Although unexposed infants demonstrated increases in cytokine levels between unstimulated and BP stimulated blood, these increases were not significant. Despite this lack of significance between unstimulated and BP-stimulated cells, U BF infants produced significantly higher amounts of cytokine at week 15 compared to D4-7. E FF infants also had increased levels of cytokine expression at week 7 and week 15 compared to D4-7. In contrast, when compared to D4-7 E BF infants had higher levels of cytokine expression at week 36.

Within the CD8+ T-cell compartment, pertussis-specific cytokine responses were also evident, as noted by the higher cytokine response in the BP stimulated blood over background at most time points post-vaccination. These were significantly higher at week 15 in both HIV-exposed and unexposed breasted infants. Peak cytokine expression for U BF and E FF infants were found at week 7. However, the background cytokine expression at week 7 was also higher than other time points in E FF infants, suggesting that this peak is likely in response to other in vivo antigenic stimuli. Consistent with the CD4+ T-cell responses, CD8+ T-cells at D4-7 in the HEU breast feeding group showed strong BP responses which masked any significant responses detectable after aP vaccination (weeks 7, 15 and 36). This is an interesting finding, as infants have not yet been vaccinated at D4-7, but they were able to respond to BP antigen stimulation. Collectively, these baseline T cell responses suggest that these may be due to environmental and/or maternal factors that may have been transferred to infants transplacentally and via breast-feeding.

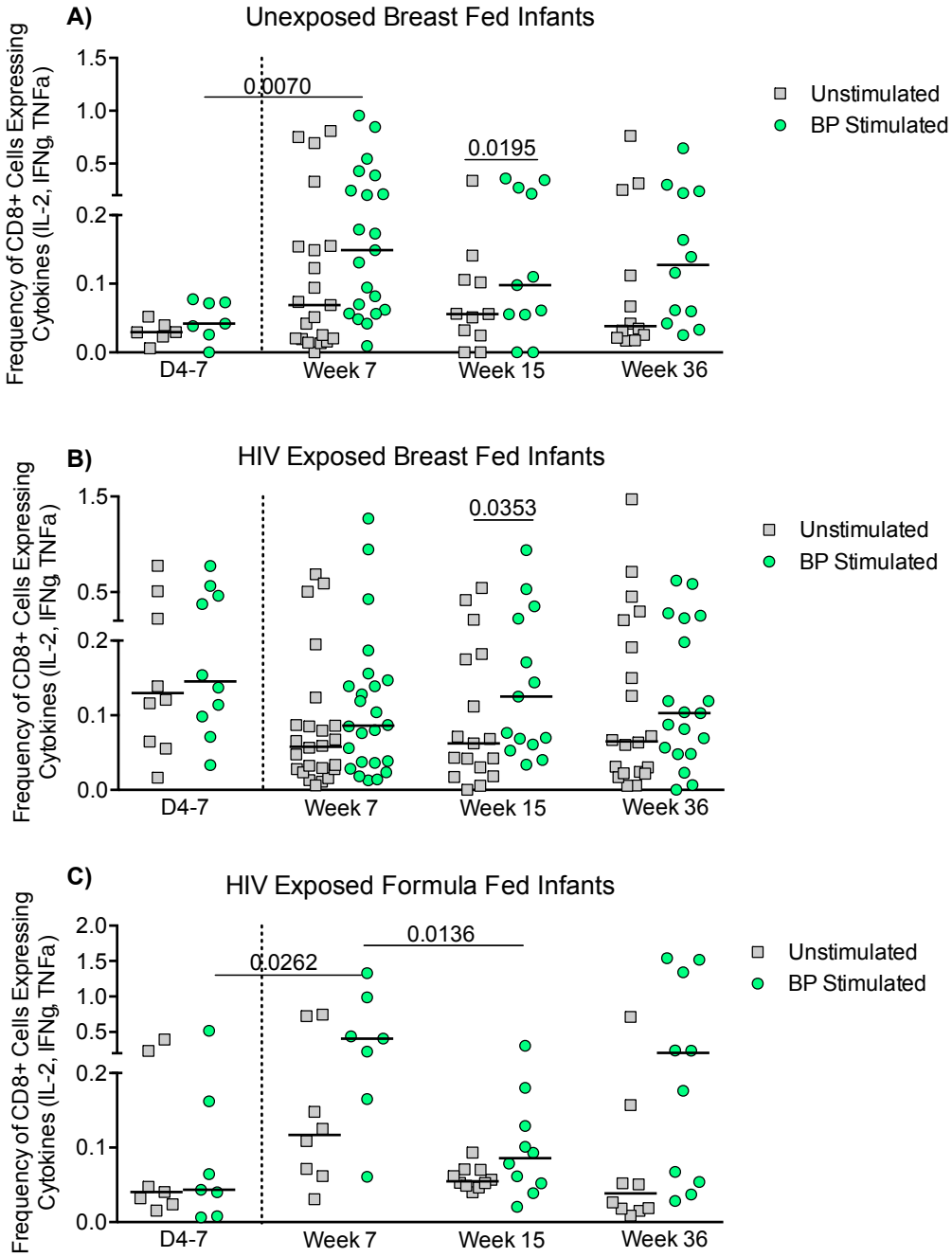


Figure 4.3.7: Comparison of changes in Unstimulated and Bordetella Pertusssis specific CD8+ cytokine expression over time. Panel A, B and C represent different feeding groups of infants. Green circles represent BP specific cytokine responses. Gray squares represent unstimulated CD8+ cytokine responses. Horizontal bars depict medians. P-values were generated using a Wilcoxon signed rank test comparing unstimulated responses to BP specific responses. A Kruskal-wallis test comparing changes across time points within each group was also done, however no significant differences were detected.

CHANGES IN IL-2 EXPRESSION OVER TIME

After assessing overall cytokine expression, individual cytokine responses were assessed starting with IL-2.

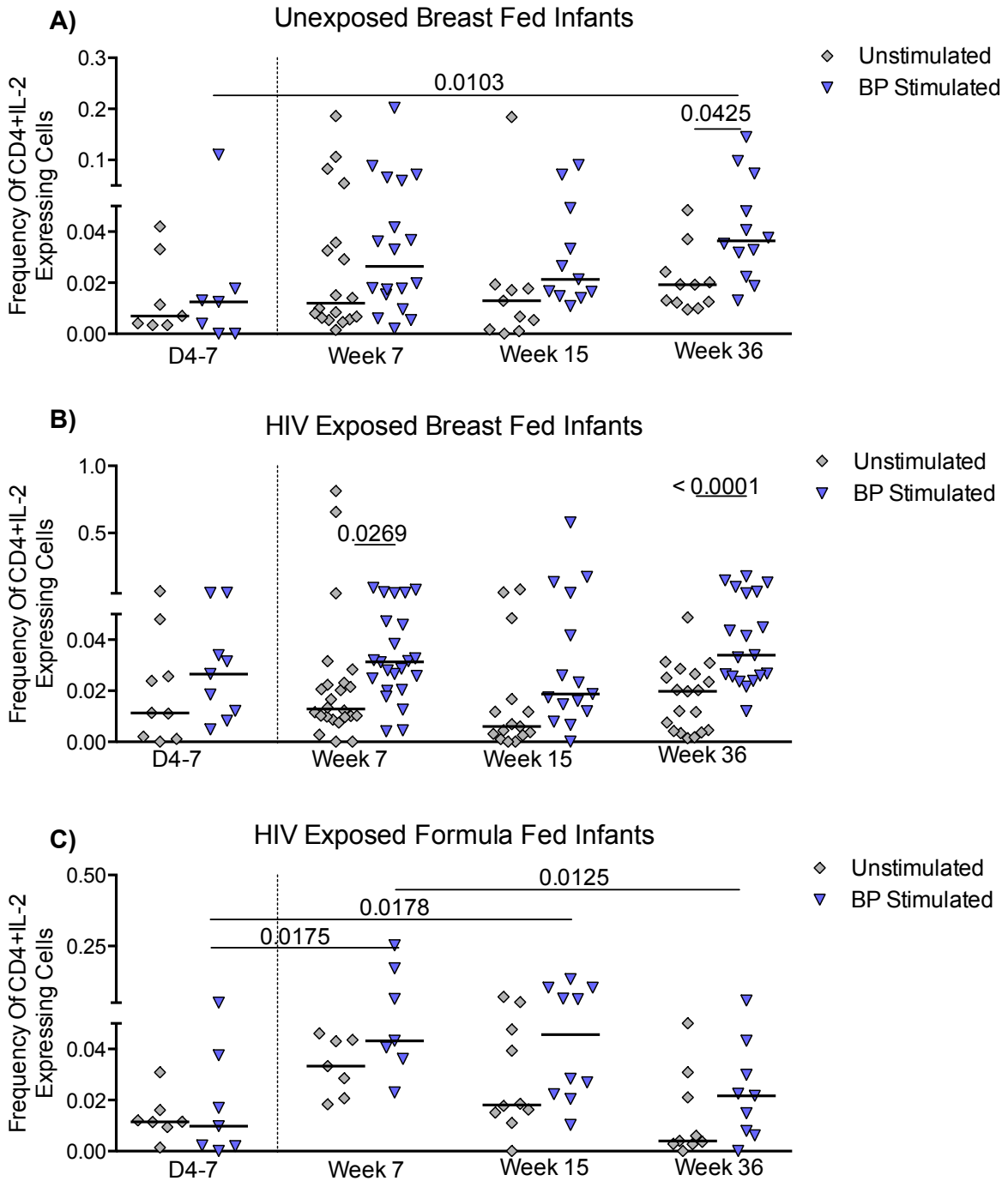


Figure 4.3.8: Comparison of changes in Unstimulated and Bordetella Pertusssis specific CD4+ T-Cell IL-2 expression over time. Panel A, B and C represent different feeding groups of infants. Green circles represent BP specific IL-2 responses. Gray squares represent unstimulated CD4+ T-cell IL-2 responses. Horizontal bars depict medians. P-values were generated using a Wilcoxon signed rank test comparing unstimulated responses to BP specific responses. A Kruskal-wallis test comparing changes across time points within each group was also done, however no significant differences were detected.

Within the CD4+ T-cell compartment, U BF infants had higher expression of IL-2 at week 36 compared to D4-7 (Fig 4.3.8). Also, at week 36 the IL-2 expression was significantly higher in the BP stimulated blood compared to unstimulated blood. This suggests that improved IL-2 responses in U BF infants develop after increasing numbers of aP booster doses. The BP specific IL-2 response in HIV exposed breast fed infants were significantly higher than background levels at week 7 and week 36. There were also no changes in IL-2 expression between time points. E FF infants did not seem to have an IL-2 response above background at any time point. However, at all time points IL-2 responses were significantly higher that D4-7. However, background IL-2 levels were also elevated at week 7. At week 15 and week 36, in contrast, the IL-2 responses to aP were well above background (although insignificantly) suggesting that cytokine production peaks at week 15 in E FF infants. As with CD4 memory responses, infants seem to have IL-2 responses at baseline. This is especially true for breast fed infants and is likely maternal responses within the infant.

In the CD8 compartment, none of the 3 infant groups seemed to produce levels of IL-2 that was significantly above background levels, making it difficult to tease out vaccine specific IL-2 expression (Fig 4.3.9). Given that backgound is caused by a variety of factors, it is possible that some backgroud responses are aP specific but stimulation with BP antigens is unable to enhance this respons in a 12 hour assay. However, the highest IL-2 expression in BP stimulated cells was found at week 7 in both U BF and E FF infants.

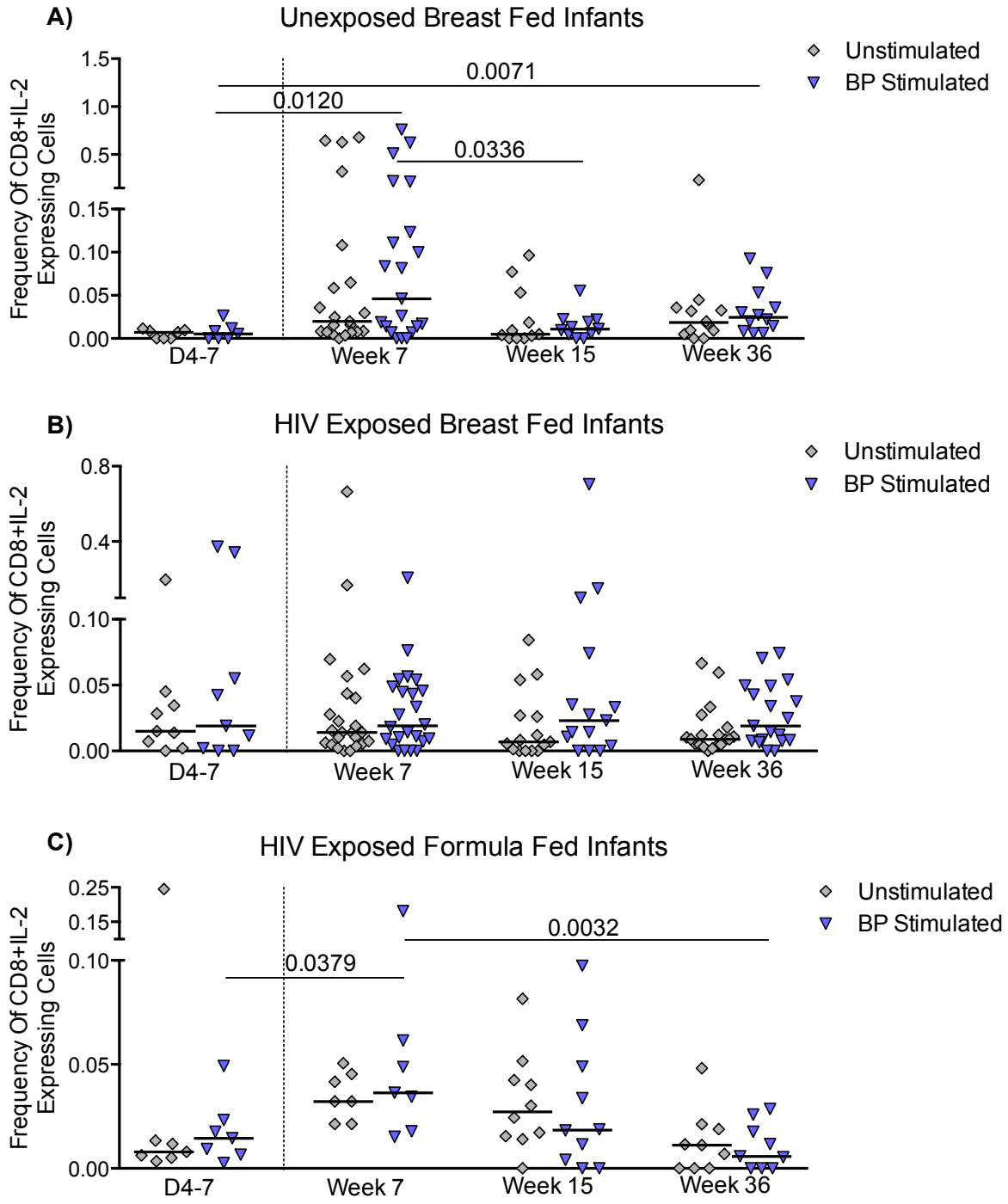


Figure 4.3.9: Comparison of changes in Unstimulated and Bordetella Pertusssis specific CD8+ IL-2 expression over time. Panel A, B and C represent different feeding groups of infants. Green circles represent BP specific IL-2 responses. Gray squares represent unstimulated CD8+ IL-2 responses. Horizontal bars depict medians. P-values were generated using a Wilcoxon signed rank test comparing unstimulated responses to BP specific responses. A Kruskal-wallis test comparing changes across time points within each group was also done, however no significant differences were detected.

CHANGES IN TNF-A EXPRESSION OVER TIME

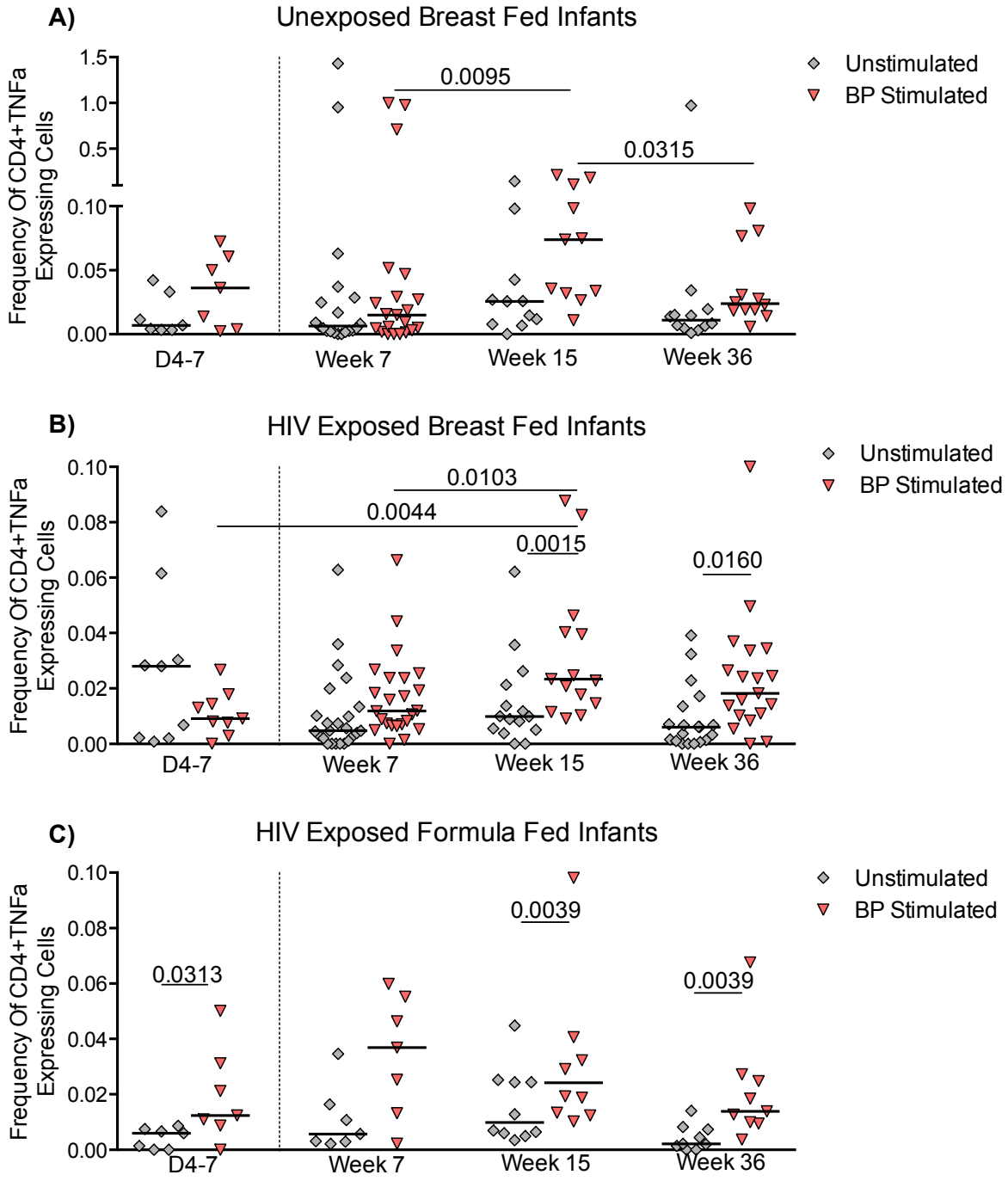


Figure 4.3.10: Comparison of changes in Unstimulated and Bordetella Pertusssis specific CD4+ T-cell TNFα expression over time. Panel A, B and C represent different feeding groups of infants. Green circles represent BP specific TNFα responses. Gray squares represent unstimulated CD4+ T-cell TNFα responses. Horizontal bars depict medians. P-values were generated using a Wilcoxon signed rank test comparing unstimulated responses to BP specific responses. A Kruskal-wallis test comparing changes across time points within each group was also done, however no significant differences were detected.

The second cytokine analysed was TNFα (Figure 4.3.10). At week 15 and week 36 both HIV exposed infant groups demonstrated TNFα responses to BP stimulation. This was not as evident in U BF infants. In addition, TNFα levels seem to peak at week 15 in both breast-fed infant groups, suggesting that week 15 is the likely peak time point of CD4+ T-cell TNFα responses to aP vaccination.

CD8+ TNFα responses were only found in E FF infants (Figure 4.3.11). E BF and U BF infants seem to have similar levels of TNFα in unstimulated and BP stimulated blood, across all time points. This suggests that some aspect of formula feeding may alter the ability of CD8+ T cells to respond to BP in culture and suggests that these infants have a better cellular response to aP vaccination. Alternatively, some aspect of breast-feeding may be suppressing these responses.

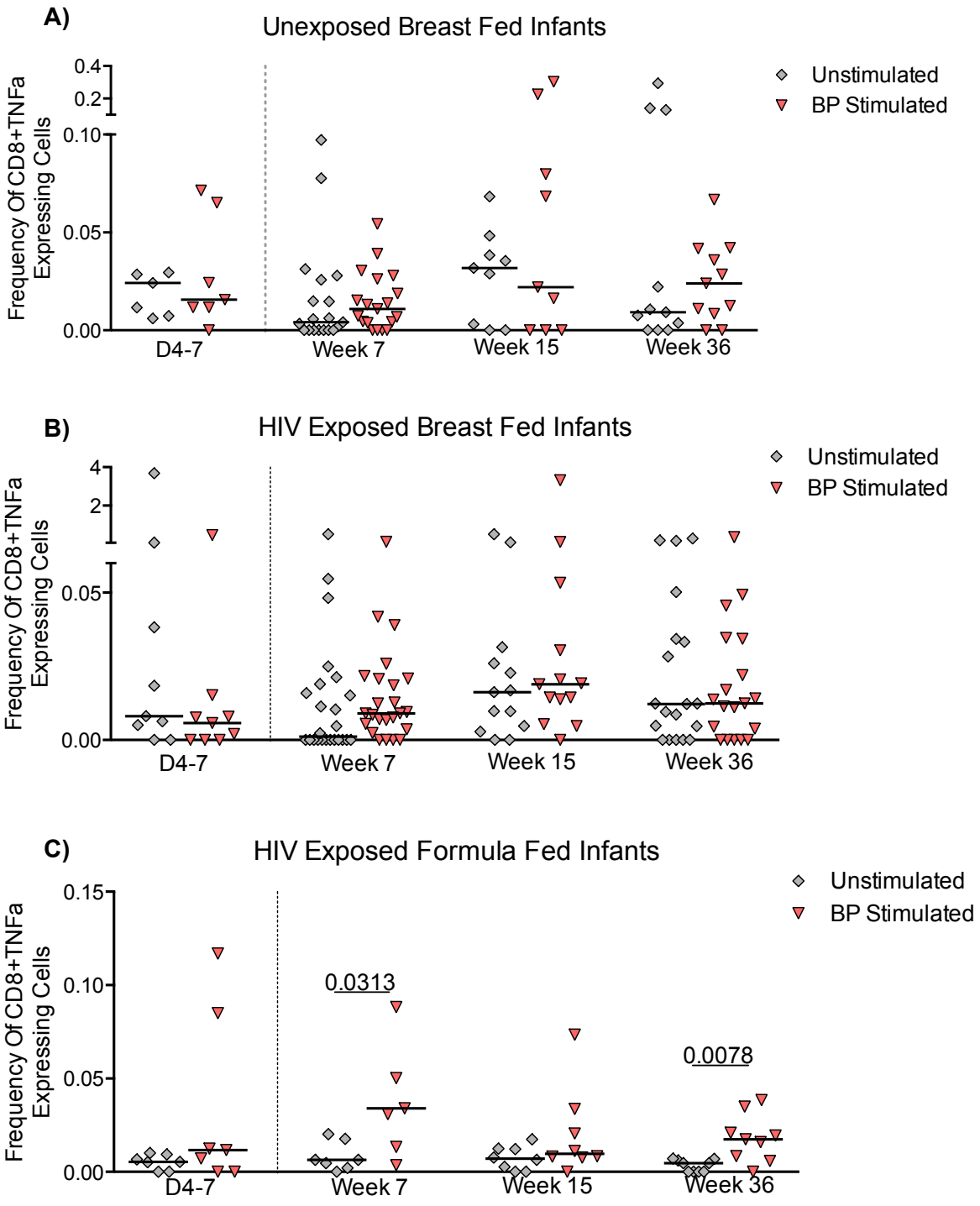


Figure 4.3.11: Comparison of changes in Unstimulated and Bordetella Pertusssis specific CD8+ TNFα expression over time. Panel A, B and C represent different feeding groups of infants. Green circles represent BP specific TNFα responses. Gray squares represent unstimulated CD8+ TNFα responses. Horizontal bars depict medians. P-values were generated using a Wilcoxon signed rank test comparing unstimulated responses to BP specific responses. A Kruskal-wallis test comparing changes across time points within each group was also done, however no significant differences were detected.

CHANGES IN IFN-γ EXPRESSION OVER TIME

The third cytokine analysed was IFNγ. No significant differences were found between BP stimulated and unstimulated blood across all time points in HIV exposed infants.

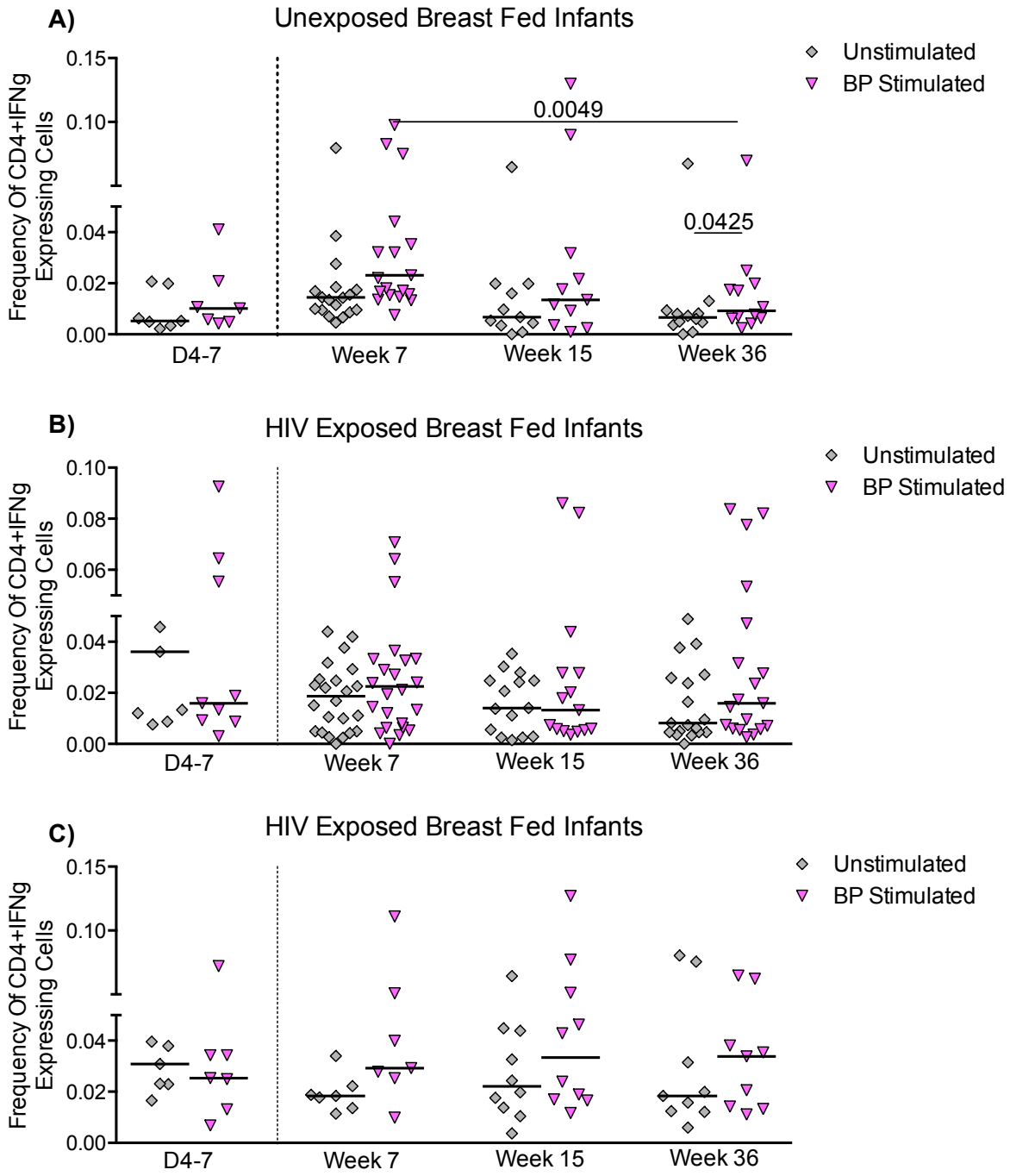


Figure 4.3.12: Comparison of changes in Unstimulated and Bordetella Pertusssis specific CD4+ T-cell IFNγ expression over time. Panel A, B and C represent different feeding groups of infants. Green circles represent BP specific IFNγ responses. Gray squares represent unstimulated CD4+ T-cell IFNγ responses. Horizontal bars depict medians. P-values were generated using a Wilcoxon signed rank test comparing unstimulated responses to BP specific responses. A Kruskal-wallis test comparing changes across time points within each group was also done, however no significant differences were detected.

In the HIV unexposed group there was a BP CD4 IFNγ response above background levels at week 36 (Figure 4.3.12). However, when compared to other time points week 36 also had the least amount of IFNγ production. The decreased level of IFNγ found at week 36 in BP stimulated blood was also found in unstimulated blood. This implies that U BF infants have reducing levels of CD4+ T-cell IFNγ expression with time.

In contrast, the CD8+ T-cell IFNγ response to in vitro BP stimulation before and after aP vaccination was markedly different (Figure 4.3.13). In U BF infants, IFNγ responses increased with age, albeit not significantly, with a BP response above background at week 7. In both HIV exposed feeding groups, positive responses were identified only at week 15. However, while this is the time point at which there is a BP response it is also the time point at which IFNγ levels are the lowest in E FF infants.

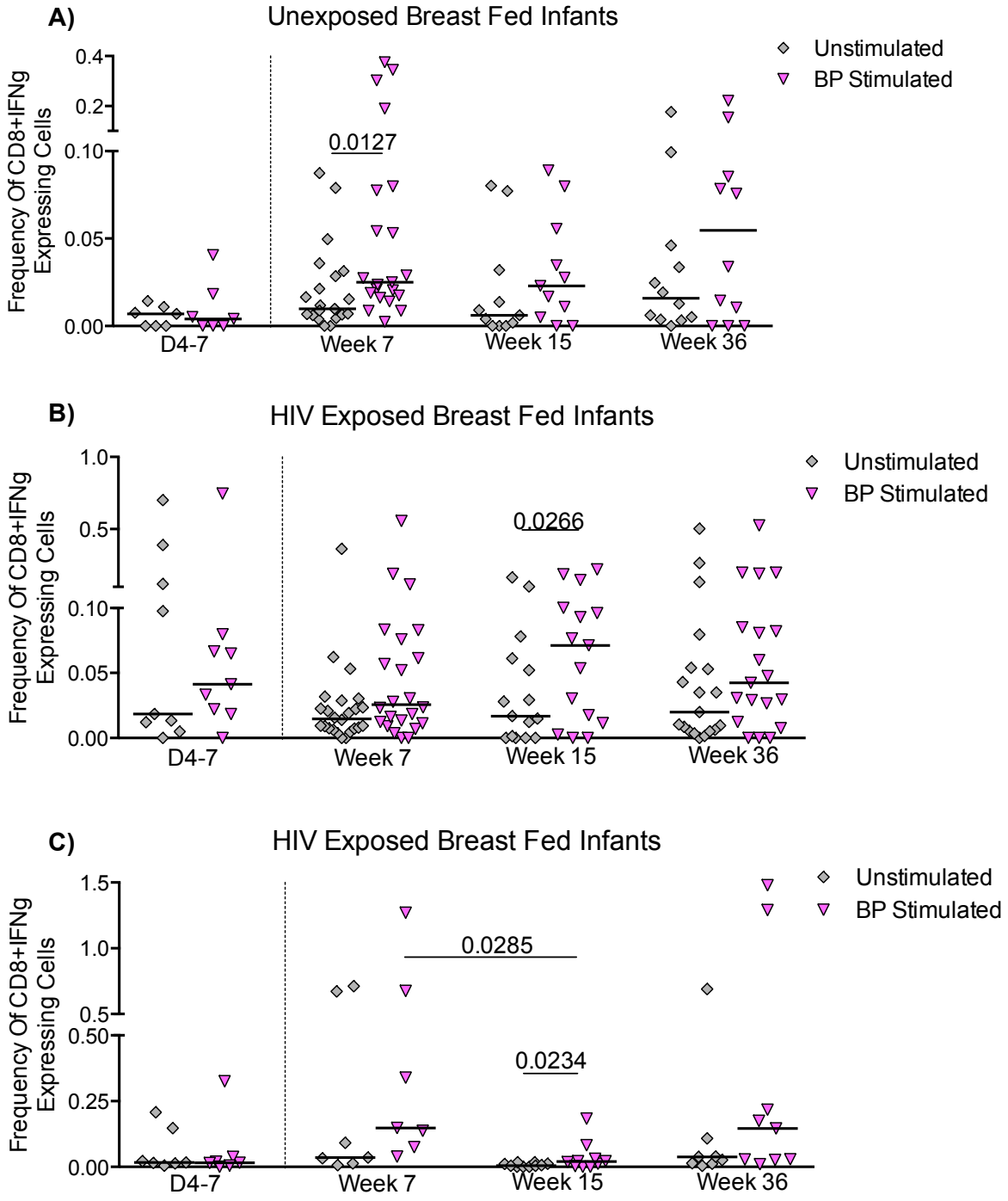
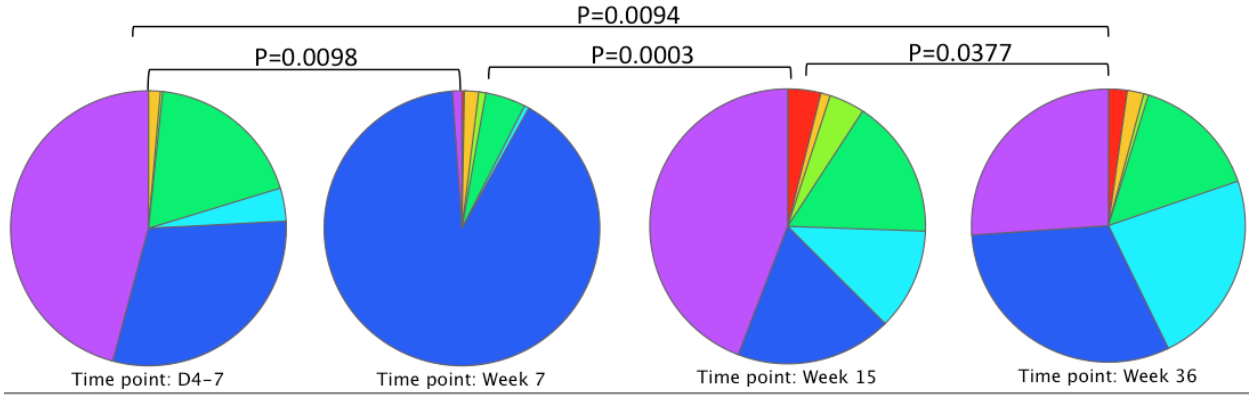
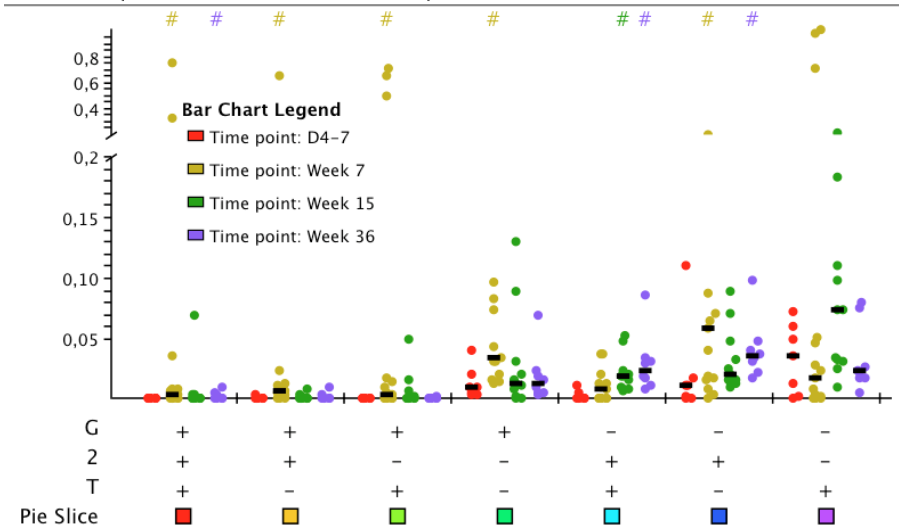


Figure 4.3.13: Comparison of changes in Unstimulated and Bordetella Pertusssis specific CD8+ IFNγ expression over time. Panel A, B and C represent different feeding groups of infants. Green circles represent BP specific IFNγ responses. Gray squares represent unstimulated CD8+ IFNγ responses. Horizontal bars depict medians. P-values were generated using a Wilcoxon signed rank test comparing unstimulated responses to BP specific responses. A Kruskal-wallis test comparing changes across time points within each group was also done, however no significant differences were detected.

CHANGES IN BORDERTELLA PERTUSSIS SPECIFIC CD4+ AND CD8+ POLYFUNCTIONAL CYTOKINE RESPONSES ACROSS TIME

In addition to the overall and single cytokine response, polyfunctional cytokine kinetics were also assessed. Polyfunctional T-cells are cells that produce more than one cytokine concurrently and are thought to provide a more effective immune response. All polyfunctional assessments were done independently for each group of infants. Figures 4.3.14 and 4.3.15 below show CD4 and CD8 T-cell responses to BP stimulation in unexposed breast fed infants.

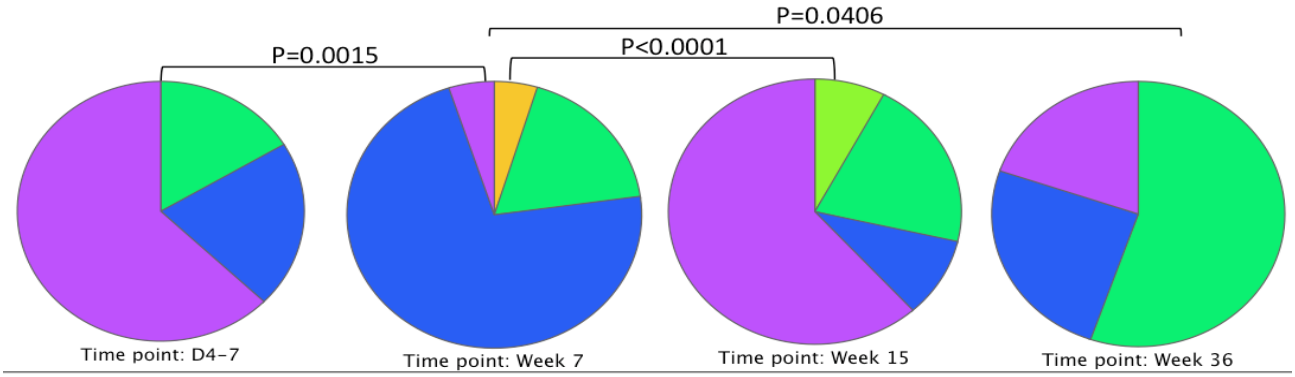


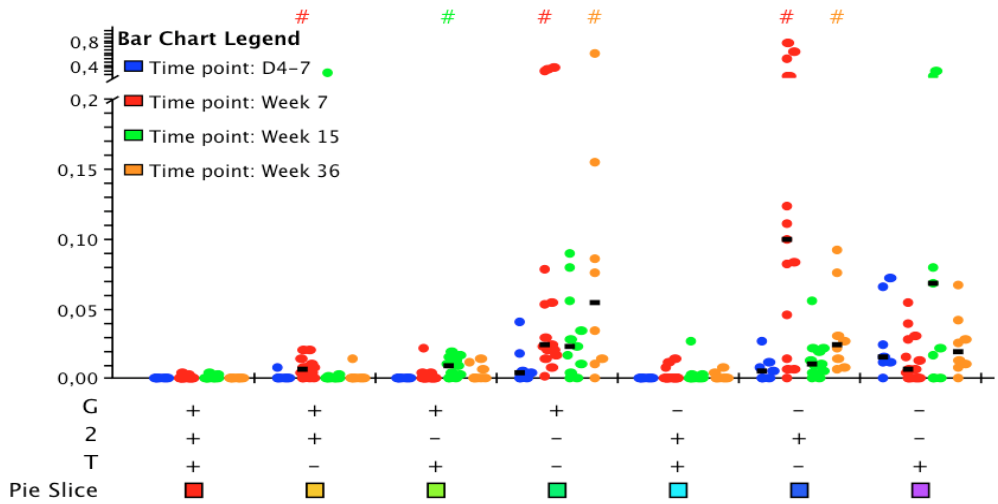


Category	Time point: Week 7	Time point: Week 15	Time point: Week 36
+ + +	0.026	0.160	0.028
+ + −	0.007	0.497	0.272
+ − +	0.026	0.113	0.355
+ − −	0.004	0.618	0.524
− + +	0.231	0.002	0.002
− + −	0.032	0.063	0.021
− − +	0.549	0.113	0.728

Figure 4.3.14: Polyfunctional Cytokine production of BP-specific CD4+ T-cells in Unexposed breast fed infants across time. The figure demonstrates the frequency of cytokines produced in different combinations by CD4+ T-cells. Black bars represent medians and # represents a signifcant difference based on a Wilcoxon Rank Test. Highlighted in red on the table are the p-values which correspond to the #.

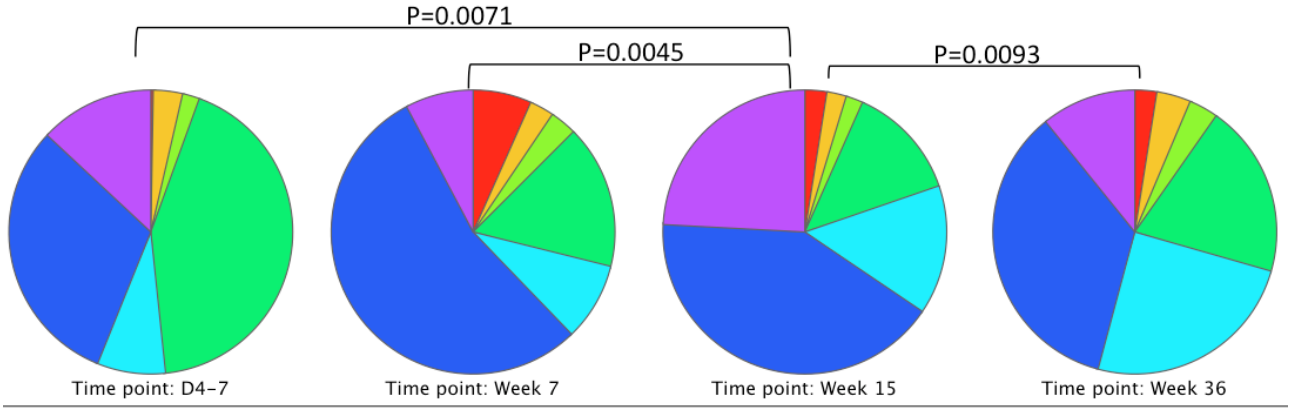
As shown in figure 4.3.14, at D4-7 infants have prodominatly single cytokine expressing CD4 T-cells, either TNFa, IL-2 or IFNg. At week 7 after the initial dose of aP vaccination, U BF infants have an increased number of dual cytokine expressing CD4 cells, but are mostly dominated with IL-2 expressing CD4+ T-cells. By week 15 and 36, triple cytokine expressing CD4+ T-cells are formed. At D4-7 CD8+ T-cells also have a predominantly single cytokine expressing CD8+ T-cell phenotype. However unlike the CD4 T-cell population, CD8 T-cells (Figure 4.3.15) remain mainly of a single cytokine expressing phenotype with a few dual cytokine expressing cells. The polyfunctional profile of CD8 T-cells were most different to baseline at week 7. CD4 polyfunctional profiles were different at week 7 and week 36 compared to baseline.

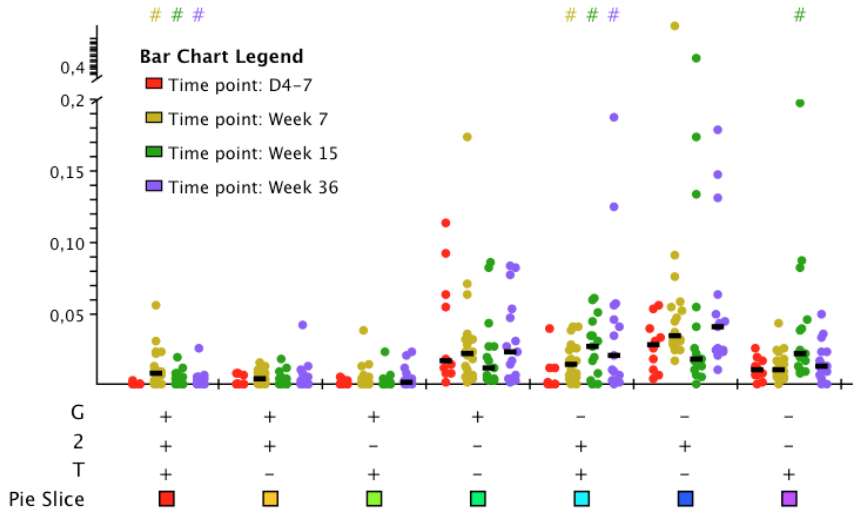




Category			Time point: Week 7	Time point: Week 15	Time point: Week 36
+	+	+	0.622	0.526	1.000
+	+	–	0.026	0.684	1.000
+	–	+	0.459	0.011	0.224
+	–	–	0.008	0.113	0.043
–	+	+	0.459	0.205	0.418
–	+	–	0.005	0.258	0.015
–	–	+	0.148	0.277	0.862

Figure 4.3.15: Polyfunctional Cytokine production of BP-specific CD8+ T-cells in Unexposed breast fed infants across time. The figure demonstrates the frequency of cytokines produced in different combinations by CD4+ T-cells. Black bars represent medians and # represents a signifcant difference based on a Wilcoxon Rank Test. Highlighted in red on the table are the p-values which correspond to the #.

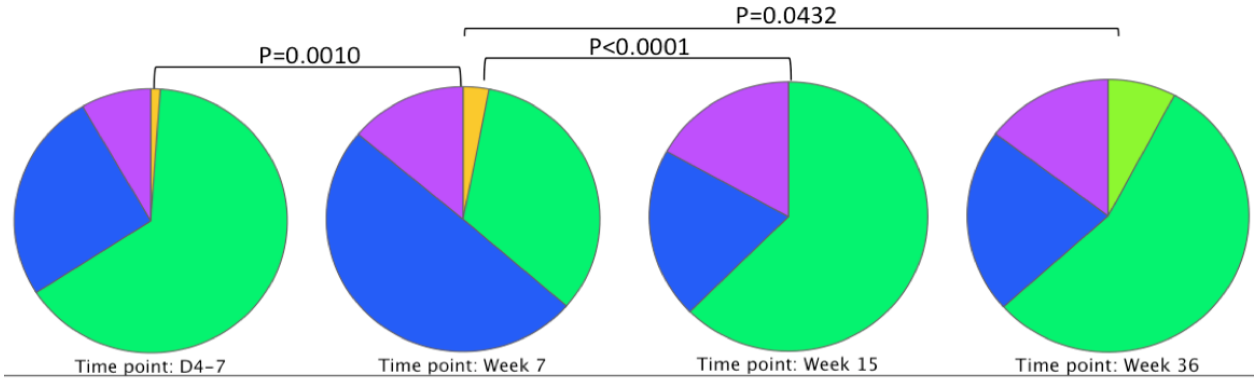


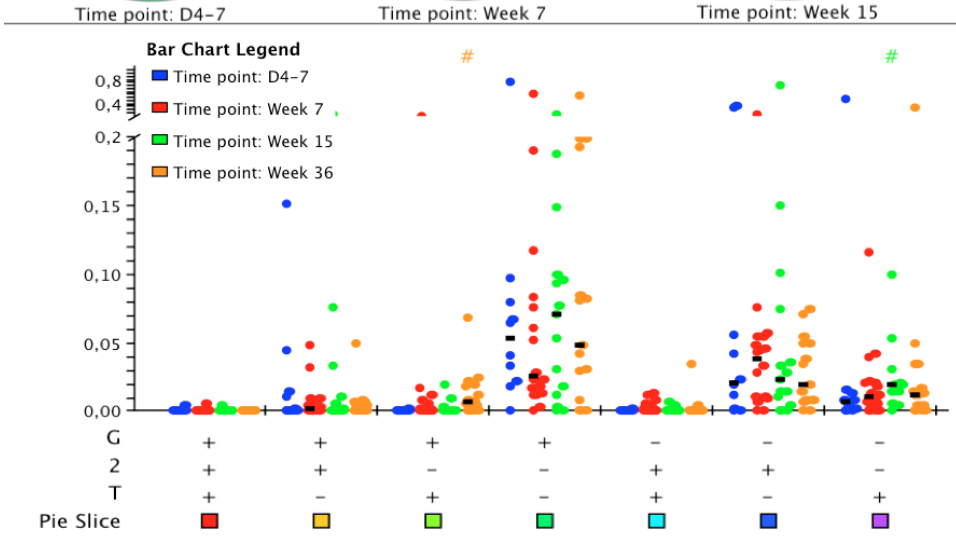


Category	Time point: Week 7	Time point: Week 15	Time point: Week 36
+ + +	0.000	0.016	0.016
+ + −	0.179	0.782	0.760
+ − +	0.231	0.677	0.134
+ − −	0.811	0.267	0.677
− + +	0.019	0.031	0.011
− + −	0.103	0.868	0.183
− − +	0.719	0.007	0.332

Figure 4.3.16 : Polyfunctional Cytokine production of BP-specific CD4+ T-cells in HIV exposed breast fed infants across time. The figure demonstrates the frequency of cytokines produced in different combinations by CD4+ T-cells. Black bars represent medians and # represents a signifcant difference based on a Wilcoxon Rank Test. Highlighted in red on the table are the p-values which correspond to the #.

In E BF infants week 15 CD4 responses and week 7 CD8 responses were different from D4-7. CD8 responses (Fig 4.3.17) were mainly of a single cytokine expressing phenotype, with predominantly IFNγ expression. CD4 cells (Fig 4.3.16) were much more polyfunctional the CD8 T-cells at all timepoints. At all timepoints post vaccination triple cytokine expressing CD4+ T-cells were present. In addition, IL-2 and TNFα, dual expressing CD4 T-cells were higher at all time points post vaccination compared to D4-7. In both CD4 and CD8 T-cells there was an increase in TNFα expression at week 15.

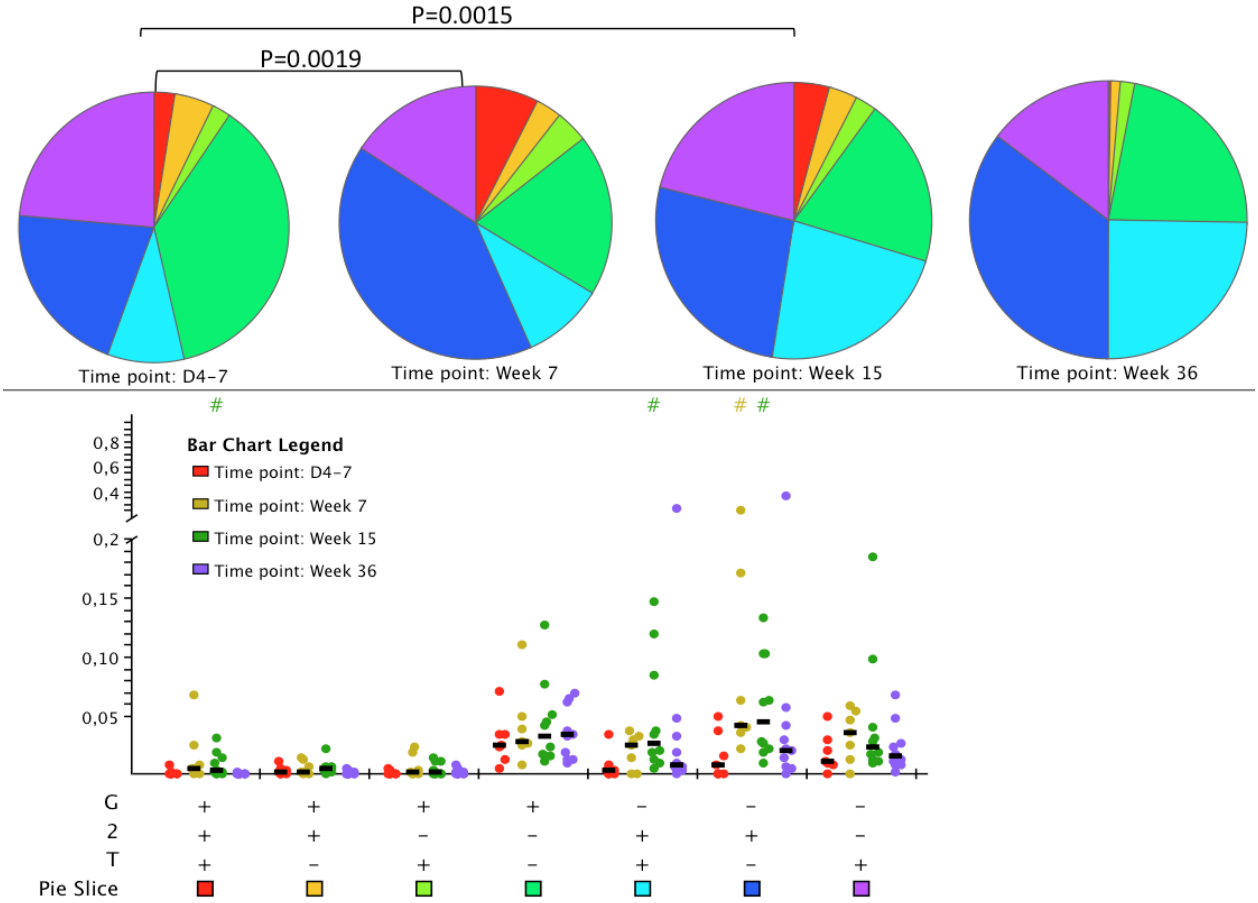




Category	Time point: Week 7	Time point: Week 15	Time point: Week 36
+ + +	0.867	0.912	0.677
+ + −	0.943	0.637	0.579
+ − +	0.157	0.618	0.008
+ − −	0.565	0.868	0.846
− + +	0.270	0.846	0.657
− + −	0.811	0.956	0.846
− − +	0.350	0.018	0.437

Figure 4.3.17: Polyfunctional Cytokine production of BP-specific CD8+ T-cells in HIV exposed breast fed infants across time. The figure demonstrates the frequency of cytokines produced in different combinations by CD8+ T-cells. Black bars represent medians and # represents a signifcant difference based on a Wilcoxon Rank Test. Highlighted in red on the table are the p-values which correspond to the #.

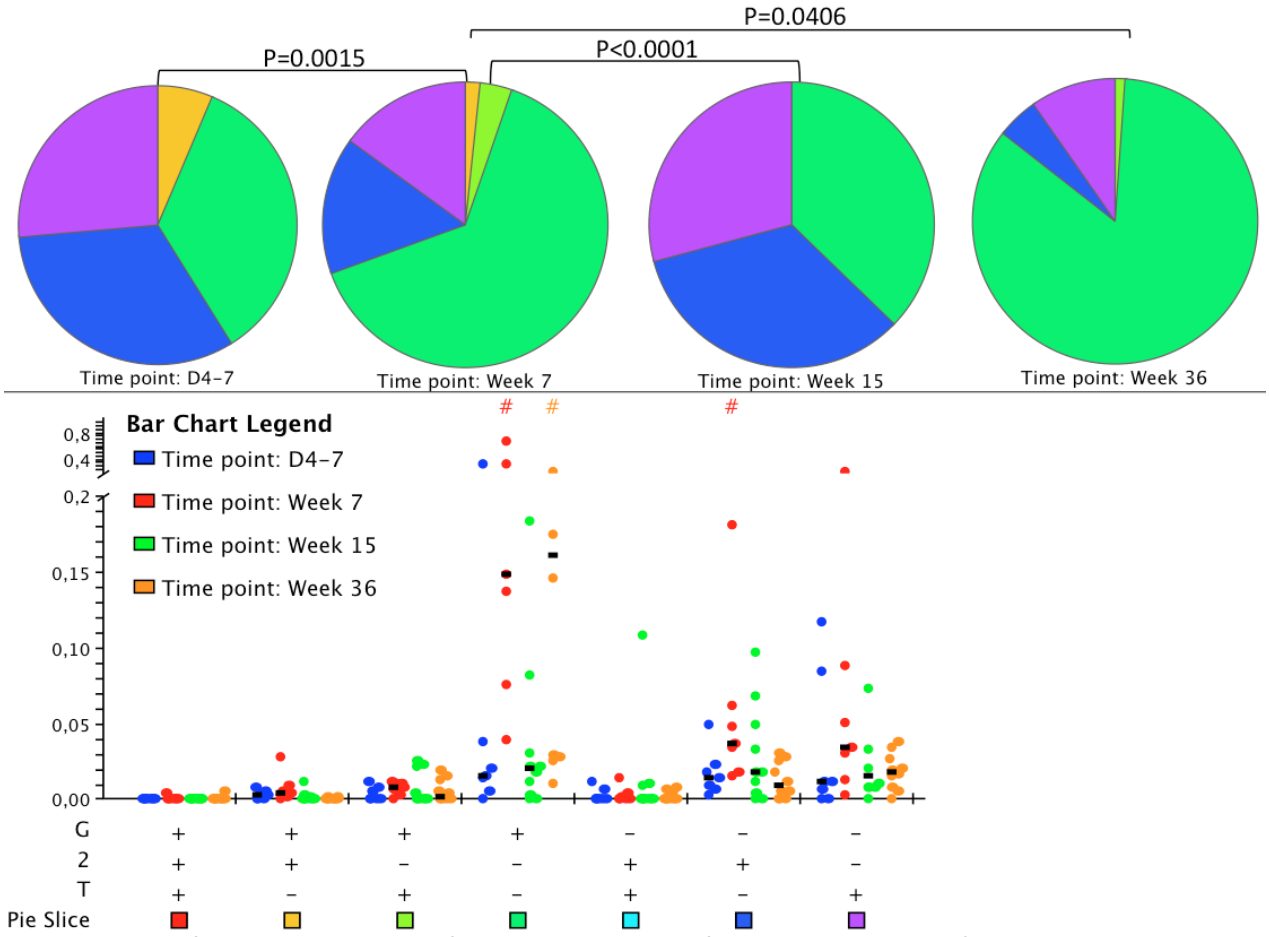
Figure 4.3.18 below shows E FF infants CD4+ polyfunctional responses. In contrast to the two breast feeding groups it seems that E FF infants have polyfunctional responses as early as D4-7. The frequencies of polyfunctional cells seem to increase from D4-7 to week 7 and week 15 compared to baseline. However, by week 36 very few polyfunctional T-cells are present.



Category	Time point: Week 7	Time point: Week 15	Time point: Week 36
+ + +	0.064	0.045	0.435
+ + −	0.338	0.079	0.407
+ − +	0.110	0.242	0.558
+ − −	0.406	0.558	0.558
− + +	0.110	0.006	0.118
− + −	0.018	0.019	0.306
− − +	0.142	0.188	0.626

Figure 4.3.18: Polyfunctional Cytokine production of BP-specific CD4+ T-cells in HIV exposed formula fed infants across time. The figure demonstrates the frequency of cytokines produced in different combinations by CD4+ T-cells. Black bars represent medians and # represents a signifcant difference based on a Wilcoxon Rank Test. Highlighted in red on the table are the p-values which correspond to the #.

Again, CD8+ T-cells are predominantly single cytokine expressing cells (Figure 4.3.19). IFNγ expressing CD8 T-cells seem to increase in frequency from D4-7 to week 7 and week 7 to week 36. Additionally, IL-2 expression was higher that D4-7 at week 7.



Category	Time point: Week 7	Time point: Week 15	Time point: Week 36
+ + +	0.655	1.000	0.733
+ + −	0.371	0.354	0.097
+ − +	0.180	0.845	0.696
+ − −	0.009	1.000	0.040
− + +	0.749	0.884	0.961
− + −	0.035	0.626	0.464
− − +	0.180	0.495	0.558

Figure 4.3.19: Polyfunctional Cytokine production of BP-specific CD8+ T-cells in HIV exposed formula fed infants across time. The figure demonstrates the frequency of cytokines produced in different combinations by CD8+ T-cells. Black bars represent medians and # represents a significant difference based on a Wilcoxon Rank Test. Highlighted in red on the table are the p-values that correspond to the #.

CONCLUSION

In general, total CD4+ T-cell cytokine responses were detected, this was especially true for HIV exposed infants, where all BP stimulated responses were higher then unstimulated responses as all time points post vaccination. In addition all infants had a general decrease in naïve like memory cell frequencies across time with corresponding increases in differentiated memory populations. Together with increased frequencies of differentiated memory cells, CD4+ polyfunctional response also increased from D4-7, peaking at week 15 HEU infants and week 36 in HU infants. Furthermore BP specific memory CD4 cells were present at D4-7, prior to vaccination.

In E BF infants, peak total cytokine responses were found at week 36, as this was the only time point at which CD4+ cytokine responses were higher that pre-vaccination responses. There was a corresponding increase in LD and TD memory CD4 T-cells from D4-7 to week 15 in E BF infants that was maintained until week 36. In addition the polyfunctional profile of E BF infants was also significantly different from baseline at week 15 suggesting that aP vaccine responses in E BF infants peak at week 15.

In contrast, EFF infants had responses higher CD4 cytokine responses at week 7 and week 15 compared to D4-7. ED and LD memory responses in E FF infants also peaked at week 15. In addition, E FF infants had higher frequencies of polyfunctional cells at week 15 compared to D4-7. LD memory responses remained elevated while ED memory responses decreased by week 36.

U BF infants also had peak CD4 cytokine responses at week 15. In addition U BF infants had the highest frequencies LD and TD memory cells at week 36.

Majority of the cytokines expressed by CD4 T-cells in al groups of infants were TNFα and IL-2, very little IFNγ was expressed. In general CD8+ T-cells expressed less cytokines than CD4+ T-cells with the exception of IFNγ

In U BF infants, BP specfic cytokine expression was highest at week 7, however in vitro unstimulated cytokine responses were also highest at this time point. In contrast, at week 15 unstimulated cytokine responses were at a similar level to cytokine levels found at D4-7. In addition BP specific cytokine responses were signifcally higher than unstimulated responses at week 15, suggesting that this is the peak CD8 cytokine response aP specific CD8+ T-cells. Similarly to U BF infants, E FF infants also had elevated cytokine responses both in BP stimulated and unstimulated CD8+ T-cells. While not significant, peak CD8+ cytokine responses seemed to be at week 36 in E FF infants. Uniquely in E BF infants, D4-7 responses were the highest, as a result all post vaccination responses were similar to responses found pre-vaccination. The high D4-7 responses were likely due to maternal factors as this was prior to aP vaccination. Additionally, week 15 was the only time point at which BP stimulated cytokine responses were higher than unstimulated responses. However CD8 polyfuctional responses in all infants seem to be best at week 7. Taken together, it seems that overall CD4+ cytokine responses peak at week 15 while CD8+ cytokine responses peak at week 7.

SECTION 4.4

THE IMPACT OF HIV EXPOSURE ON AP T-CELL RESPONSES

As previously mentioned, HIV exposed infants have an increased risk of morbidity and mortality compared to unexposed infants (Mussi-Pinhata et al., 2007). These infants have also been shown to exhibit an increased expansion of Treg cells and have altered T-cell activation compared to HU infants (Legrand et al., 2006). Therefore, one of the aims of this study was to assess the effects that HIV exposure may have on aP vaccine responses. To complete this aim, direct comparisons of T-cell responses between HIV exposed and HIV unexposed infants were performed. Only breast-fed HIV exposed and breast-fed unexposed infants were included in this comparison to avoid any impact, as shown in earlier sections between different feeding modes.

THE EFFECTS OF HIV EXPOSURE ON MEMORY DIFFERENTIATION

The first analysis completed was a comparison of memory differentiation between HIV exposed and unexposed infants. Figure 4.4.1 and 4.4.2 below demonstrate the CD4+ memory T-cell differentiation found at D4-7 and week 7 in unstimulated blood of aP vaccinated infants. Comparisons were also completed at week 15 and week 36, however no significant differences were found at these time points therefore these figures were put into appendix 2, page 183. Unstimulated blood was used because there were no significant differences between unstimulated and BP stimulated blood.

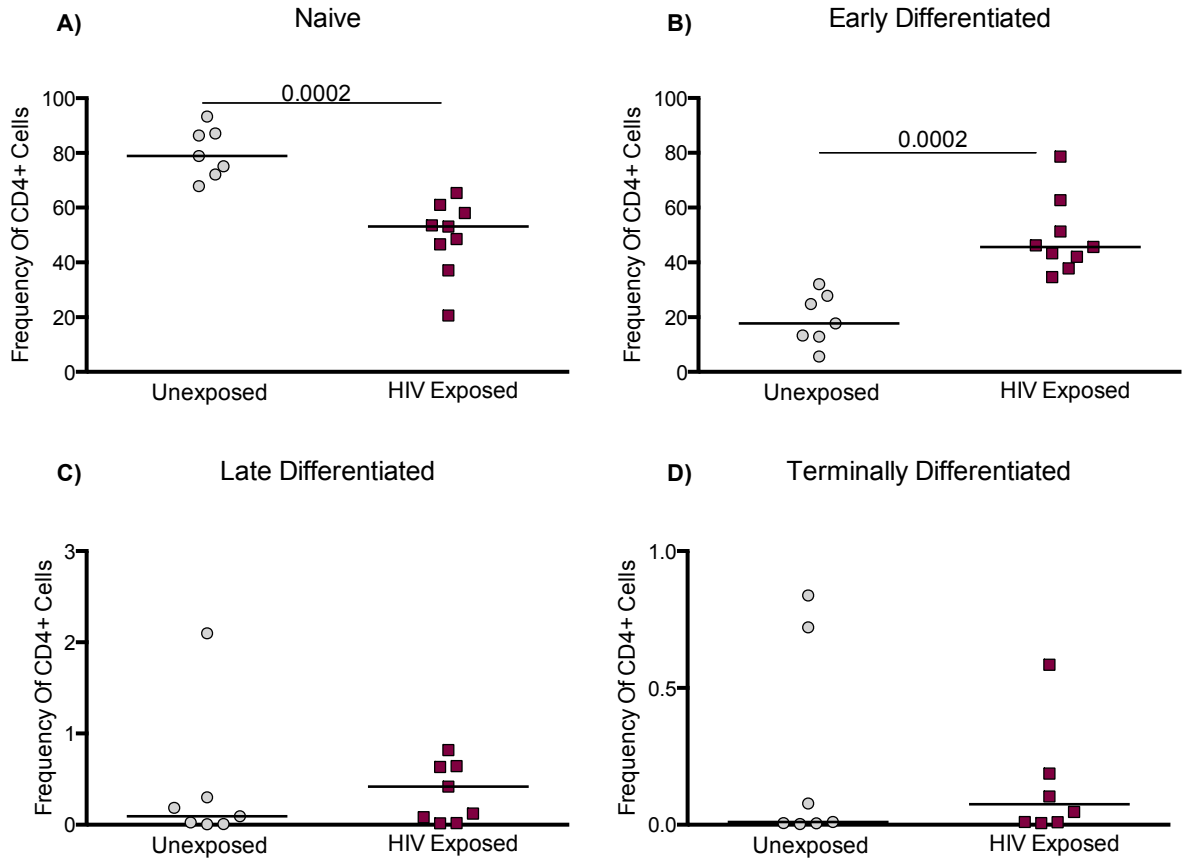


Figure 4.4.1: D4-7 CD4+ Memory T-cell Differentiation in HIV Exposed and Unexposed Infants. Horizontal bars depict medians. P-values were generated using a Mann-Whitney U test.

As evident in figure 4.4.1 and 4.4.2 HIV exposed infants have significantly elevated frequencies of ED CD4 and CD8 T-cells and reduced naïve like cell frequencies at D4-7 compared to unexposed infants. In addition LD CD8+ T-cells were also significantly elevated in HEU infants compared to HU infants. This suggests that exposure to HIV causes T-cells to differentiate, resulting in a more differentiated phenotype of memory cells in HEU infants compared to HU infants.

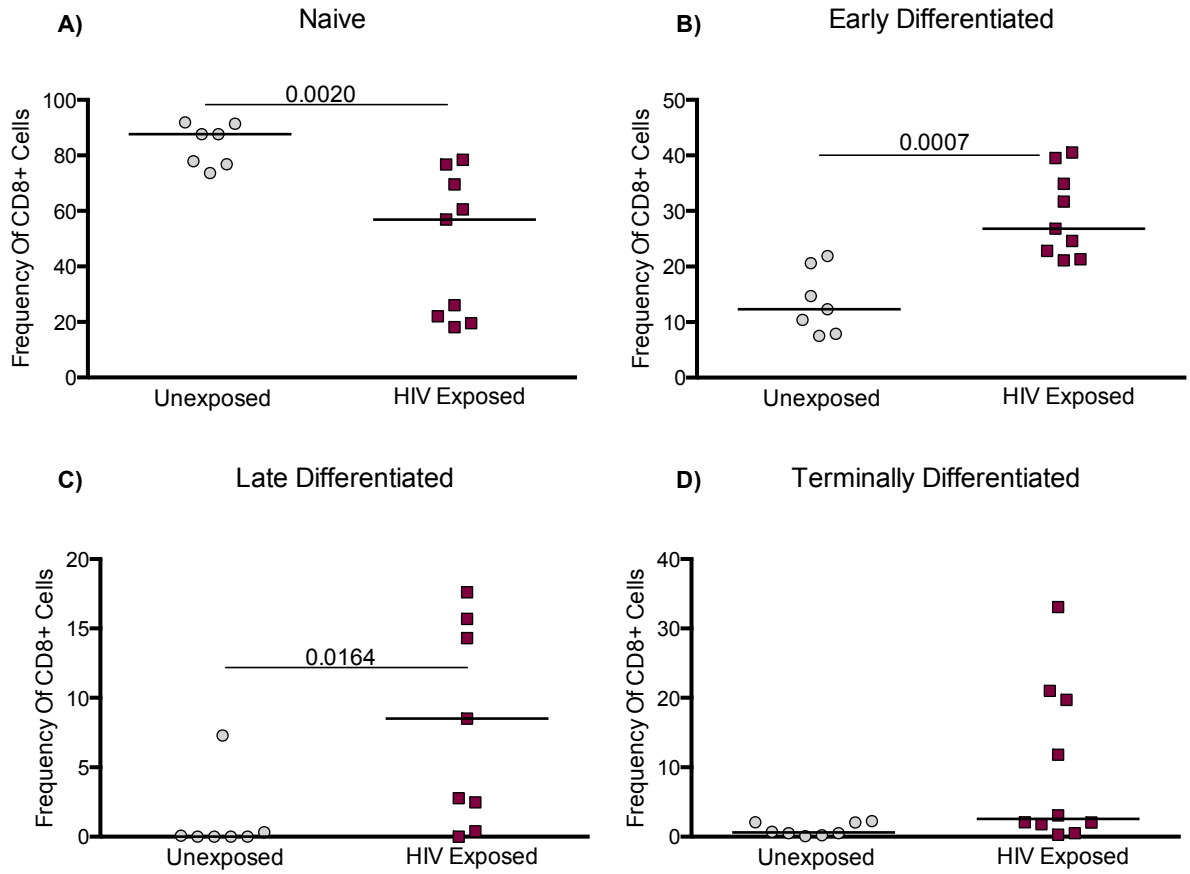


Figure 4.4.2: D4-7 CD8+ Memory Differentiation in HIV Exposed and Unexposed Infants. Horizontal bars depict medians. P-values were generated using a Mann-Whitney U test.

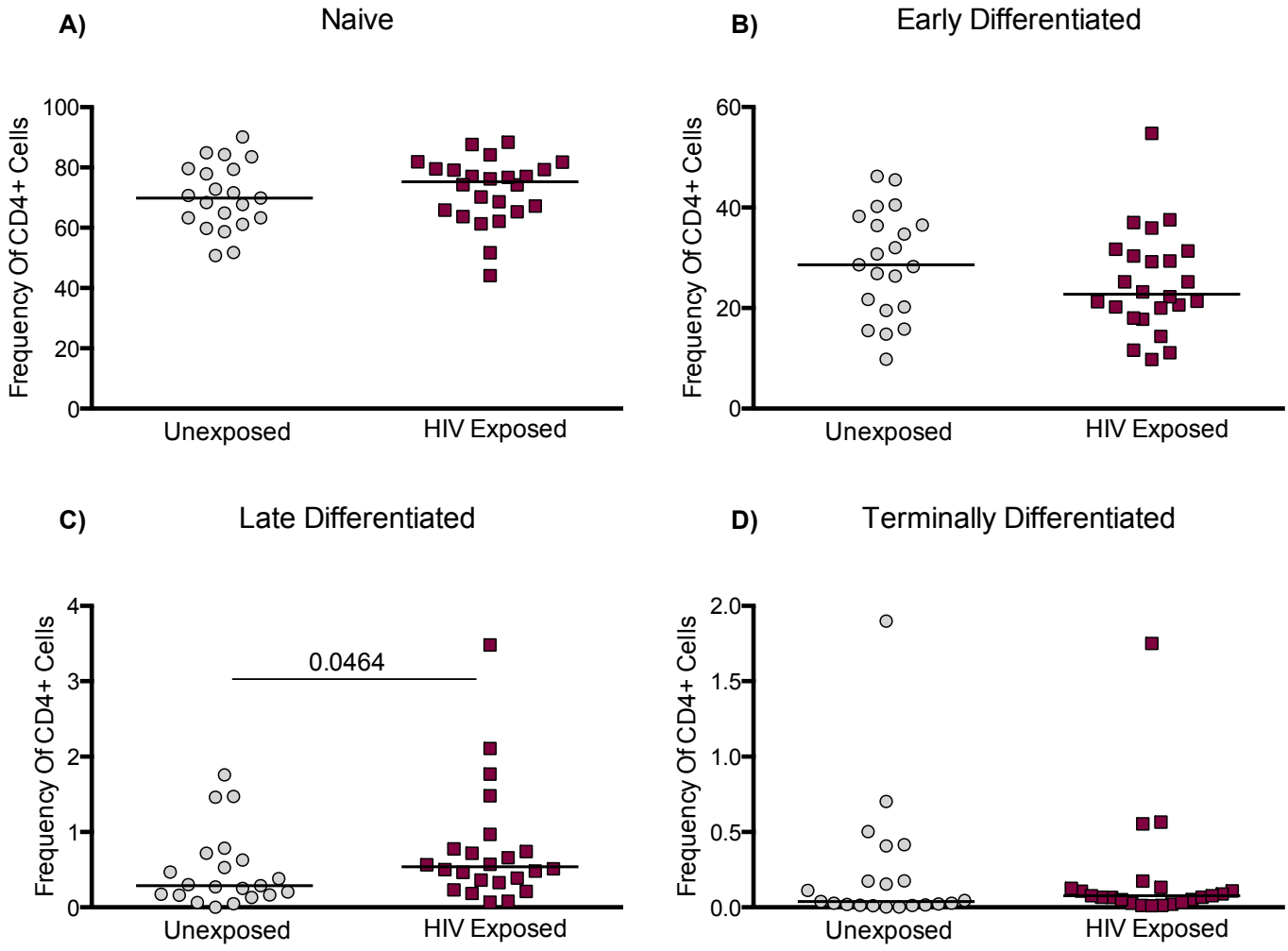


Figure 4.4.3: Week 7 CD4+ Memory T-cell Differentiation in HIV Exposed and Unexposed Infants. Horizontal bars depict medians. P-values were generated using a Mann-Whitney U test.

By week 7 HIV exposed infants had significantly higher proportions of LD memory CD4+ T-cells but less CD8+ ED memory T-cells. This further suggests that exposure to HIV causes T-cells to differentiate.

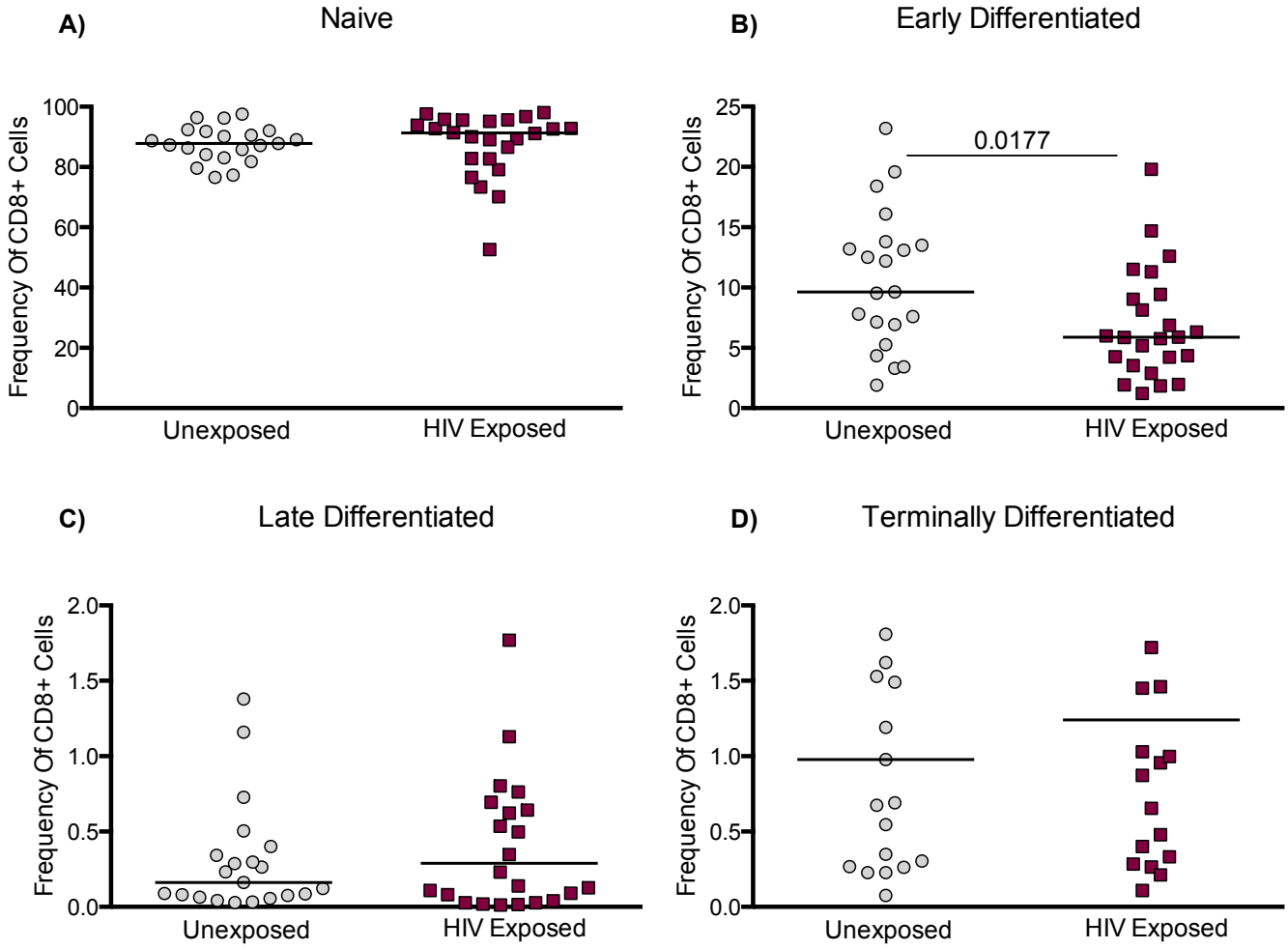


Figure 4.4.4: Week 7 CD8+ Memory Differentiation in HIV Exposed and Unexposed Infants. Horizontal bars depict medians. P-values were generated using a Mann-Whitney U test.

THE EFFECTS OF HIV EXPOSURE ON CYTOKINE PRODUCTION

Figure 4.4.5 below is a representative flow plot of the analysis used to assess differences in cytokine expression by CD4+ and CD8 T-cells in HIV exposed and unexposed infants. It also gives an indication of the changing ratios of CD8 and CD4+ T-cells between week 7 and week 36 of life.

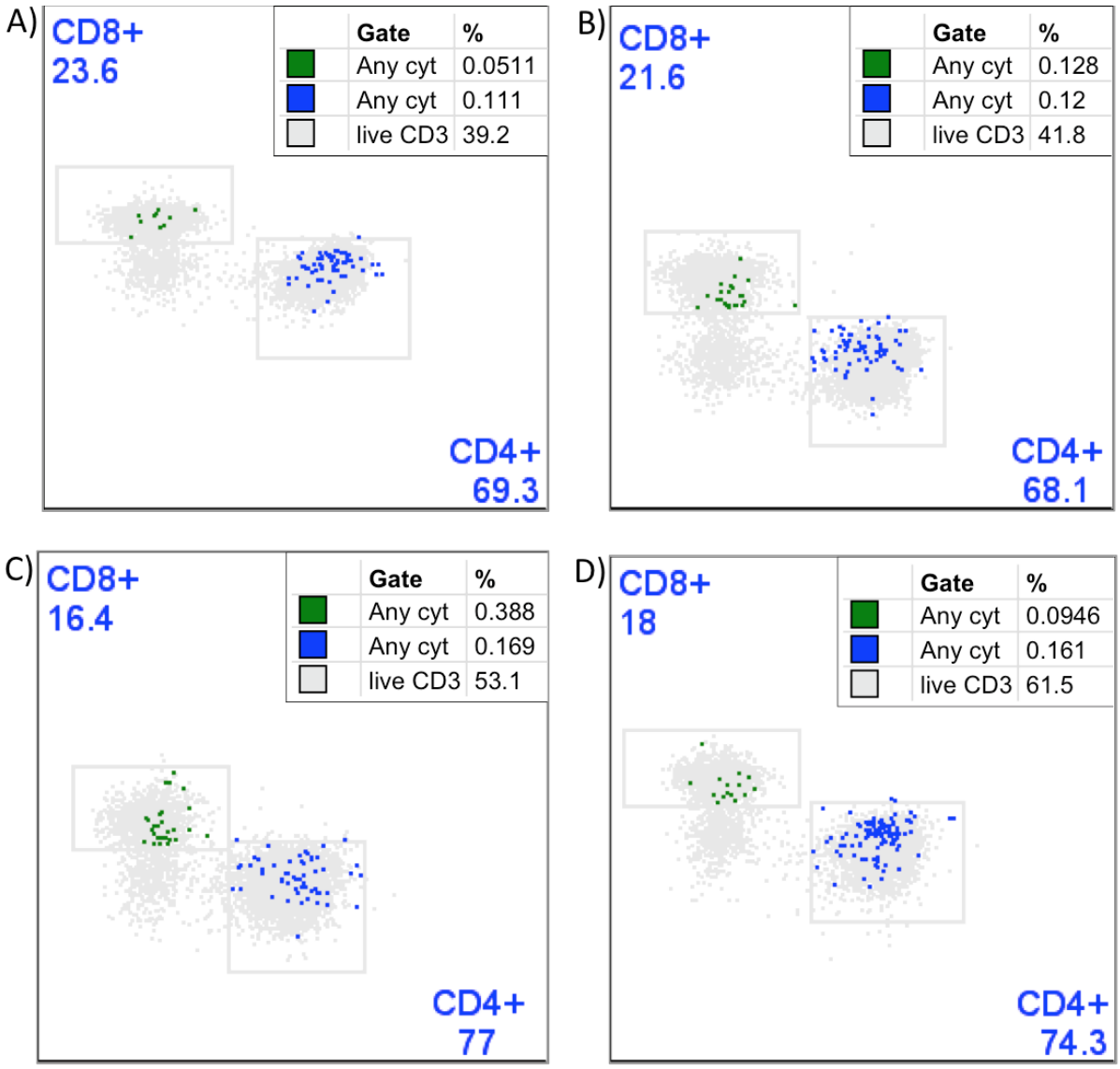


Figure 4.4.5: Representative Flow Plots Of BP-specific CD4+ and CD8+ T-cell Cytokine Responses in HIV Exposed And Unexposed Infants. Panel A and B show week 7 responses from an HIV unexposed and HIV exposed infant respectively. Panel C and D represents week 36 responses from an HIV unexposed and exposed infants respectively. On the X-axis is the frequency of CD4+ T-cells. On the y-axis is CD8+ T-cells. Green dots represent CD8+ T-cells expressing any cytokines (IFNγ , IL-2 and TNFα). Blue dots represent CD4+ T-cells expressing any cytokine (IFNγ, IL-2 and TNFα).

The next analysis was the comparison of cytokine expression in HIV exposed and unexposed infants. Figures 4.4.6 and 4.4.7 below demonstrate the differences in net CD4+ and CD8 T-cells cytokine production between HIV-exposed and unexposed infants. Net responses were used so as to account for the variation in background cytokine expression.

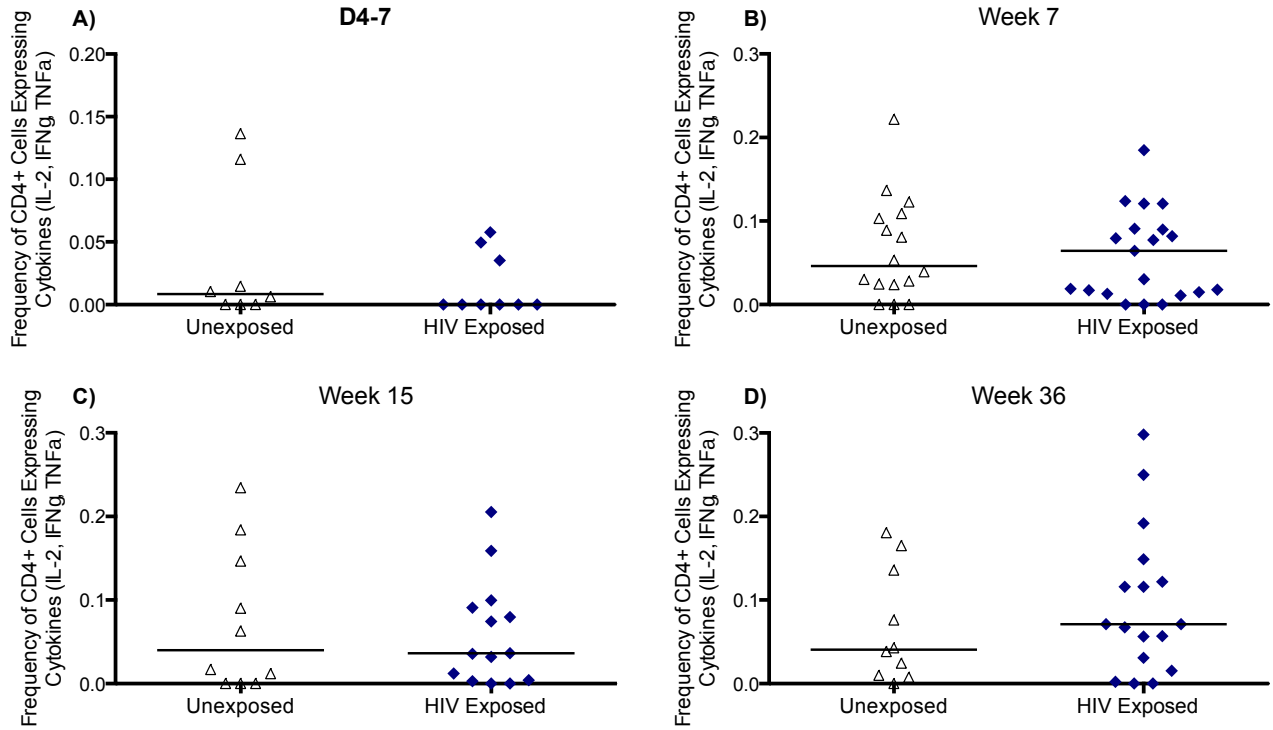


Figure 4.6.5: Representative Flow Plots Of BP-specific CD4+ and CD8+ T-cell Cytokine Responses in Breast Fed and Formula Fed Infants. Panel A and B show week 7 responses from a breast fed (left) and formula fed (right) infant respectively. Panel C and D represents week 36 responses from a breast fed and formula fed infant respectively. On the X-axis is the frequency of CD4+ T-cells. On the y-axis is CD8+ T-cells. Green dots represent CD8+ T-cells expressing any cytokines (IFNγ , IL-2 and TNFα). Blue dots represent CD4+ T-cells expressing any cytokine (IFNγ, IL-2 and TNFα).

No significant differences in CD4+ T-cells were found between the two groups. The only significant difference found in CD8+ T-cells was at week 7, where unexposed infants produced more cytokines than HIV exposed infants. This suggests that exposure to HIV limits CD8+ T-cells cytokine expression at early time points, but this limitation disappears with age.

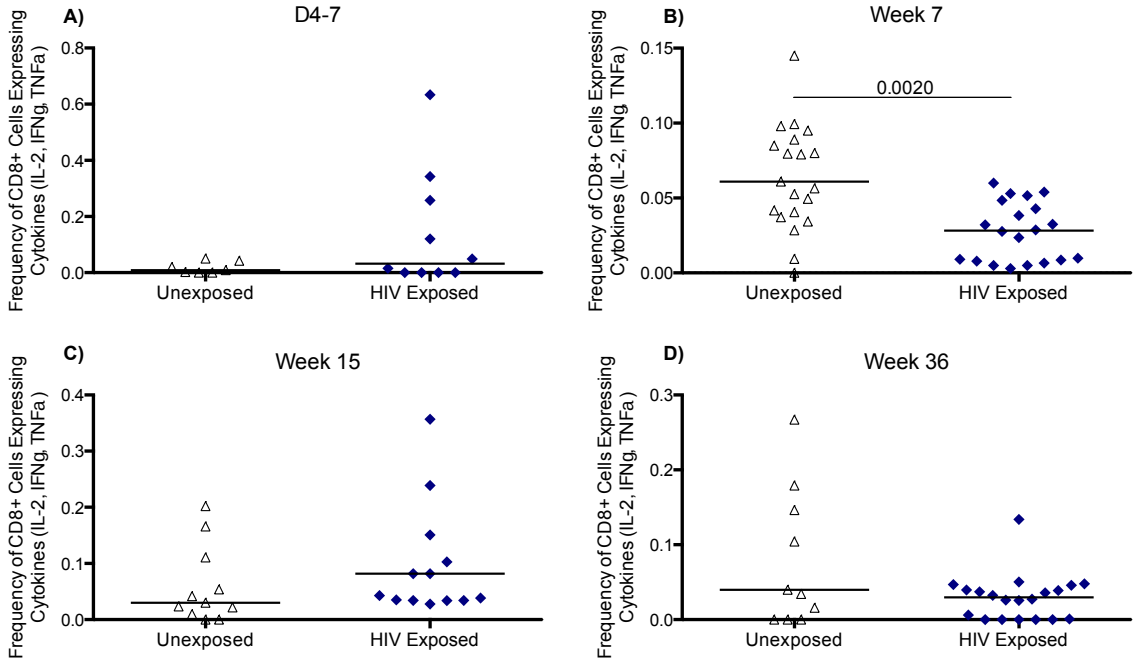


Figure 4.4.7: Comparison of CD8+ T-cell Cytokine Expression In HIV Exposed and Unexposed Infants. Horizontal bars depict medians. Comparisons between HIV exposed and unexposed infants were done with a Wilcoxon signed rank test.

Monofunctional cytokine expression between HIV exposed and unexposed infants were also assessed. Net cytokine expression was used again. Monofunctional cytokine responses were not assessed at D4-7, as the frequencies of these cells were to low to reliably assess at D4-7.

No significant difference in cytokine expression was found in CD4+ T-cells at all time points (appendix 3, page 185). Figure 4.4.8 below demonstrates CD8+ T-cell responses. As seen in the figure no differences were found at week 7 and week 36. However, at week 15 HIV exposed infants have higher expressions of IL-2 and IFNγ compared to unexposed infants. These results, together with the total cytokine analysis suggest that exposure to HIV only limits cytokine expression at very early ages of life, such as that found at week 7. However, by week 15 it seems that HIV exposed infants are able to produce much more cytokine in response to BP stimulation, suggesting that they have better CD8+ IL-2 and IFNγ responses compared to unexposed infants.

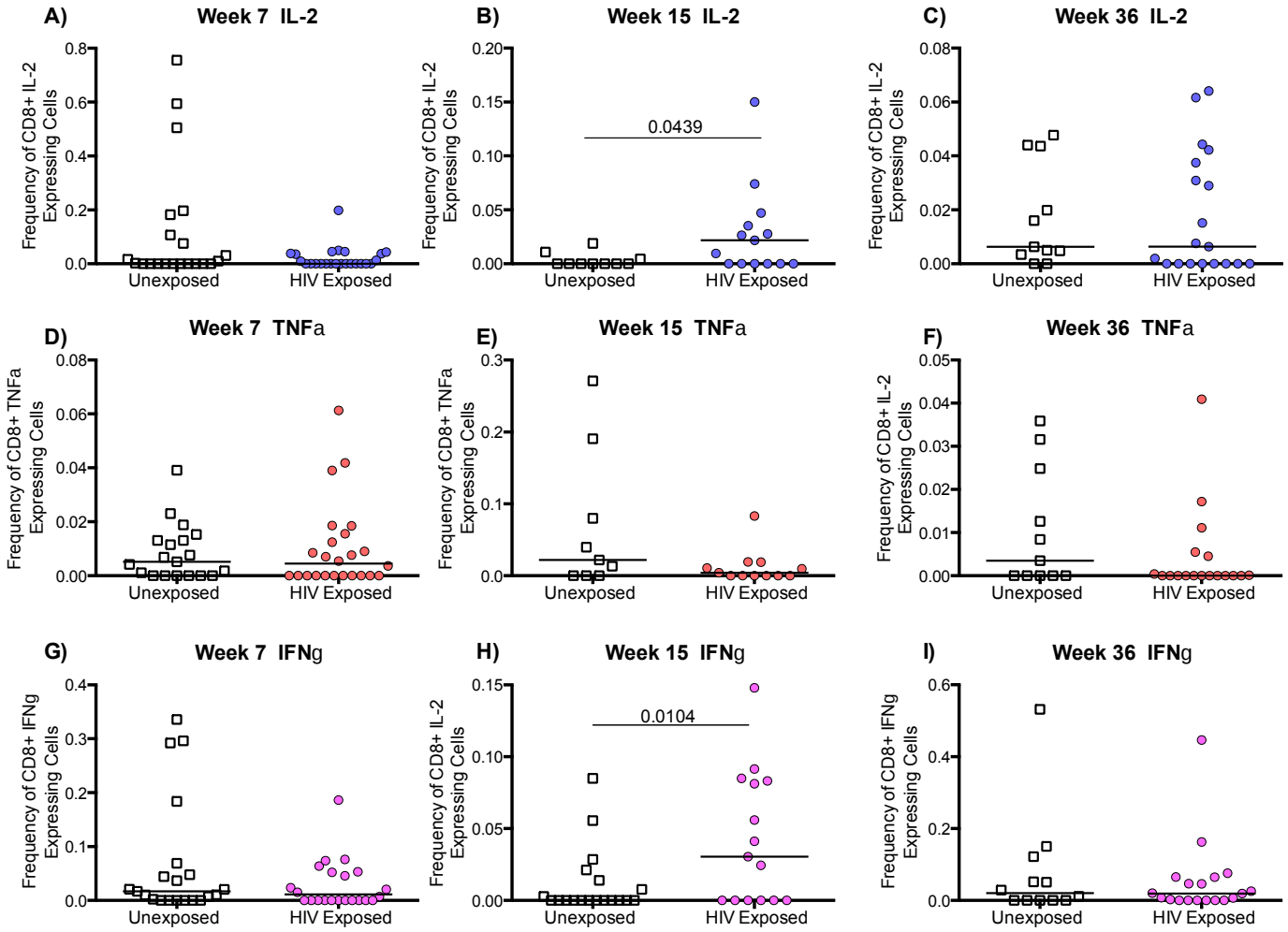


Figure 4.4.8: The Effects of HIV Exposure On CD8+ T-cell Single Cytokine Expression. Open squares represent unexposed infants while coloured circles represent HIV exposed infants. Panels A,D and G demonstrate week 7 responses, panels B,E and H represent week 15 responses and panels C,F and I represent week 36 responses. Row one, two and three demonstrate IL-2, TNFα and IFNγ responses respectively.

In addition to the total and single cytokine analysis, polyfunctional cytokine analyses were also conducted. As discussed in the previous section, polyfunctional analyses provide important information on the functional capacity of a single cell. The data thus far indicated that cytokine responses as well as activation and proliferation peak at week 15. Week 15 is also one-week post completion of the aP vaccine schedule. For this reason, all polyfunctional comparisons were synchronized at week 15 for breast feeding HEU and HU infants.

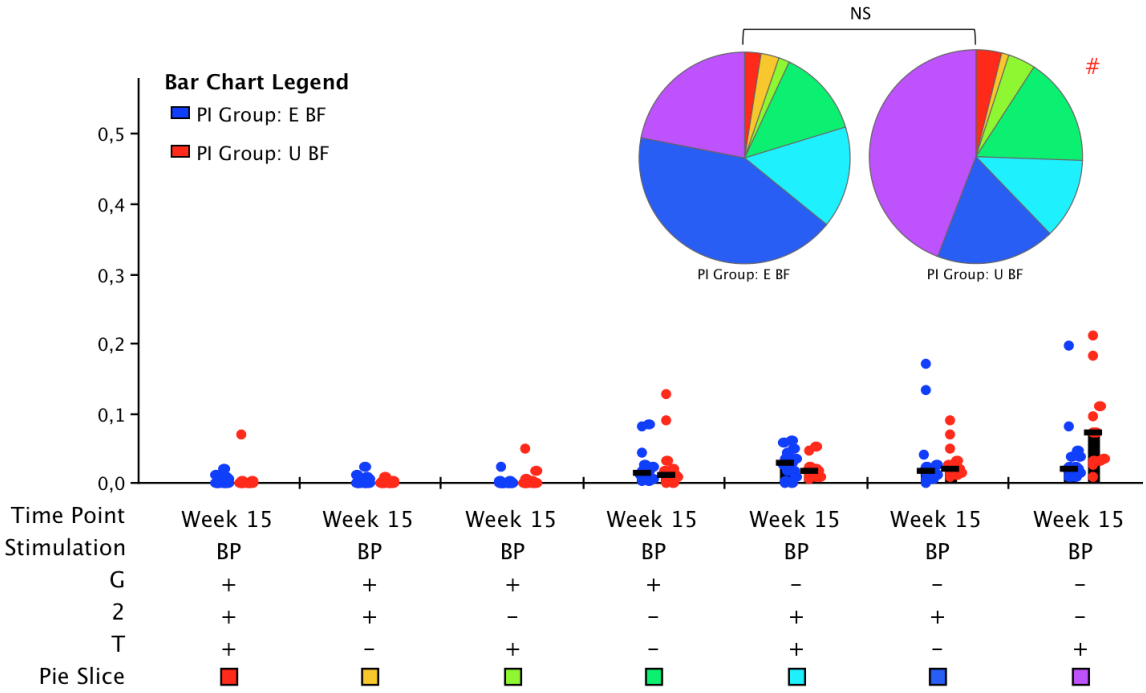


Figure 4.4.9: Cytokine production of BP-specific CD4+ T-cells at week 15. The figure demonstrates the frequency of cytokines produced in different combinations by CD4+ T-cells in U BF (red) and E BF(blue) infants. Solid bars represents medians and # indicates a significant difference based on a Wilcoxon rank test.

HIV unexposed infants expressed significantly higher levels of CD4+ TNFα than HIV exposed infants (Figure 4.4.9). TNFα was also the only CD8+ cytokine from the 3 cytokines analyzed that was not significantly elevated HEU infants when comparing single cytokine responses. Both CD4 and CD8 TNFα responses together suggest that exposure to HIV has overall negative impacts on TNFα expression.

In addition, figure 4.4.10 demonstrates that unexposed infants have more CD8+ T-cells that are simultaneously expressing IFNγ and TNFα. This also supports the theory that HIV exposure limits TNFα responses thus they have fewer cells producing TNFα with other cytokines as well.

While TNFα and co-expressing IFN and TNFα responses are significantly different between HEU and HU infants, overall polyfunctional responses were not different as demonstrated by the pie graphs.

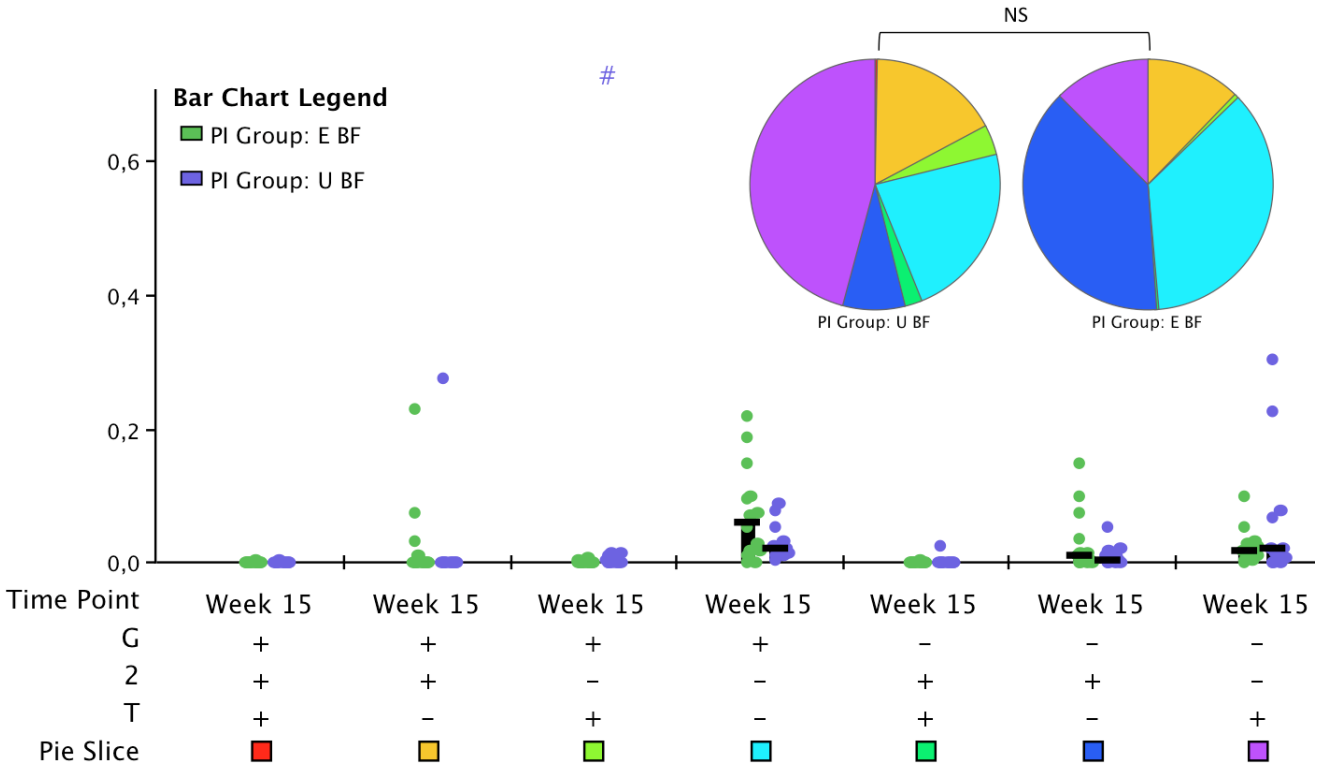


Figure 4.4.10: Cytokine production of BP-specific CD8+ T-cells at week 15. The figure demonstrates the frequency of cytokines produced in different combinations by CD8+ T-cells in U BF (purple) and E BF(green). Solid bars represents medians and # indicates a significant difference based on a Wilcoxon rank test.

THE EFFECTS OF HIV EXPOSURE ON ACTIVATION AND PROLIFERATION

A direct comparison of total CD4+ and CD8+ T-cell activation and proliferation between HIV exposed and unexposed infants was conducted. Figures are shown in appendix 4, page 186. No significant differences between the two groups were found, suggesting that HIV exposure does not have any effect on aP vaccine induced activation or proliferation.

CONCLUSION

Exposure to HIV had minimal effects on activation, proliferation and polyfunctional responses of CD4+ and CD8+ T-cells but did alter memory and cytokine responses. HIV exposure causes infants to have more differentiated memory CD4 and CD8 within the first week of life. Some of these differences are maintained until week 7 but disappear by week 15. Additionally, HIV exposed infants expressed more CD8+ IFNγ and IL-2 but less TNFα at week 15. Furthermore, HEU infants have less overall CD8+ T-cell cytokines at week 7. Together the data in this section suggests that exposure to HIV impacts T-cell responses early in life, however it seems that by week 36, no differences remain between the 2 groups, implying that exposure does not have long-term effects on infant immunity.

SECTION 4.6

THE IMPACT OF FEEDING MODE ON ACELLULAR PERTUSSIS T-CELL RESPONSES

In addition to HIV exposure, breast or formula feeding has been shown to have different impacts on infant immunity (Lawrence & Lawrence, 2010; Slade & Schwartz, 1987). Breast milk contains a host of immunological factors that are essential to providing protection against infectious diseases early in life. However, these factors have also been known to reduce vaccine immunogenicity of some vaccines (S. S. Moon et al., 2010). While many studies have looked at the impact that breast-feeding has on BP vaccines (Quinello, Quintilio, Carneiro-Sampaio, & Palmeira, 2010), very few studies focused on T-cell responses. Thus, the final aim of this chapter was to assess the effects of feeding modality on T-cell responses to aP vaccination. For all analyses completed in this study, only HIV exposed infants were included. No unexposed infants were included to minimize any confounding effects of HIV exposure on the results. While the data in this section overlaps with section 4.2, the key differences between the sections is that section 4.2 assess changes over time within different groups of infants, while this section focuses on the statistical differences between HEU breast fed and HEU formula infants at each time point.

THE EFFECTS OF FEEDING MODE ON MEMORY DIFFERENTIATION

To determine the effects that feeding mode had on memory differentiation, HIV exposed breast-fed and formula fed infant memory T-cell phenotypes were compared at each time point. Only unstimulated memory responses were shown as BP stimulation had minimal affects on the response. As previously mentioned total CD4+/CD8+ T-cell memory differentiation was measured, as it was not possible to look at vaccine specific cells due to the low frequency of cytokine positive cells. Thus, BP stimulated differentiation mimicked the differentiation found in the total memory populations.

At D4-7 E FF infants had higher frequencies of naïve and TD memory CD4+ T-cells compared to E BF infants. In addition both CD4 and CD8 ED memory cells were elevated in breast fed infants.

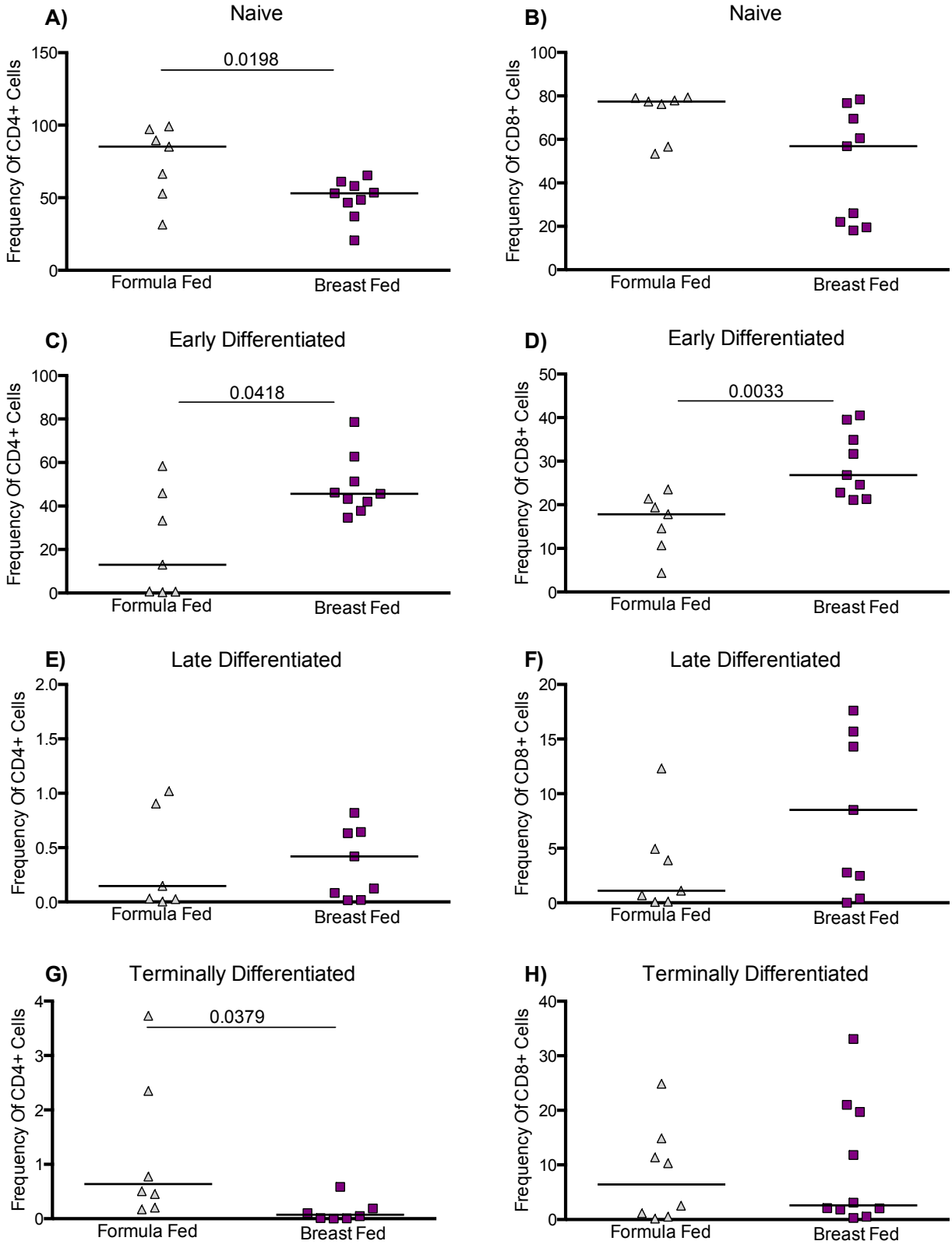


Figure 4.6.1: The Impact Of Different Feeding Modes On CD4+ and CD8+ T-cell Memory Differentiation at D4-7. Purple squares represent breast fed infant responses while gray triangles represent formula fed infants. Horizontal bars depict medians. Panels A,C,E and G represent CD4 differntiation while panels B,D,F and H represent CD8 differentiation. Comparisons between breast and formula fed infants were completed with a Mann-Whitney test and P-values were plotted on the graph.

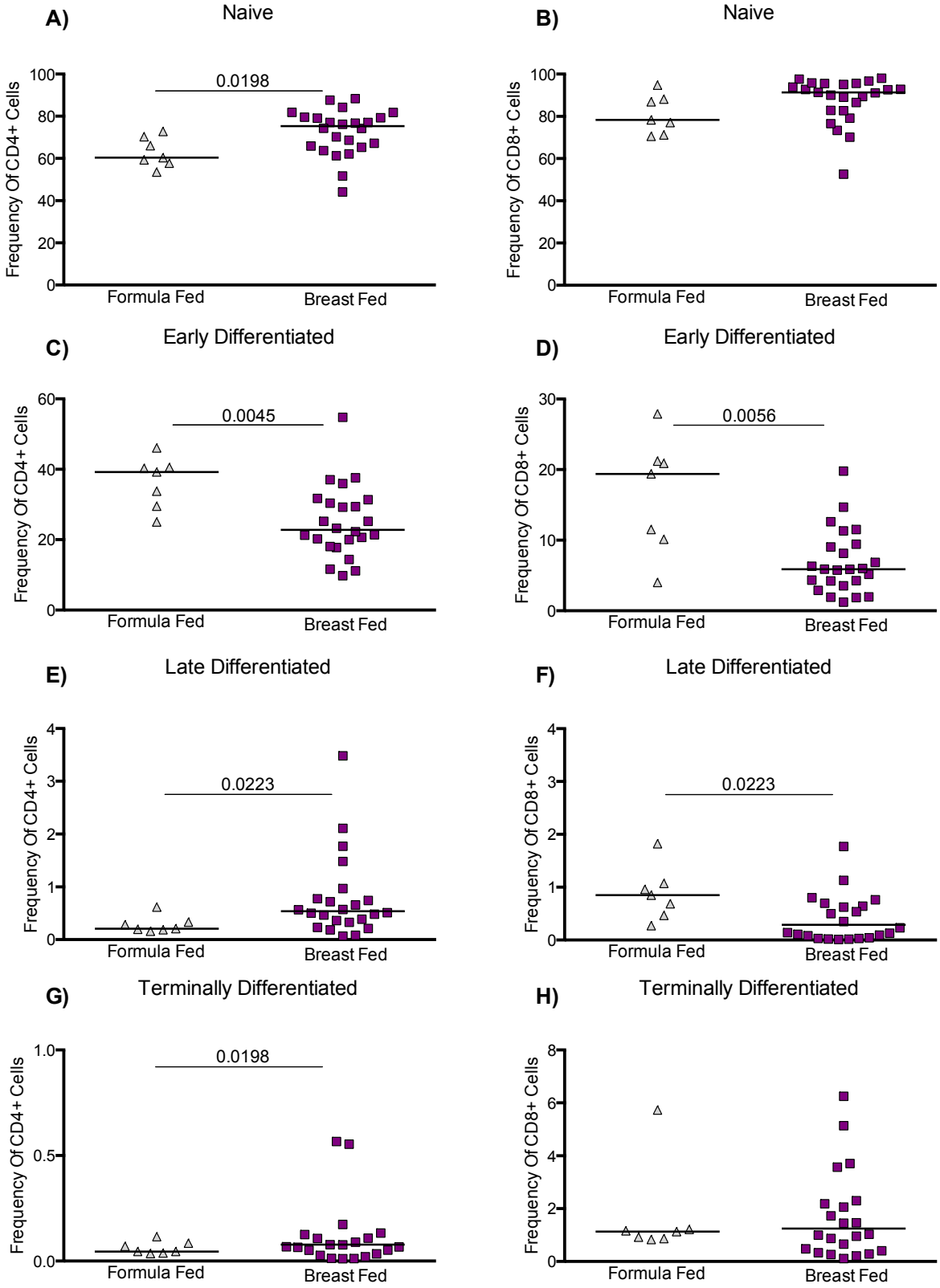


Figure 4.6.2: The Impact Of Different Feeding Modes On CD4+ and CD8+ T-cell Memory Differentiation at Week 7. Purple squares represent breast fed infant responses while gray triangles represent formula fed infants. Horizontal bars depict medians. Panels A,C,E and G represent CD4 differntiation while panels B,D,F and H represent CD8 differentiation. Comparisons between breast and formula fed infants were completed with a Mann-Whitney test and P-values were plotted on the graph.

As evident in the figure 4.6.2 breast fed infants had more naïve, LD and TD memory CD4+ T-cells by week 7. This is an inversion of the D4-7 memory populations. In addition breast fed infants had less ED memory CD4+ and CD8+ T-cells. In contrast with LD CD4+ memory cells breast-fed infants had less LD CD8+ memory cells compared to formula fed infants. These findings suggest that breast fed infants have more differentiated CD4+ memory cells but less differentiated CD8+ memory cells compared to formula fed infants at week 7.

At week 15 the differences found in LD and TD memory cells, both CD4+ and CD8+ seem to balance out (Figure 4.6.3). This maybe due to fact that by week 15 many of the breast fed infants start to mix feed, thus the immune system of these infants start to resemble that of formula fed infants. The only difference that persists is that formula fed infants had higher proportions of ED CD4+ and CD8+ memory cells, as well as more naïve CD4+ memory cells than formula fed infants.

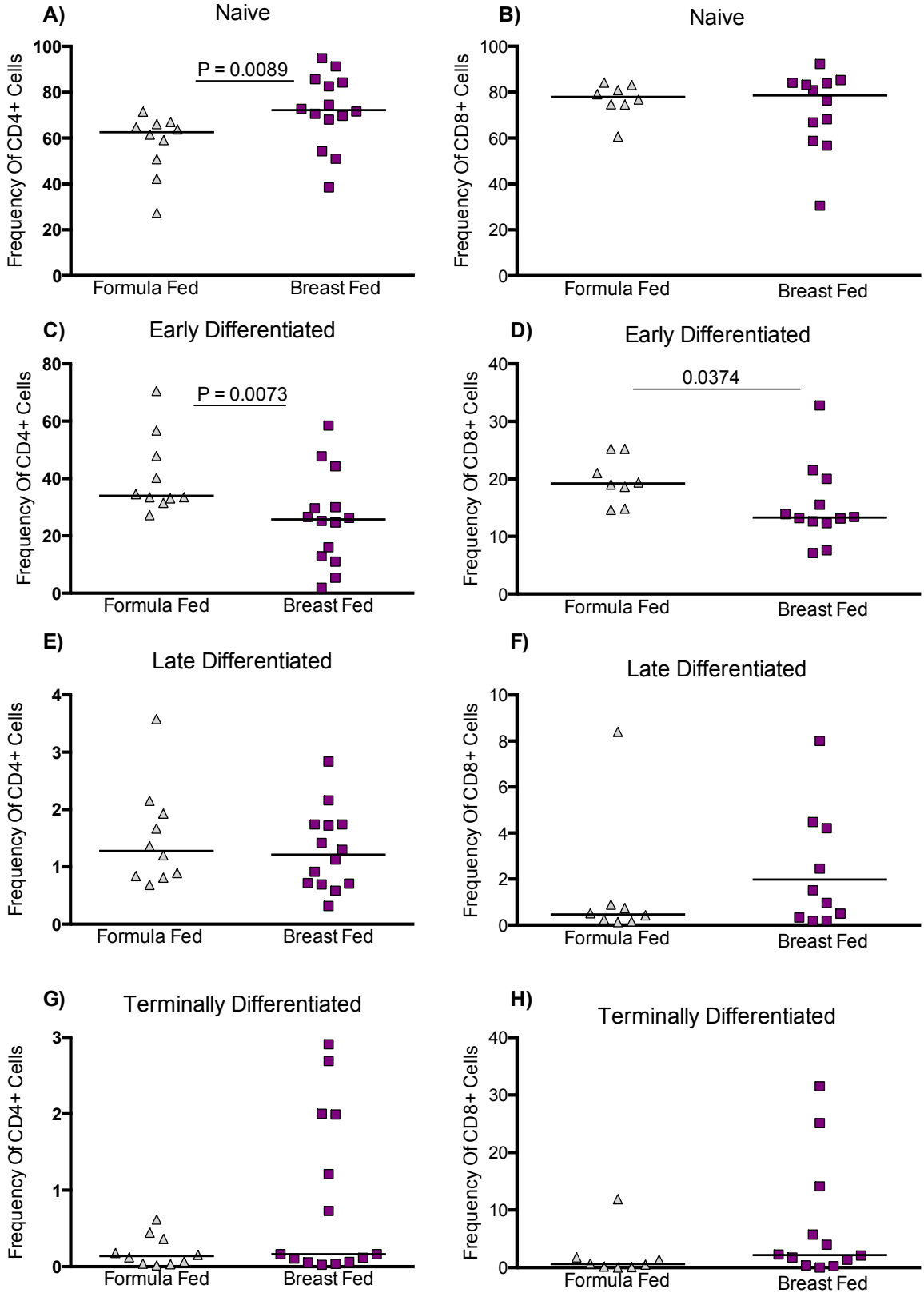


Figure 4.6.3: The Impact Of Different Feeding Modes On CD4+ and CD8+ T-cell Memory Differentiation at Week 15. Panels A, B, C and D represent naïve like, early, late and terminally differentiated memory populations respectivetly. Purple squares represent breast fed infant responses while gray triangles represent formula fed infant responses. Horizontal bars depict medians. Comparisons between breast and formula fed infants were completed with a Mann-Whitney test and P-values were plotted on the graph.

By week 36 there seems to be another inversion of memory cell populations. Breast fed infants have less naïve CD8+ T-cells compared to formula fed infants (Figure 4.6.4). In addition, it seems that breast fed infants also have less naïve CD4+ T-cells and increased ED CD4+ T-cells compared to formula fed infants, however this is not statistically significant. Furthermore, LD CD4+ T-cells and TD CD8+ T-cells are significantly higher in breast fed infants compared to formula fed infants. It also looks like breast fed infants have more LD CD8+ memory T-cells, albiet not significant. Taken together this data suggest that formula feeding alters memory T-cell differentiation, especially short term CD4+ memory differentiation but long term CD8+ memory differentiation.

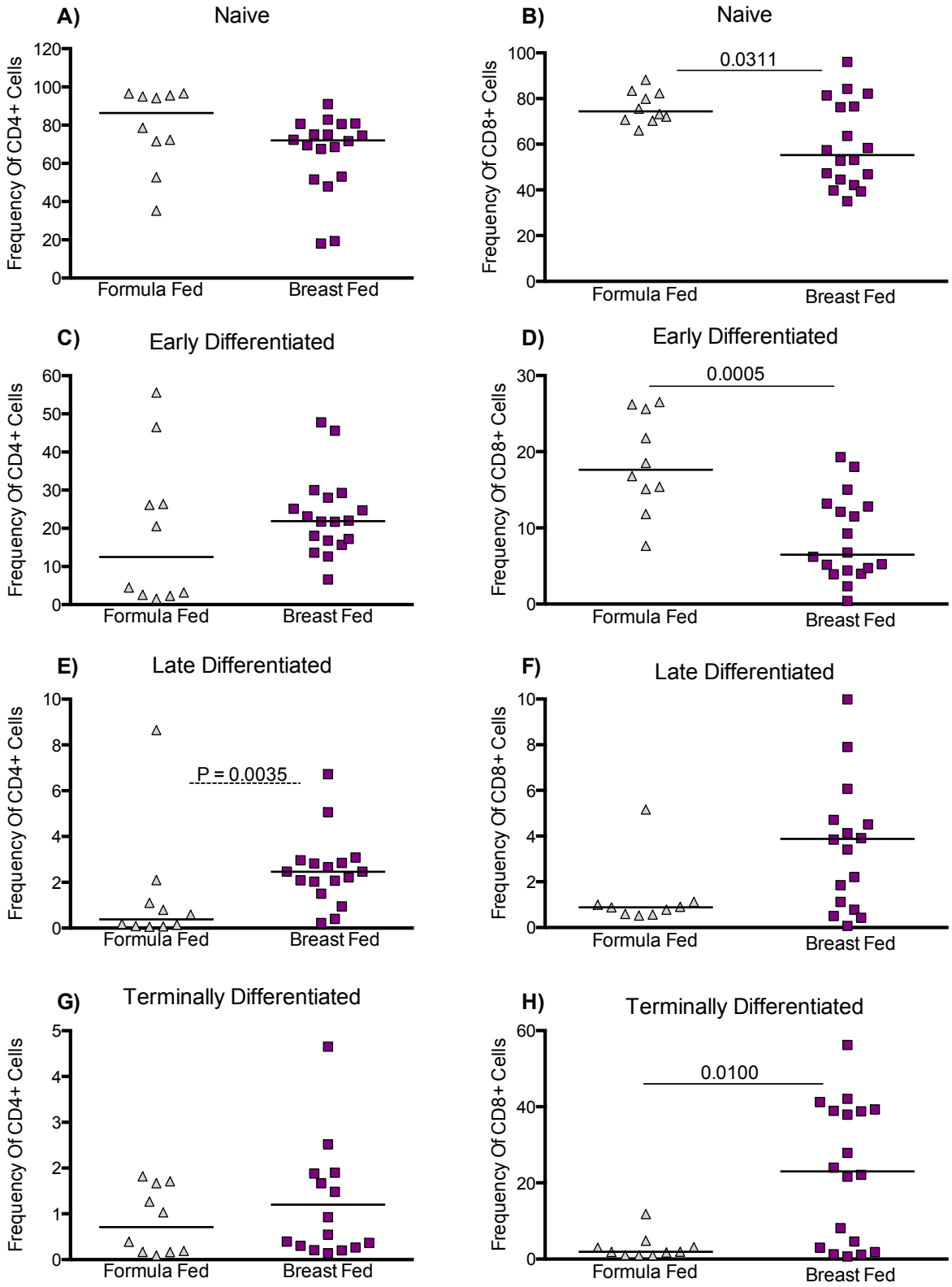


Figure 4.6.4: The Impact Of Different Feeding Modes On CD8+ T-cell Memory Differentiation at Week 36. Panels A, B, C and D represent naïve like, early, late and terminally differentiated memory populations respectively. Purple squares represent breast fed infant responses while gray triangles represent formula fed infant responses. Horizontal bars depict medians. Comparisons between breast and formula fed infants were completed with a Mann-Whitney test and P-values were plotted on the graph.

THE EFFECTS OF FEEDING MODE ON CYTOKINE PRODUCTION

Figure 4.6.5 below demonstrates a representative flow plot of cytokine positive CD4+ and CD8 T-cells between breast-fed and formula fed infants.

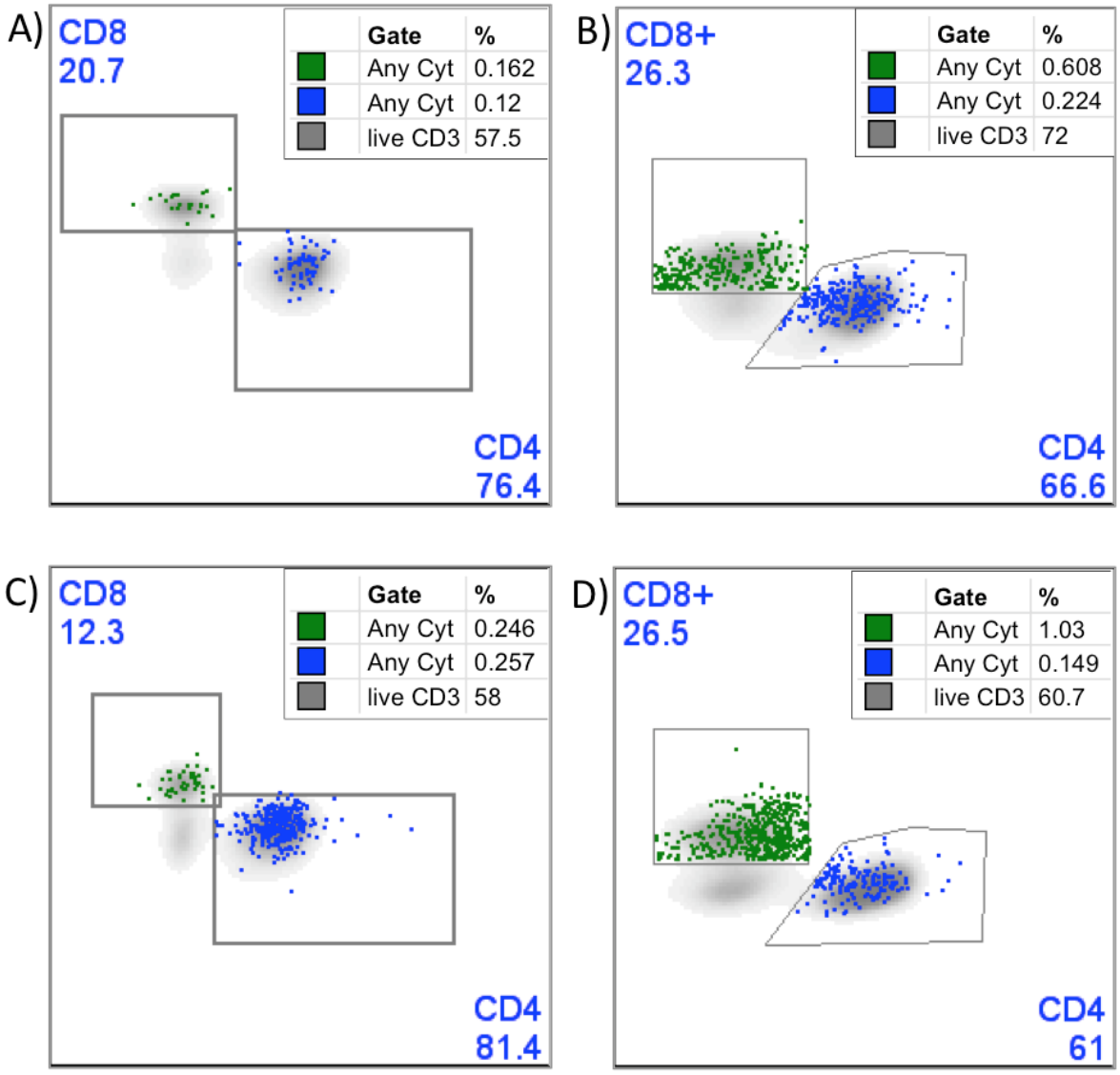


Figure 4.6.5: Representative Flow Plots Of BP-specific CD4+ and CD8+ T-cell Cytokine Responses in Breast Fed and Formula Fed Infants. Panel A and B show week 7 responses from a breast fed (left) and formula fed (right) infant respectively. Panel C and D represents week 36 responses from a breast fed and formula fed infant respectively. Green dots represent CD8+ T-cells expressing any cytokines (IFNγ , IL-2 and TNFα). Blue dots represent CD4+ T-cells expressing any cytokine (IFNγ, IL-2 and TNFα).

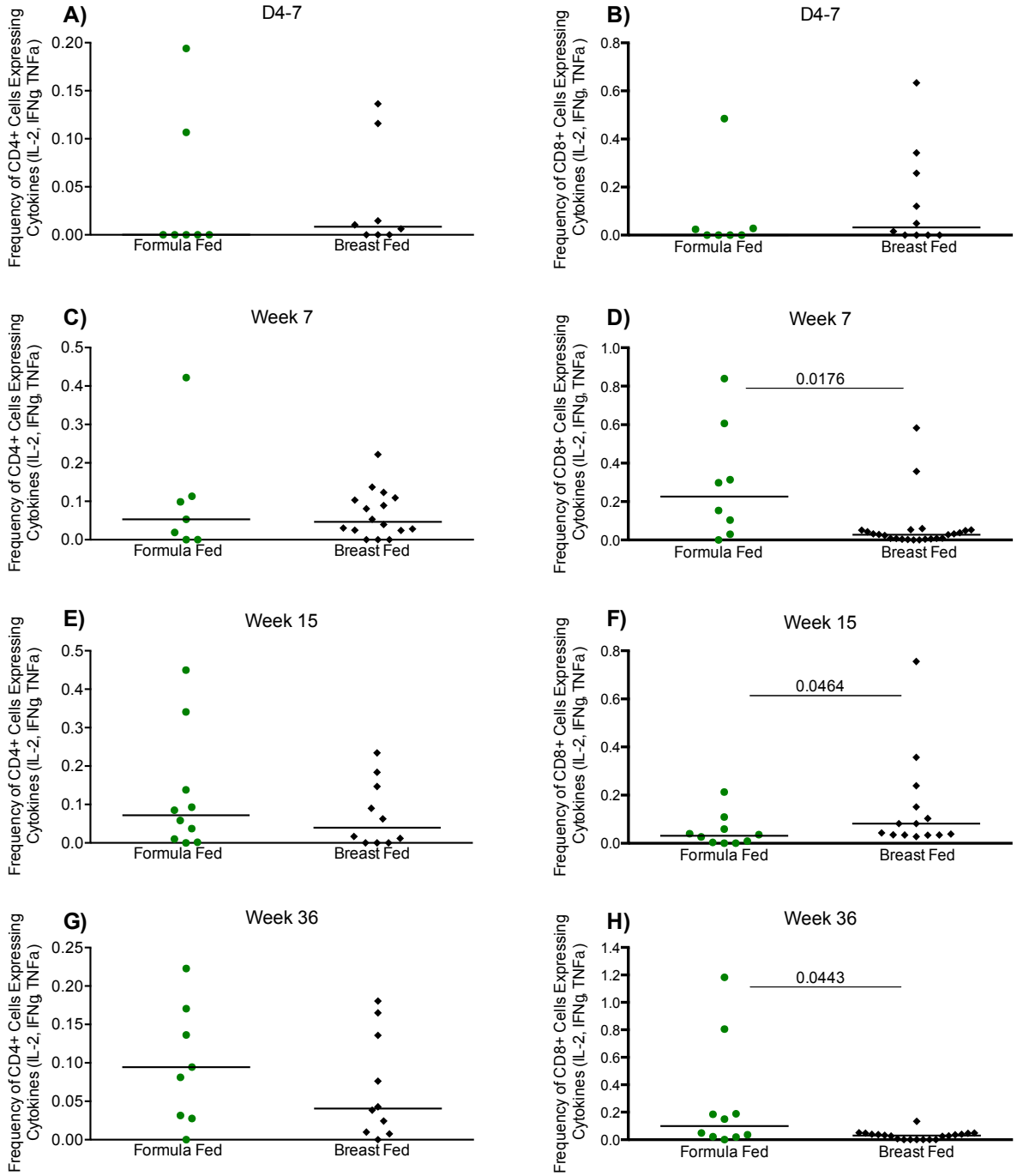


Figure 4.6.6: Impact of Feeding Mode On CD4+ and CD8+ T-cell Cytokine Production. Panels A, C and D represent net CD4+ T-cell cytokine responses, while panels B, D and F show net CD8+ T-cell cytokine responses. Horizontal bars depict medians. Comparisons between breast fed and formula fed infants were done using a Mann-Whitney test.

No significant differences were found between formula fed and breast fed infants CD4+ T-cells suggesting that feeding practise does not drastically affect CD4+ T-cell cytokine expression. However, formula fed infants expressed significantly more CD8+ T-cell cytokines at week 7 and week 36 compared to breast fed infants. In contrast, at week 15 breast fed infants expressed more CD8+ T-cell cytokines

compared to formula fed infants. Thus, feeding practise alters CD8+ T-cell cytokine responses however, the impacts this alteration has on infant health is not clear.

Single cytokine responses were also assessed. As mentioned previously, at D4-7 there were to low frequencies of cytokine expressing cells to reliably analyse. No significant differences were found between breast fed and formula fed infants IL-2 and IFNγ expression as shown in appendix 5, page 188. Figure 4.6.7 below depicts net TNFα responses found in formula fed and breast fed infants at all time points post vaccination.

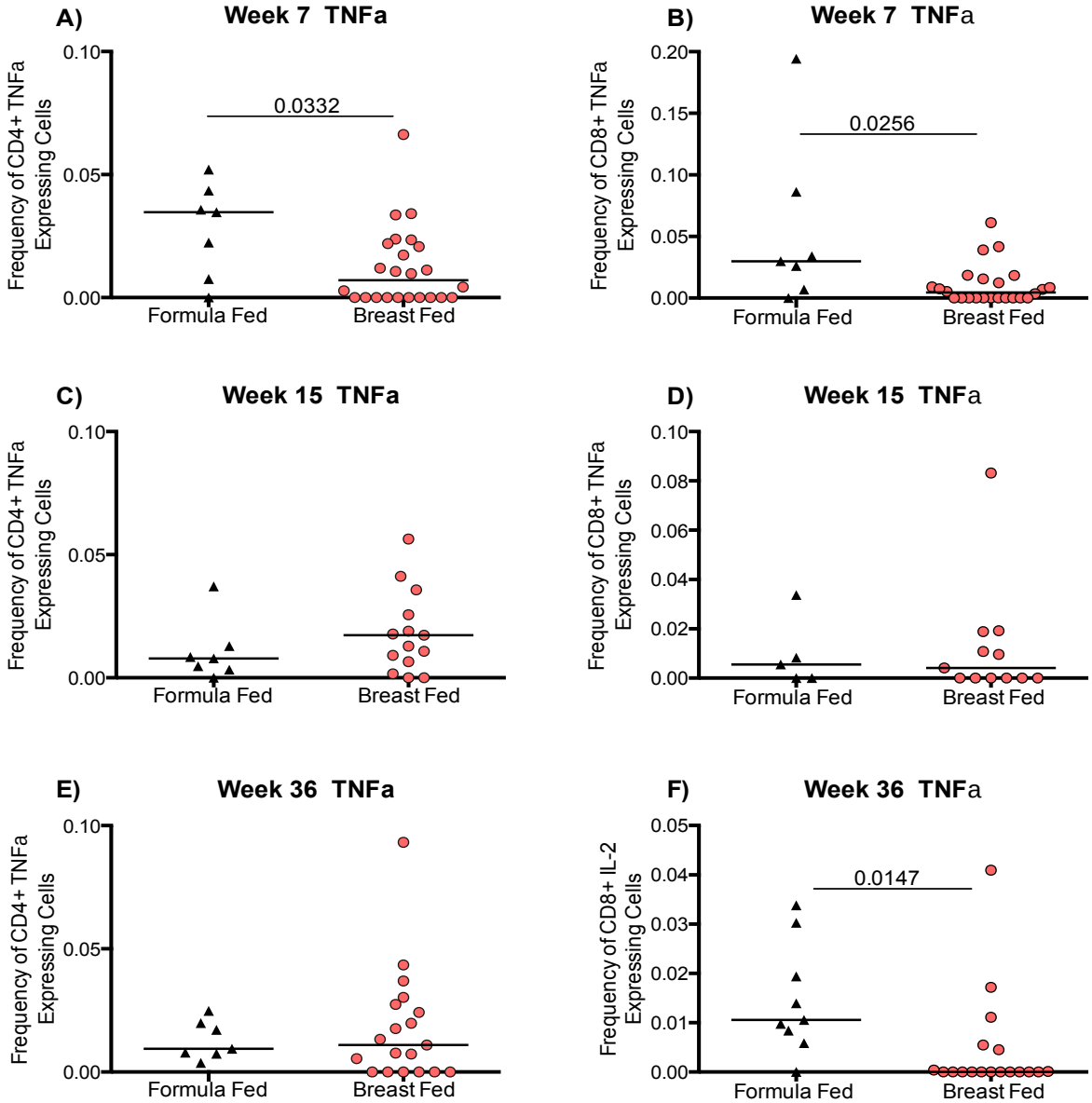


Figure 4.6.7: Net BP Specific CD4+ and CD8+ T-cell Expression of TNFα at Week 7, 15 and 36. Black triangles represent formula fed infant responses while peach circles represent breast fed infant responses. Panels A,C and E demonstrate CD4+ T-cell responses and panels B,D and F represent CD8+ T-cell responses. Horizontal bars depict medians. Comparisons between breast fed and formula fed infants were done using a Mann-Whitney test.

As seen in figure 4.6.7, formula fed infants have high expression of both CD4+ and CD8+ T-cells TNFα at week 7 and higher expression of CD8+ TNFα at week 36. Previous studies have found similar results and suggests that this difference is due to the anti-inflammatory immune system that breast feeding promotes (Kainonen, Rautava, & Isolauri, 2013).

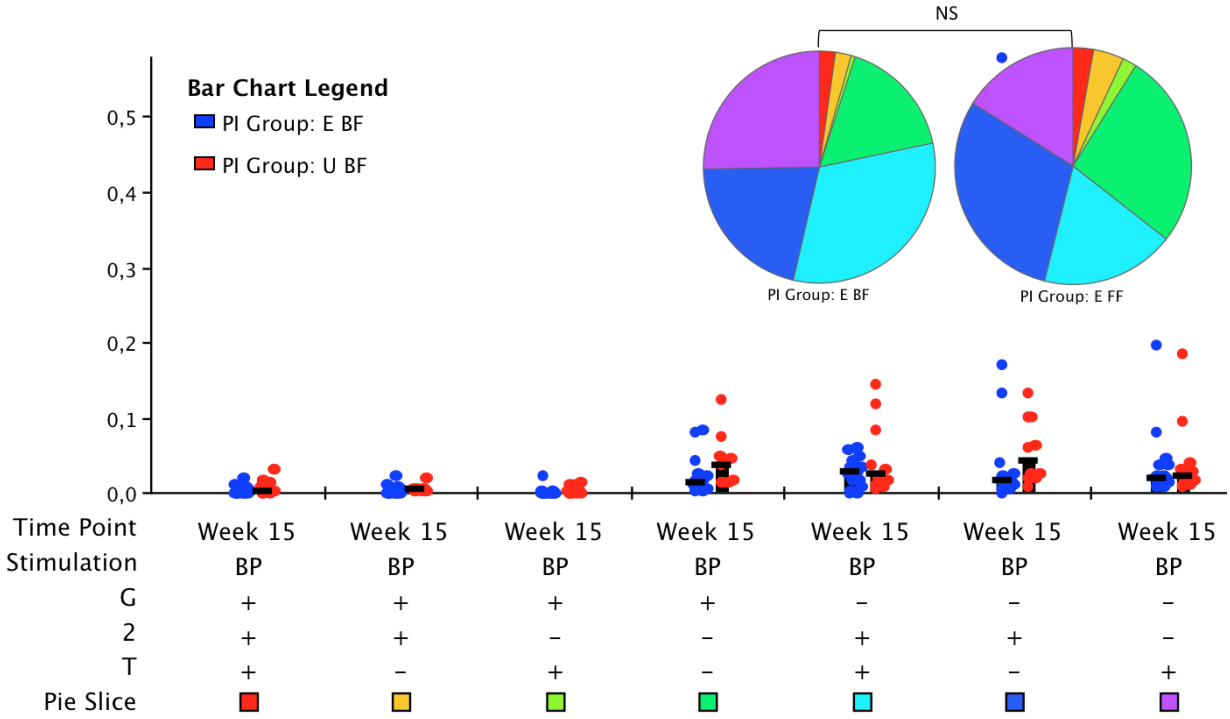


Figure 4.6.8: Polyfunctional Cytokine production of BP-specific CD4+ T-cells at week 15. The figure demonstrates the frequency of cytokines produced in different combinations by CD4+ T-cells in U BF (red) and E BF(blue) infants. Solid bars represents medians and # indicates a significant difference based on a Wilcoxon rank test.

As with HIV exposed and unexposed infants, polyfunctional responses between breast and formula fed infants were also analysed at week 15. No significant differences were found in CD4+ or CD8 T-cell responses, suggesting that feeding practise does not singificantly alter polyfunctionality of T-cell responses.

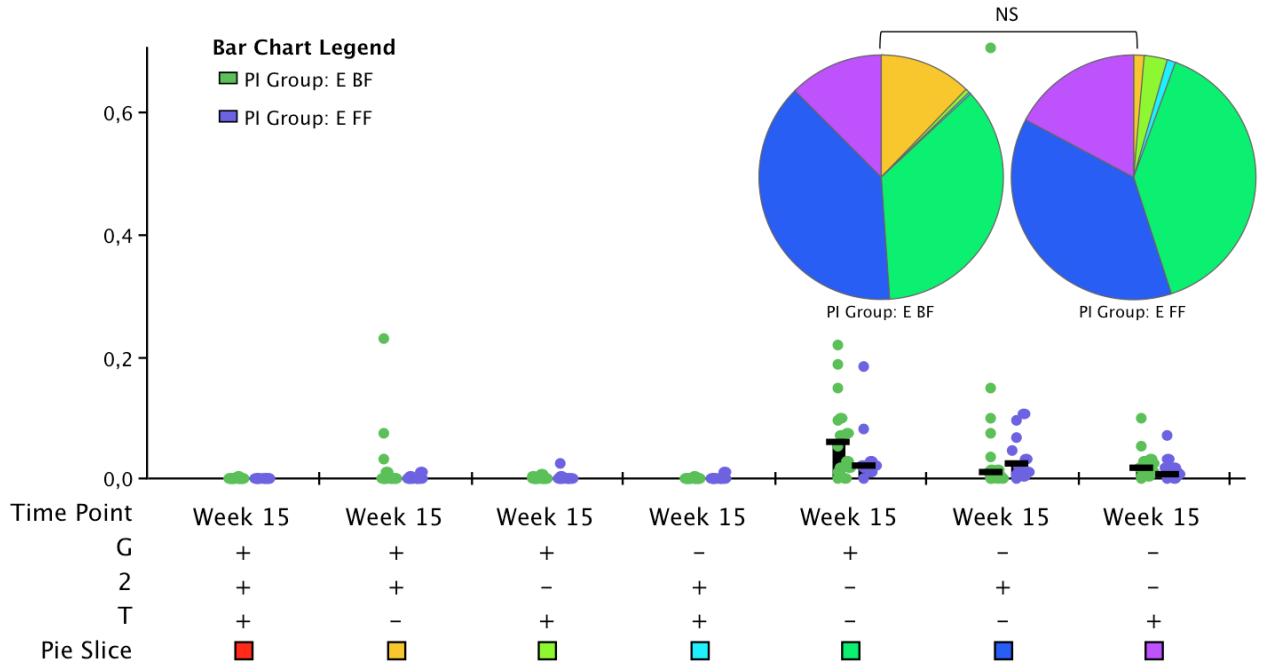


Figure 4.6.8: Polyfunctional Cytokine production of BP-specific CD8+ T-cells at week 15. The figure demonstrates the frequency of cytokines produced in different combinations by CD4+ T-cells in U BF (red) and E BF(blue) infants. Solid bars represents medians and # indicates a significant difference based on a Wilcoxon rank test.

THE EFFECTS OF FEEDING MODE ON CD4+ AND CD8+ T-CELL ACTIVATION AND PROLIFERATION.

The final analysis of this section was to assess the impacts of feeding mode on activation and proliferation of both CD4+ and CD8 T-cells. CD4+ responses are shown in appendix 6, page 90. The only significant difference found, was that formula fed infants had a higher level of activation (p-value = 0.0256) when stimulated with BP antigen at week 7 compared to breast fed infants.

Figure 4.6.9 below demonstrates the CD8 activation in unstimulated and BP stimulated blood. At D4-7 breast fed infants had higher levels of HLA-DR than formula fed infants but by week 7, formula fed infants express higher levels of HLA-DR compared to breast fed infants. This is also true for week 36 BP stimulated blood, where formula fed infants have more activation then breast fed infants.

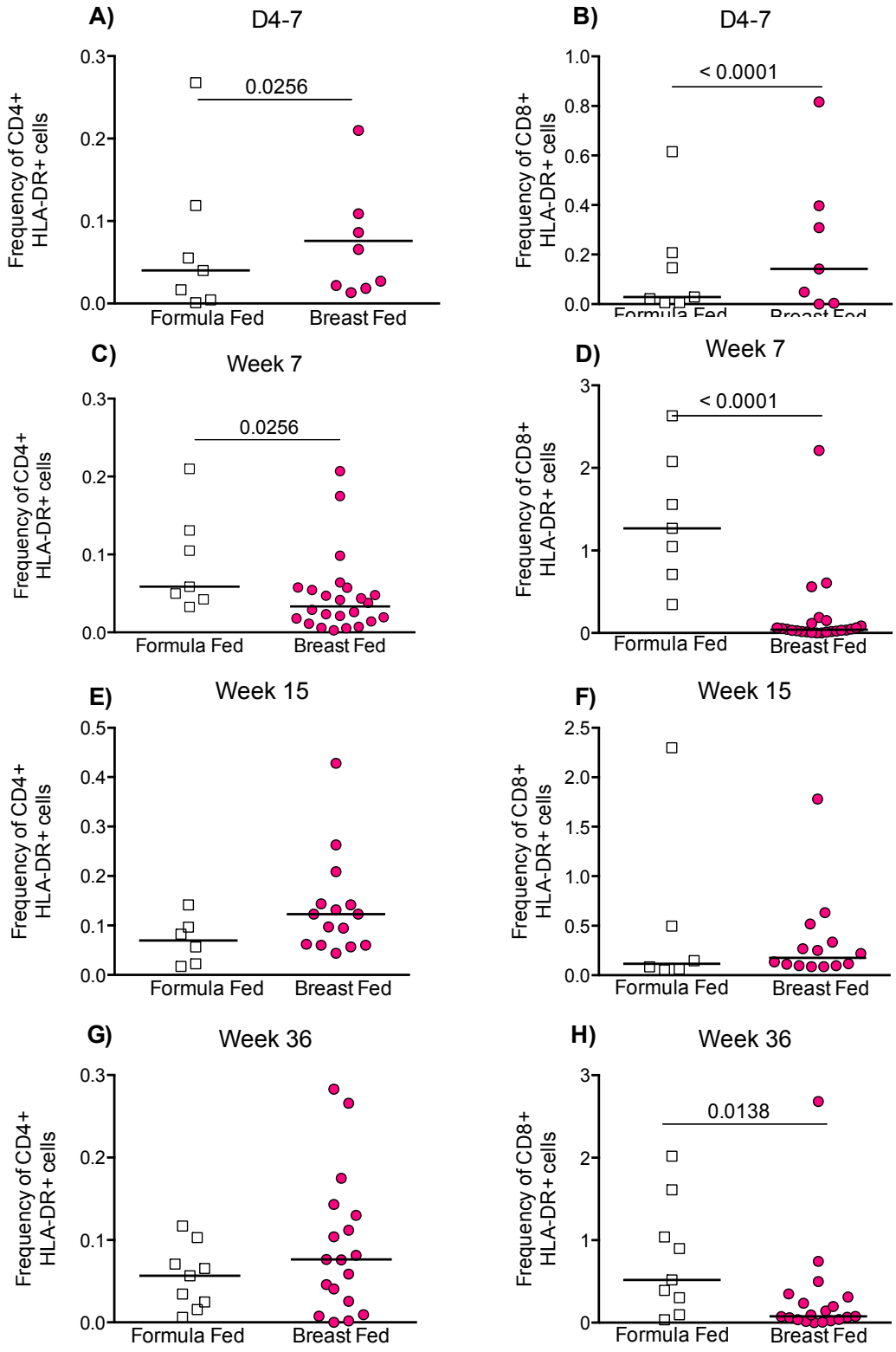


Figure 4.6.9: Impact Of Feeding Mode On CD4+ and CD8+ T-cell Activation. Open squares represent formula fed infants and pink circles represent breast fed infants. Horizontal bars depict medians. Comparisons between formula fed and breast fed were done with a Mann-Whitney test.

T-cells proliferative potential was also compared between breast and formula fed infants (appendix 6, page 190). No significant differences were found between these

two groups of infants, suggesting that T-cell proliferation is negligibly altered by feeding practises.

CONCLUSION

In summary, the data in this section suggest that formula feeding results in several alterations in the immune sytem of infants. These alterations include increased levels of activation post vaccination and less memory differentiation at D4-7 and week 36 but more differentiation of memory cells at week 7. Formula fed infants also had elevated CD8+ vaccine specific cytokine responses at week 7 and week 36. Furthermore TNFα responses were also significantly higher in formula fed infants. Thus, we may conclude that formula fed infants have better aP vaccine responses compared to breast fed infants.

SECTION 4.7

DISCUSSION

IMMUNE RESPONSE TO B.PERTUSSIS INFECTION

B. pertussis (BP) is generally considered an extracellular pathogen, but is capable of infecting respiratory epithelial cells and macrophages (Lamberti, Hayes, Perez Vidakovics, Harvill, & Rodriguez, 2010; Paddock et al., 2008). Alveolar macrophages and dendritic cells are the first cells at the site of infection. Recognition of BP pattern-associated molecular patterns (PAMPs) and virulence factors results in maturation and cytokine expression by macrophages and dendritic cells(DCs) (Nishida et al., 2010; Z. Y. Wang et al., 2006). Mature DCs then express IL-12 and IFNg, which stimulate TH1 responses (Higgins et al., 2003). Additionally, DCs activated by intrinsic BP virulence factors have been shown to also enhance TH17 responses (Spensieri et al., 2006). Following the recruitment of DCs and macrophages, there is an infiltration of NK cells, neutrophils and γδT-cells in the lung. γδT-cells orchestrate early trafficing of innate immune cells by expressing IL-17 (Zachariadis, Cassidy, Brady, & Mahon, 2006). Neutrophils conduct antibody-mediated phagocytosis, intracellular killing of bacteria and formation of extracellular traps (Andreasen & Carbonetti, 2009; Eby, Gray, & Hewlett, 2014). In addition, IL-12 stimulated NK cells express IFNγ, enhancing the antimicrobial activity of macrophages.

αβT-cell responses occur much later on during infection and develop relatively slowly in a primary response. However, these T-cells are crucial for bacterial cleareance. Murine studies have shown that persistent and lethal infections may occur in mice that are αβ T-cell deficient (Mills et al., 1993). Adoptive transfer of T-cells to deficient mice resulted in bacterial clearance within 14-21 days post challenge. Figure 4.7.1 shows the course of the immune response to natural infection. Whole cell pertussis (wP) vaccines have been shown to induce immune responses that closely mimick immune responses to natural infection, with a dominant TH1/TH17 (IFNγ/IL-17) response (Brummelman, Wilk, Han, van Els, & Mills, 2015; Ross et al., 2013). In contrast, aP vaccines seem to induce a Th2/Th17 immune response (Ross et al.,

2013; Warfel, Zimmerman, & Merkel, 2014) with predominant IL-4, IL-5 and IL-17 expression, but low IFN-γ expression.

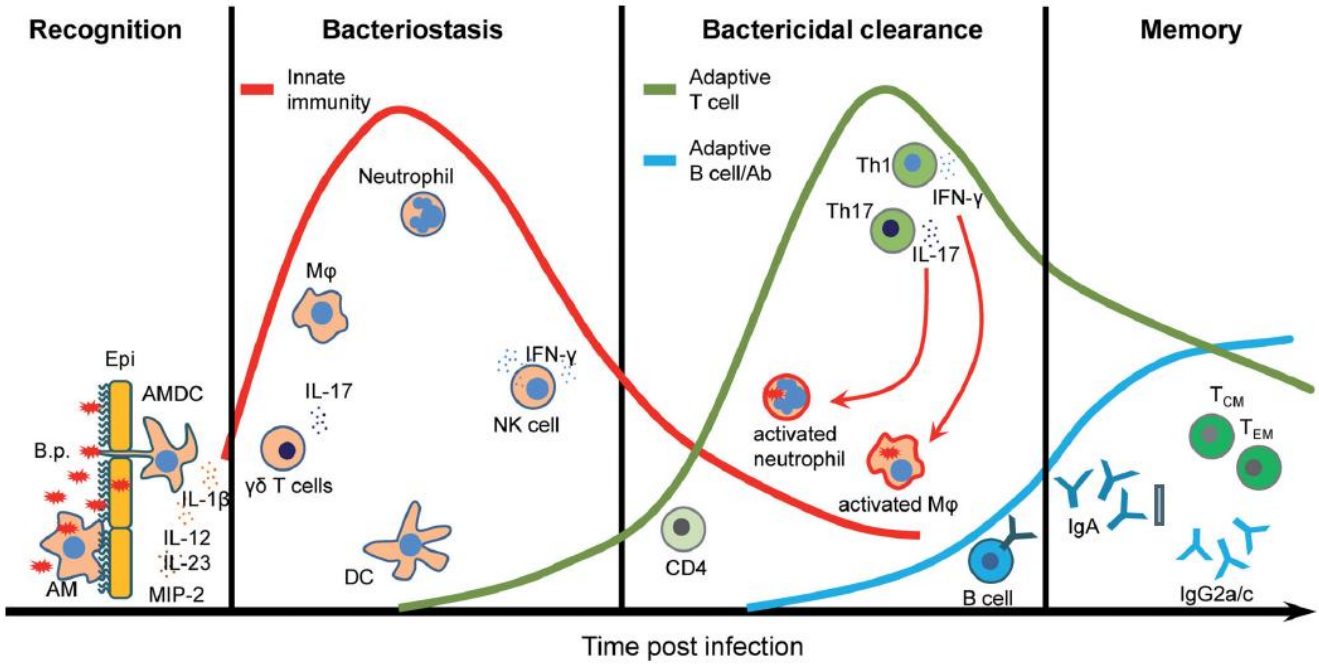


Figure 4.7.1: Immune response to natural Bordetella Pertussis infection and the development of memory cells (Brummelman et al., 2015)**.** B.p., *Bordetella pertussis*; Epi, epithelium; AMs, alveolar macrophages; AMDCs, airway mucosal dendritic cells; DCs, dendritic cells; Mφ, macrophages; Neu, neutrophils; Th, T helper cells; T_{CM}, central memory T cells; T_{EM}, effector memory T cells.

Furthermore, a study assessing the memory subtypes of T-cells induced by both vaccines found that wP induced a higher proportion of central memory and effector memory T-cells, while aP vaccines induced higher proportion of terminally differentiated T-cells (de Rond et al., 2015).

MEMORY T-CELLS DIFFERENTIATION

In this study, we found an overall increase of LD and TD memory CD4+ T-cells in all three groups of infants. Similarly, CD8+ T-cells for both groups of breast fed infants but not formula fed infants. This is important to note, as different types of memory cells may exhibit different functional abilities and therefore confer differing levels of immune protection. However, the detected memory responses were found in total CD4 and CD8 T-cells not just BP specific T-cells, and unstimulated blood showed similar patterns of increasing LD and TD memory populations with age. In fact,

unstimulated blood exhibited more significant increases in LD and TD cell populations than BP stimulated blood. This suggests that the level of LD and TD memory T-cells detected in BP stimulated blood is simply reflecting the overall maturation of the infant immune system.

The only significant changes observed in BP specific CD4+ T-cells was an overall increase in TD memory cells from D4-7 to week 36. This was found in all groups of infants, suggesting that all infants are responsive to aP vaccination. These results support findings by *de* Rond et al, who also found that aP vaccines induced high proportions of terminally differentiated memory cells (de Rond et al., 2015). However a large number of end-stage memory cells may indicate that aP induced T-cell responses are only capable of excecuting short-term immunity but may become exhausted resulting in sub-optimal protection in the long term (Brummelman et al., 2015). This may attribute to the the early waning of immunity found in aP vaccinated individuls. Many studies have demonstrated that wP(DTP) vaccines are much more effective than aP (DTaP) vaccines. One study conducted in Califonia showed that even after 5 doses of DTaP, children showed waning immunity to Bordertella Pertussis within 6 years after completion of the vaccine schedule (Clark, Messonnier, & Hadler, 2012).

In contrast to the proportions of LD and TD memory cells, total and cytokine specific ED CD4+ memory cells in breast fed infants were consistent at all time points. Since ED memory cells are the first type of memory cells to develop in an immune response, it makes sense that infants, being constantly exposed to new antigen/vaccines would have a high number of ED memory cells. Repeated antigen exposure is needed for the production of LD and TD memory cells, thus these cells take time to develop. However, repeated antigen exposure may also result in activation of naïve and stem-cells which would make more ED memory cells as the pre-exsisting memory cells form LD and TD memory. Thus, infants are likely to have an unchanging proportion of ED memory cells within there circulation. CD8+ ED memory responses in E BF infants seem to peak at week 15, while similar patterns are observed in U BF infants, it is not at a significant level. This possible occurs due

to the large number of vaccines infants receive at week 14, causing multiple T-cells to differentiate into ED memory cells at a faster rate then normal.

Formula fed infants had different CD4 and CD8 memory profiles in the total T-cell population compared to breast fed infants. Unstimulated CD8 naïve, ED and LD memory populations remained unchanged over time. This may be due to the fact that formula contains multiple antigens, which can stimulate the immune system very early in life. This may explain the observation that 4-7 day old infants possesed a large proportion of differentiated memory cells. Thus changes from D4-7 to week 36 are not as marked relative to changes observed in breast fed infants. Formula fed infants also had increased naïve and TD memory cells at week 36 with decreased LD and ED memory cells. This supports the notion that formula feeding results in immune stimulation causing terminal memory differentiation which may lead to exhaustion and death. As explained earlier, prolonged exposure to antigen favours apoptosis. Thus by week 36 most formula specific CD4+ T-cells would have died and the infants immune system would have built a regulatory response to the antigens in formula resulting in a decrease in ED and LD memory cells with an increased number of naïve like cells. However, changes in BP specific CD4+ T-cells across time was the same as that found in breast fed infants, suggesting that the discrepancies found in the general T-cell memory pool has minimal effects on aP vaccine specific CD4+ memory differntiation.

Taken together these findings suggests that breast milk plays a crucial role in preserving memory population by restricting exhaustive differentiation. A varity of anti-inflammatory factors in breast milk, have been shown to induce a tolerogenic enviroment in infants, which may contribute to the differences in memory differentiation between breast and formula fed infants (Cerini & Aldrovandi, 2013). It would explain the high proportion of naïve T-cells at D4-7 in breast fed infants and the gradual increase in memory cells with time. At week 15 and week 36 most of the infants in this cohort were being mixed fed. This would infer that they are drinking less breast milk hence, receiving fewer tolerogenic signals which would allow for the development of memory cells. In addition the intake of solid foods may cause different antigenic stimuli that outweigh the tolerance signals from breast milk.

Direct comparisons of BP specific CD4+ T-cells showed no differences between memory populations, with the exception of ED CD4+ T-cells at week 15, which suggests that dispite the differences in total T-cell differentiation, both formula and breast fed infants have similar memory responses to aP vaccination. In addition to the differences in phenotype of blood lymphocytes, we show that breast and formula fed infants exhibit differences in vaccine responses and immune function, similar to findings in other studies (Pabst et al., 1997).

CD4+ AND CD8+ T-CELL ACTIVATION AND PROLIFERATION

One of the immune functions assessed in this study was T-cell proliferation. In all groups of infants D4-7 proliferative responses were higher than post-vaccination time points suggesting that infants have rapid T-cell turnover in first week of life. Proliferation remained at a constant level at all time points post vaccination in breast fed infants. The constant baseline levels of proliferation through the first 9 months of life may be attributed to the fact that infants are in a stage of life where they encounter many new antigens. Each antigen exposure stimulates the immune system, causing T-cell proliferation to remain elevated in infants. Formula fed infants had peak T-cell proliferation at week 15, which is probably due to the high volume of vaccines received at week 14 causing a stimulation of the immune system. However, direct comparisons showed no significant differences in proliferation were found among the 3 infant groups at any time point.

Furthermore, one of the main hypothesies of this study was that HEU infants would have a higher level of activation compared to unexposed infants during early time points, which we observed at D4-7. Both HEU groups of infants had the highest expression of HLA-DR at D4-7. In support of our findings literature has shown that exposure to HIV-antigens or other opportunistic pathogens in the fetus of a HIV infected women results in chronic activation and inflammation in her infant (Halapi et al., 1999; Kou et al., 2000; Rich, Siegel, Jennings, Rydman, & Landay, 1997). In our cohort however activation was acute not chronic, as activation in HEU infants. With current advances in PTMCT, infants likely have little to no antigen exposure in utero, which may account for the normalisation of actiavtion levels at week 7.

Post-vaccination T-cell activation in breast fed infants peaked at week 15 post-vaccination. This peak in activation was found regardless of stimulation and therefore is not BP antigen specific. Peak activation in breast fed infants maybe attributed to increased likelihood of introduction of non-breastmilk foods starting at or before week 15. In contrast to the week 15 peak in activation found in breast fed infants, formula fed infants seem to have peak T-cell activation earlier, at week 7, which is consistent with previous evidence demonstrating that formula feeding results in systemic immune activation (Pabst et al., 1997). This implies that food antigens may be inducing immune activation. As with memory differentiation these findings suggests that formula feeding has a negative impact on immune function since there are potentially fewer naïve T- cells available for response to vaccines and pathogens.

In addition, individuals with elevated immune activation are at higher risk of acquiring HIV then those with a quiescent immune system (Cohen et al., 2010). This is probably due to an increase in the number of activated CD4+ T-cells, which are targets for HIV infection, found in an activated immune system (Kaul et al., 2008). The relative deficiency of tolerance-inducing factors found in breast milk may additionally explain why formula fed infants have a higher level of activation at week 7 which gradually decreases. The decrease in activation with age in this group is likely due to the infants' immune system adapting to antigens found in formula milk. While immune activation is a necessary precursor for most cytokine production, excessive amounts of activation may not only lead to increased susceptibility to HIV and reduced vaccine responses but, may also cause immune exhausion and tissue damage through inflammation (Watkins, Maier, & Goehler, 1995). Therefore, we may conclude that formula feeding is not as good as breast feeding for immune ontogeny.

CYTOKINE RESPONSES TO BP STIMULATION IN AP VACCINATED INFANTS

In addition to activation and proliferation, cytokine responses to BP stimulation were also assessed. Th1 cytokines were predominantly measured, which have been previously noted to be induced by aP vaccination. Upon assessment of total cytokine responses, it was found that HIV exposed infants produced significantly higher levels of CD4 cytokines when stimulated with BP compared to unstimulated blood. This was

not seen in U BF infants. However, when compared side-by-side, there were no signficant differences in CD4+ Th1 cytokine responses between E BF and U BF infants. In the CD8 compartment, both groups of breast fed infants expressed levels of cytokines above background but formula fed infants did not.

It is difficult to interpret exactly what significance over backgroud might mean, as background cytokine expression may be affected by multiple factors. Co-infections, maternal influences, diet, environment, other vaccines etc, may all impact backgound cytokine levels. In addition, some of the background maybe aP specific responses, as vaccines were only given one week prior to conducting this analysis. Therefore infants may still have activated T-cells producing cytokines to the vaccine without stimulation, resulting in a marginally increases in cytokine production upon stimulation with BP antigen. Therefore a cytokine response which is not significantly higher than background does not necessarily indicate a lack of vaccine response. However, given that a good vaccine should induce memory cells capable of elobrating vast amounts of cytokines upon antigen exposure, it can be expected that infants who secrete more cytokines in response to BP stimulation have better immune responses. The fact that cytokine responses were significantly higher than background levels as early as 7-weeks of age in some infants suggests that these responses are indeed real vaccine specific response, as week 7 is only one week post priming and using significance over background is a reliable approach to assessing cytokine responses.

Further analyses of cytokine changes over time demonstrated that BP specific CD4+ T-cell responses peaked at week 36 in E BF infants and at week 7 in E FF infants. CD8+ T-cell responses peaked at week 7 for U BF and E FF infants, but no obvious changes across time was observed for E BF infants. Side-by-side analysis of net CD8+ responses showed that HU infants had better CD8 TH1 cytokine responses at week 7 compared to HEU infants. This suggests that HU infants are able to mount aP specific responses more rapidly than HEU infants. However, since no differences are observed between HU and HEU infants at week 15 and week 36, imply that differences seen at week 7 are not long-lasting and that both groups of infants will have similar levels of protection againt Bordertella Pertussis by week 15 of age.

Assessment of single cytokine responses showed that BP specific CD4+ and CD8+ IL-2 cytokine production was higher at one or more time points post-vaccination compared to D4-7 in U BF infants. In E FF infants BP specific CD4+ and CD8+ IL-2 expression increased from D4-7 to week 7 but then dropped again at week 36. The spike in IL-2 expression observed at week 7 is expected as IL-2 is an early cytokine mainly expressed by naïve-like and ED memory cells. As LD and TD memory cells begin to form with time, these cells probably start to express other cytokines and IL-2 expression decreases. Had we acquired sufficient numbers of cytokine expressing T-cells, the polyfunctional nature of different specific memory cells could have been analysed.

In U BF infants the presence of breast milk is likely dampening immune responses to aP vaccination at early time points, thus there is a gradual increase in IL-2 expression. This is likely benefical to the infant as it prevents excessive activation and inflammation stimulated by IL-2. In contrast to U BF and E FF infants, E BF infants did not show any changes in cytokine production with age, however at week 7 and week 36 their CD4+ IL-2 expression was significantly higher in BP stimulated blood compared to unstimulated blood. This suggests that these infants are responsive to aP vaccination but that the magnitude of their IL-2 response does not change with time.

Furthermore CD4+ TNFα responses peaked at week 15 for both groups of breast fed infants but this was not seen in E FF infants, also suggesting that breast fed infants develop better memory responses. However, HIV exposed infants, both breast an formula fed expressed CD4 TNFα at significantly higher levels in BP stimulated blood compared to unstimulated blood at week 15 and 36. This was not seen in U BF infants, suggesting that HIV exposure actually allows for better CD4+ TNFα responses to aP vaccination. However side-by-side comparisons of TNFα between HEU and HU infants showed no significant differences between groups. Additionally E FF infants also had CD8+ TNFα responses above background levels at week 7 and week 36. Side-by-side comparisons further supported this findings, revealing that formula fed infants had better TNFα responses then breast fed infants. A paper by

B.Winkler et al showed that formula feeding promotes TH1 responses, including the expression of TH1 cytokines such as TNFα. Without the intake of formula, infants immune systems are naturally TH2 skewed, limiting the production of TH1 cytokines an infants may express. Thus, it seems that intake of formula balances the TH1/TH2 skewing allowing formula fed infants to express higher levels of TNFα in response to aP vaccination.

In addition to IL-2 and TNFα, IFNγ was assessed. Analysis of IFNγ responses showed that U BF infants showed a decrease in CD4+ IFNγ expression from week 7 to week 36, and in the CD8 arm E FF infants showed a decrease from week 7 to week 15. Moreover, background CD4 IFNg expression was similar to vaccine induced IFNg expression. CD8 IFNγ expression was higher then background levels at week 7 in U BF infants and at week 15 in HIV exposed infants. This supports many findings which have shown that aP induces TH2 type immune responses with reduced IFNg production (Ross et al., 2013). This is disadvantageous as IFNg is necessary for clearence of BP infections (Mahon et al., 1997). IFNγ is expressed by late differentiated memory cells and thus should increase with time. However, the opposite seems to be true for U BF infants, whos CD4 IFNγ expression decreased from week 7 to week 36. One explanation for this may be that, maternal factors are driving the spike in IFNγ expression at week 7. Alternatly, it may imply that U BF infants have a good balance of ED and LD memory cells which are capable of sustaining long term immunological memory while rapidly expressing IFNγ in response to stimulation.

Comparative assessments between groups showed that feeding practise did not alter IFNγ responses but that HEU infants had better CD8+ IFNγ responses than HU infants at at week 15. In addition HEU infants also had better CD8 IL-2 responses at week 15. These findings imply that exposure to HIV, permits better peak CD8+ IL-2 and IFNγ responses to aP vaccination. Literature by Jones et al, found similar results, where HEU infants had better aP vaccine responses compared to HU infants (Jones, 2013). Taken together these findings suggest that HIV exposed infants may have better aP vaccine responses compared to unexposed infants.

Finally, few differences in polyfunctional responses were found between different infant groups. CD8 polyfunctional responses were best detected at week 7 while CD4

polyfunctional responses looked best at week 15. Most infant responses were of a single cytokine expressing phenotype, with few dual-cytokine producing phenotypes. CD8+ T-cell were almost exclusively single and some double cytokine expressing cells. While, CD4 cells exhibited minor populations of triple cytokine producing cells and some dual cytokine producing cells. This suggests that the quality of infant responses to stimulation is restricted, possible due to the suppressive environment of the immune system or that we were unable to detect polyfunctional responses due to the use of an inappropriate cytokine panel.

One of the major shortcomings of this study was the small number of participants enrolled. This was due to the late implementation of aP in the WBA of the parent study, and a slower than expected rate of enrolment. However, enrolment of this study is ongoing, and more data will be added to the current database to improve the statistical power of this study. Also, much of the focus of this study was on TH1 cytokines, IFNg, IL-2 and TNFa. While these cytokines have proved to be important in the immune responses to natural infection, much of the current literature suggests that TH2 responses are more important in aP vaccine responses. Thus, assessment of TH2 cytokine responses would probably be more informative than looking at TH1 cytokine responses alone.

CHAPTER 5

CONCLUSION

This dissertation has explored methodologies to identify T cell responses to vaccine responses: rotavirus and pertussis. Rotavirus cellular immunity has been shown to have an important role in preventing Rotavirus disease. However, very limited data is currently available on the manner in which T-cells contribute to Rotavirus protection after vaccination. Additionally, there is no assay available for the assessment of Rotavirus T-cell responses. This may be because it is very difficult to assess T-cell responses to rotavirus or because it has always been assumed that antibodies are the primary source of protection (Ward et al., 1992). In this study, optimization of an assay capable of detecting T-cell Rotavirus responses was attempted. However due to the difficulty in purifying antigens capable of eliciting a Rotavirus vaccine response, the assay could not be optimized. Thus, the first step for further investigation would be to try alternate methods of antigen purification. Ultracentrifugation may be tried for separation of DS-1 virus from Vero cells and other LPS removal kits maybe employed for purification of endotoxin-contaminated proteins. Given the important role T-cells seem to play in protective responses to Rotavirus infection/vaccination, optimizing an assay capable of assessing these responses would provide essential information that could help inform vaccine practices.

This dissertation also focused on measuring aP vaccine responses after in vitro BP stimulation of whole blood. Taken together, the BP stimulation data suggests that all infants, regardless of feeding mode or HIV exposure status, are able to mount a T-cell response to aP vaccination. The peak response appears to be at week 15 (how long after vaccination?). Given the differing ontogeny of responses seen in all three groups of infants lends some insight on the complex determinants of vaccine T -cell immunogenicity. In this case, age since vaccination, HIV exposure, and feeding mode resulted in apparent changes in vaccine responses. T cell memory differentiation appeared to be proportional to the age of the infant and feeding mode tended to accelerate T-cell differentiation and enhance cell activation. In addition formula feeding seem to associate with more cytokine expression by T cells, specifically with TNFα. but also pushes memory maturation to early late terminal

differentiation. HIV exposure enhanced IL-2 and IFNγ responses, but had minimal affect on TNFα responses, and also resulted in elevated T-cell activation in the first week of life. One of the fascinating findings in this study, was that majority of the BP specific T-cells producing cytokines were of a naïve phenotype. This is a phenomenon which has not previously been explored and further investigation could reveal insightful information on ways to enhance long-term aP vaccine responses in infants.

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APPENDIX 1 (EXPERIMENTAL LAYOUT)

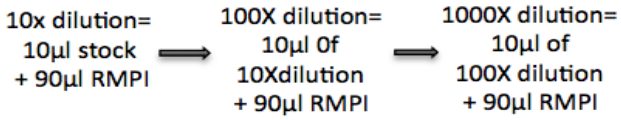
Section 3, Figure 3.3.1 and 3.3.2

For this whole blood assay experiment the batch of proteins used had the following concentrations:

1. VP4 Stock = 0.7µg/µl
LPS in VP4 stock = 4.4407902EU/ml = 0.00444079µg/µl

2. VP6 Stock = 2.84µg/µl
LPS in VP6 stock = 7. 5304424EU/ml = 0.0075304424EU/µl

VP6 was diluted as follows :



Volume of protein to add to each titration was calculated as follows:

For 0.4µg/ml stimulation

$C_1V_1 = C_2V_2$
(2.84) X = (0.0004)(250)
X = 0.035211µl VP6 stock
= 3.5µl of 1:100 dilution

$C_1V_1 = C_2V_2$

$(0.7)\ X = (0.0004)(250)$

$X = 0.142\mu l\ VP4\ stock$

For 4µg/ml stimulation

$C_1V_1 = C_2V_2$
(2.84) X = (0.004)(250)
X = 0.35211µl VP6 stock
= 3.5µl of 1:10 dilution

$C_1V_1 = C_2V_2$
(0.7) X = (0.004)(250)
X = 1.42µl VP4 stock

For 40µg/ml stimulation

$C_1V_1 = C_2V_2$
(2.84) X = (0.04)(250)
X = 3.5211µl VP6 stock

(0.7) X = (0.04)(250)
X = 14.2µl VP4 stock

LPS concentrations were only calculated for the 40µg/ml stimulation as follows:

3.25µl of VP6 stock has 0.0075304424 x 3.25 = 0.0244793EU
Therefore in a final volume of 250µ there is (0.0244793)/250
=9.789X10^{-5}EU/µl

14.2µl of VP4 stock has 0.00444079x 14.2 = 0.00630EU
Therefore in a final volume of 250µ there is (0.00630)/250
=2.522X10^{-5}EU/µl

Since the LPS concentration was so small no LPS control was done in this WBA

Section 3, Figure 3.3.3 and 3.3.4

This experiment was set up in the same manner as experiment 3, with the same batch of proteins but used at different concentrations.

The volume of protein added to each stimulation was calculated using the formula $C_1V_1 = C2(V_1+V_2)$, Therefore $V_1 = (C_2V_2)/(C_1-C_2)$

This formula was used instead of the basic "$C_1V_1 = C_2V_2$" to compensate for the large volumes of solution added, in order to obtain the required final concentration of protein.

For 20µg/ml stimulation

$V_1 = (C_2V_2)/(C_1-C_2)$
X = (20x250)(700-20)
= 7.3529 µl VP4

$V_1 = (C_2V_2)/(C_1-C_2)$
X = (20x250)(2860-20)
= 1.7605 µl VP6

For 40µg/ml stimulation

$V_1 = (C_2V_2)/(C_1-C_2)$
X = (40x250)(700-40)
= 15.15 µl VP4

$V_1 = (C_2V_2)/(C_1-C_2)$
X = (40x250)(2860-40)
= 3.5460 µl VP6

For 80µg/ml stimulation

$V_1 = (C_2V_2)/(C_1-C_2)$
X = (80x250)(700-80)
= 32.258 µl VP4

$V_1 = (C_2V_2)/(C_1-C_2)$
X = (80x250)(2860-80)
= 7.1942 µl VP6

For 160µg/ml stimulation

$V_1 = (C_2V_2)/(C_1-C_2)$
X = (160x250)(700-160)
= 74.07407 µl VP4

$V_1 = (C_2V_2)/(C_1-C_2)$
X = (160x250)(2860-160)
= 14.8148 µl VP6

For 320µg/ml stimulation

$V_1 = (C_2V_2)/(C_1-C_2)$
X = (320x250)(700-320)
= 210.5063 µl VP4

$V_1 = (C_2V_2)/(C_1-C_2)$
X = (320x250)(2860-320)
= 31.4960 µl VP6

The concentration of LPS used in the LPS control was based on the highest stimulation tube, the 320µg/ml stimulation. The concentration was calculated as follows:

In 31.4960µl of VP6 stock there is 0.0075304424 x 31.4960 = 0.2371EU
In 210.5063µl of VP4 stock there is 0.00444079x 210.5063 = 0.92789EU

Therefore the final concentration of LPS in the 320µg/ml VP4 stimulation was
= 0.92789/ (250+210.5063)
= 0.002260EU/ul
=2.26EU/ml

Stock LPS = 20.6EU/ml
$C_1V_1 = C_2V_2$
(20.6) X = (2.36)(250)
X = 27.91µl

Therefore 27.91ul of LPS stock was added into the LPS control.

Section 3, Figure 3.4.1 and 3.1.2

This was a standard WBA (as described in methods), protein and LPS calculations were as follows:

$V_1 = (C_2V_2)/(C_1-C_2)$
$X = (40x250)(700-40)$
= 15.15 µl VP4

$V_1 = (C_2V_2)/(C_1-C_2)$
$X = (40x250)(2860-40)$
= 3.5460 µl VP6

LPS concentrations were only calculated for the 40µg/ml stimulation as follows:

3.25µl of VP6 stock has 0.0075304424 x 3.25 = 0.0244793EU
Therefore in a final volume of 250µ there is (0.0244793)/250
$=9.789X10^{-5}$EU/µl

14.2µl of VP4 stock has 0.00444079x 14.2 = 0.00630EU
Therefore in a final volume of 250µ there is (0.00630)/250
$=2.522X10^{-5}$EU/µl

Since the LPS concentration was so small no LPS control was done in this WBA

Section 3, Figure 3.5.2 and 3.5.3

This was a standard WBA (as described in methods). However the concentrations of protein were severely depleted.

VP4 concentration decreased from 1200ug/ml to 1.4ug/ml
VP6 concentration decreased from 7800ug/ml to 0.8ug/ml
The volumes of VP4 and VP6 added to each stimulation tube were 8.62ul and 1.28865ul respectively.

Thus the final concentration of proteins actually added to each stimulation tube was as follows:

$C_1V_1 = C_2V_2$
$(1.4)(8.62) = (X)(250)$
X =0.048ug/ml VP4

$C_1V_1 = C_2V_2$
$(0.8)(1.28) = (X)(250)$
X =0.0041ug/ml VP6

LPS in stock VP4 was 60EU/ml and in stock VP6 was 120EU/ml. Therefore the final concentration of LPS in each tube calculated as follows:

1.28µl of VP6 stock has 0.12x1.28 = 0.1536EU
Therefore in a final volume of 250µ there is 0.1536/250= 0.0006144EU/ul

8.62µl of VP4 stock has 0.06x8.62 = 0.5172EU
Therefore in a final volume of 250µ there is 0.5172/250=0.0020688EU/ul

Total LPS in each stimulation tube = 0.0006144+0.0020688
= 0.0026832EU/ul = 2.6832EU/ml

Section 3, Figure 3.6.2, 3.6.3 and 3.6.4

This was a standard WBA (as described in methods) was done for both experiments, with the following protein concentrations:

VP4 = 1200ug/ul with 430EU/ml LPS
VP6 = 7800ug/ul with 700EU/ml LPS

10-fold serial dilutions were done on both proteins by adding 100ul stock or 100ul of the previous dilution solution to 900ul RPMI, to yield a 10X, 100X, 1000X, 10 000X ,100000X and a 1million time dilution. Additionally a 20000X and 200000X dilution of VP6 was made up by mixing 500ul of 10000X and 100000X dil to 500ul RMPI respectively.

The volumes of proteins to be added in each tube was calculated using the C_1V_1 = C_2V_2 formula to yield the following results:

1ug/ul stim = 32.05ul of 1000Xdil VP6 + 20.83ul of 100dil VP4
0.1ug/ml stim = 3.2ul of 1000x dil VP6 + 2ul of 100Xdil VP4
0.01ug/ml stim = 3.2ul of 10000Xdil VP6 + 2ul of 1000Xdil VP4
0.001ug/ml stim = 0.64ul of 20000Xdil VP6 + 2ul of 10000Xdil VP4
0.0001ug/ml stim =0.64ul of 200000Xdil VP6 + 2ul of 100000Xdil VP4

For experiment 8 (panel C and D) LPS control was done as follows:

0.03205µl of VP6 stock has 0.7x0.03205 = 0.022435EU
Therefore in a final volume of 250µ there is 0.022435/250= 0.00008974EU/ul

0.2083µl of VP4 stock has 0.43x0.2083 = 0.089569EU
Therefore in a final volume of 250µ there is 0.089569/250=0.000358276EU/ul

LPS concentration in 1ug/ml stimulation =0.000448016
LPS stock =0.034EU/ul
C_1V_1 = C_2V_2
(0.034) X = (0.000448016)(250)X = 3.294µlTherefore 3.294ul of LPS stock was added into the LPS control.

For experiment (panel A and B) LPS control was 10x less than LPS control of experiment 8.
Therefore LPS concentration in LPS control was 0.000448016
Thus 0.3294ul of LPS stock was added to LPS control

Appendix 2

THE IMPACT OF HIV EXPOSURE ON T-CELL MEMORY DIFFERENTIATION

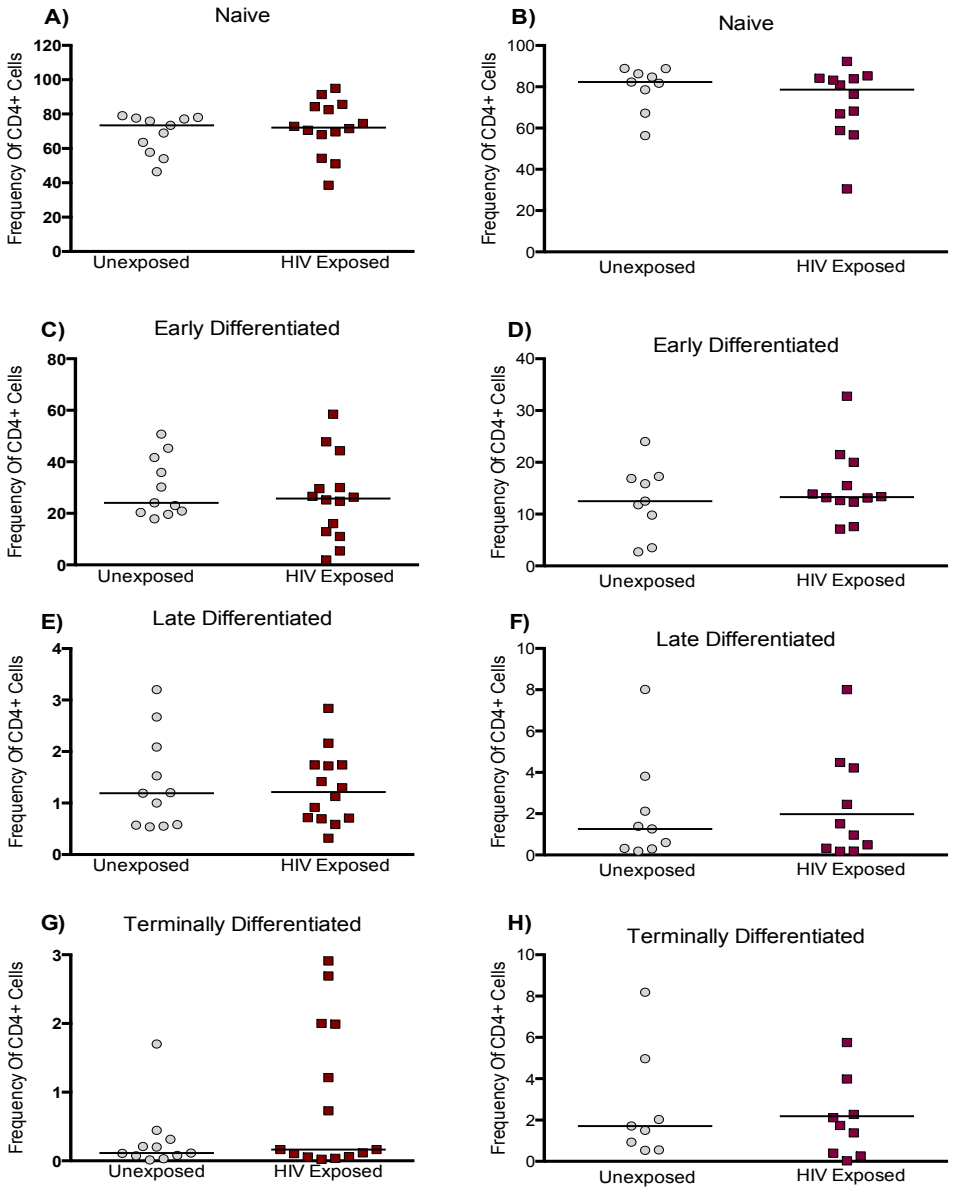


Figure A4.1: Effects of HIV Exposure on CD4 T-cell Memory Differentiation at week 15. Maroon squares represent HIV exposed infants. Panels A, C, E and G represent CD4 memory while panels B, D, F and H represent CD8 memory differerntiation. Comparisons between HIV exposed and unexposed infants were done with a Mann-Whitney test.

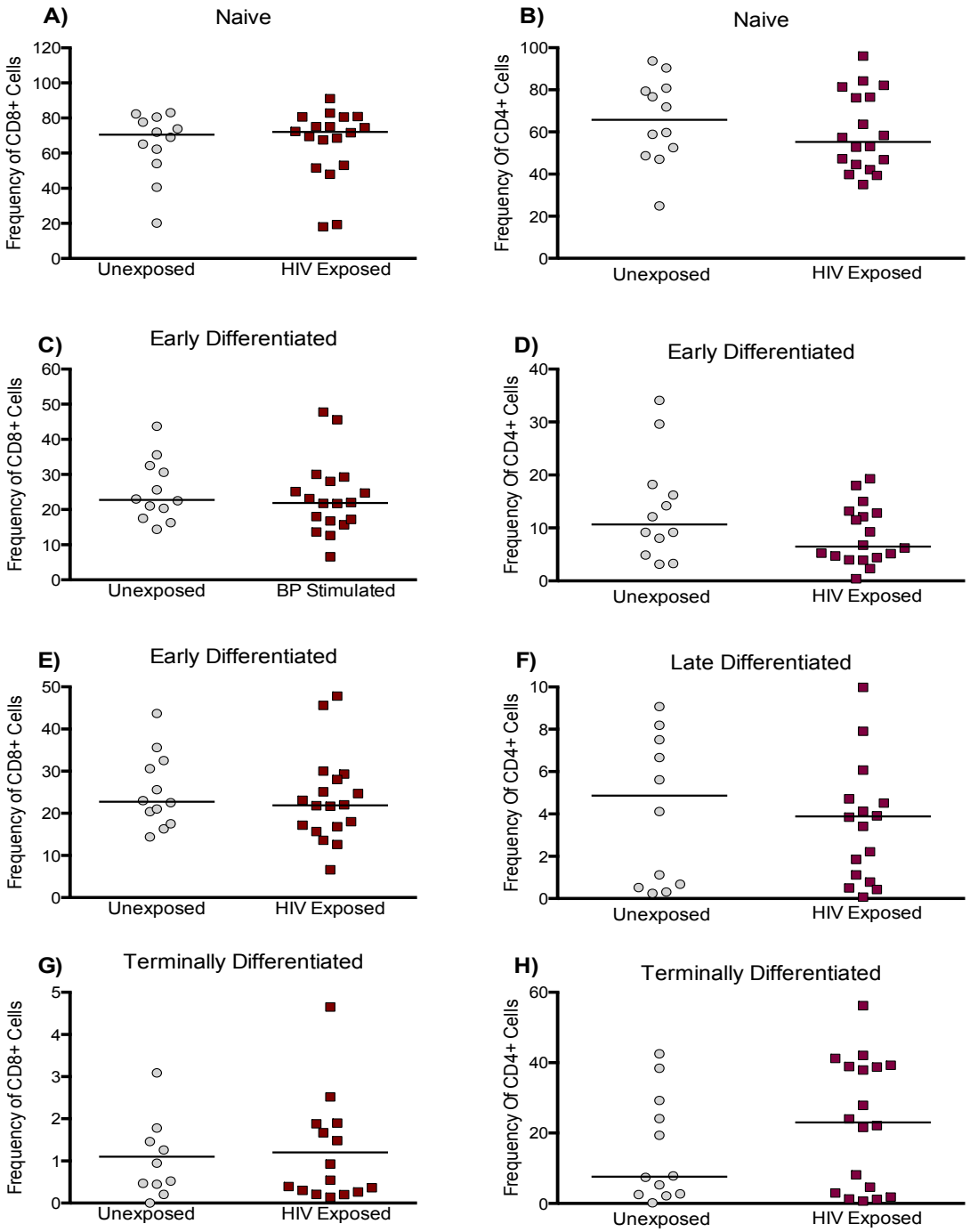


Figure A4.1: Effects of HIV Exposure on CD4 T-cell Memory Differentiation at week 36. Maroon squares represent HIV exposed infants. Panels A, C, E and G represent CD4 memory while panels B, D, F and H represent CD8 memory differerntiation. Comparisons between HIV exposed and unexposed infants were done with a Mann-Whitney test.

Appendix 3

THE IMPACT OF HIV EXPOSURE ON CD4+ T-CELL SINGLE CYTOKINE EXPRESSION

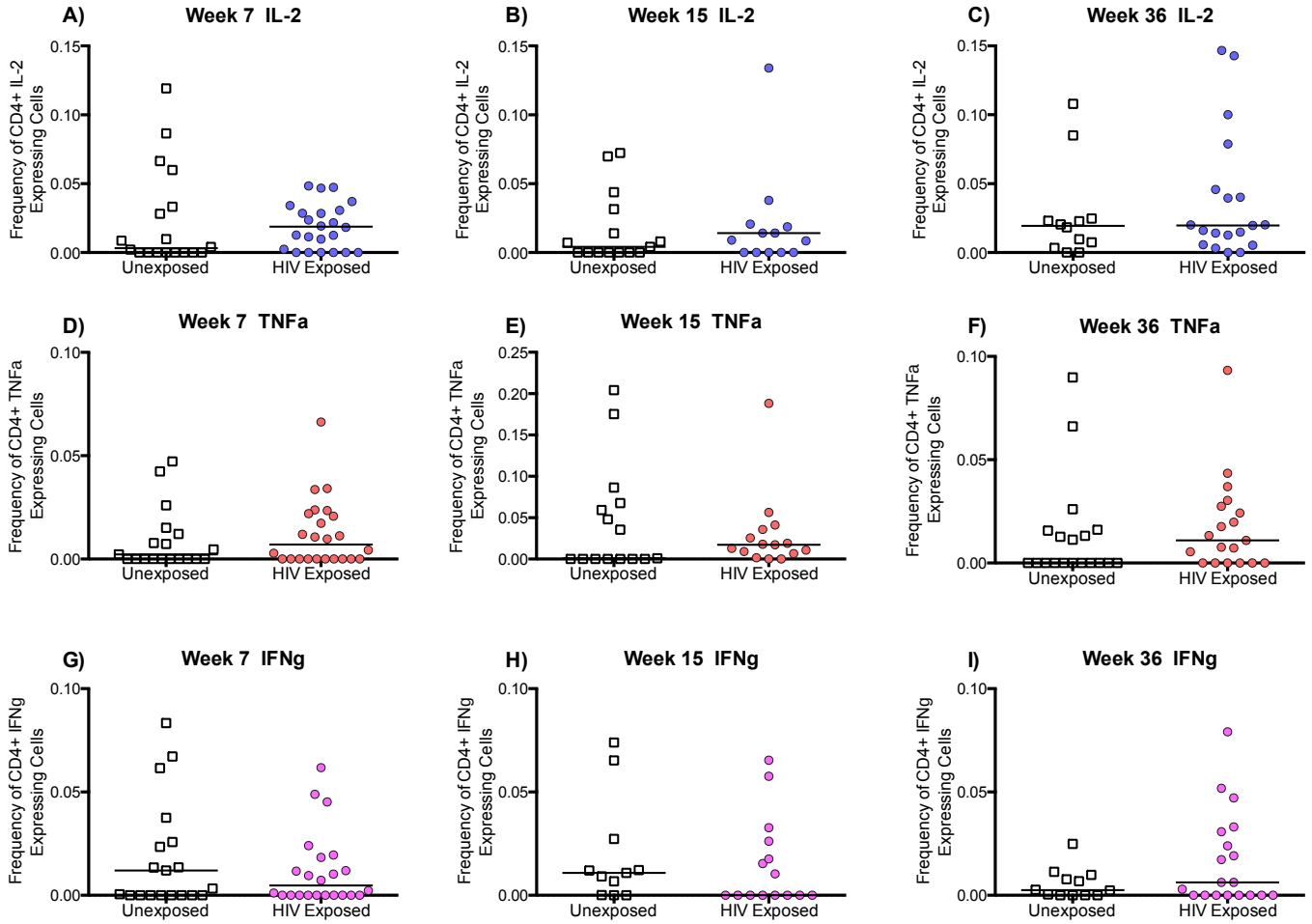


Figure A3.1: The Effects of HIV Exposure On CD4+ T-cell Single Cytokine Expression. Open squares represent unexposed infants while coloured circles represent HIV exposed infants. Panels A,D and G demonstrate week 7 responses, panels B,E and H represent week 15 responses and panels C,F and I represent week 36 responses. Row one, two and three demonstrate IL-2, TNFα and IFNγ responses respectively.

Appendix 4

EFFECTS OF HIV EXPOSURE ON T-CELL ACTIVATION AND PROLIFERATION

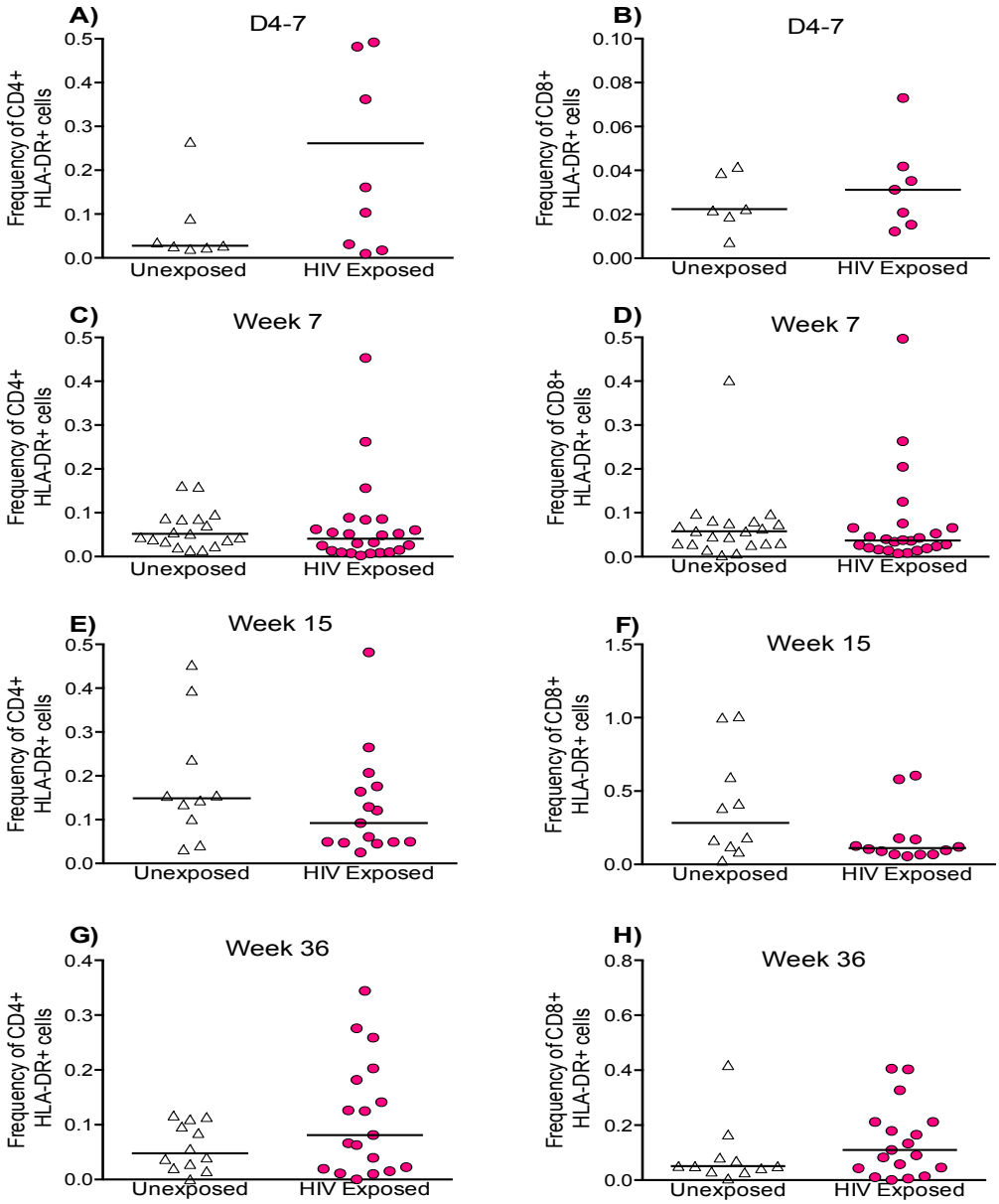


Figure A4.1: Effects of HIV Exposure on CD4+ and CD8+ T-cell Activation. Pink circles represent HIV exposed infants. Open triangles represent unexposed infants. Panels A, C, E and G represent CD4+ T-cell activation and panels B, D, F and H represent CD8+ T-cell activation. Comparisons between HIV exposed and unexposed infants were done with a Mann-Whitney test.

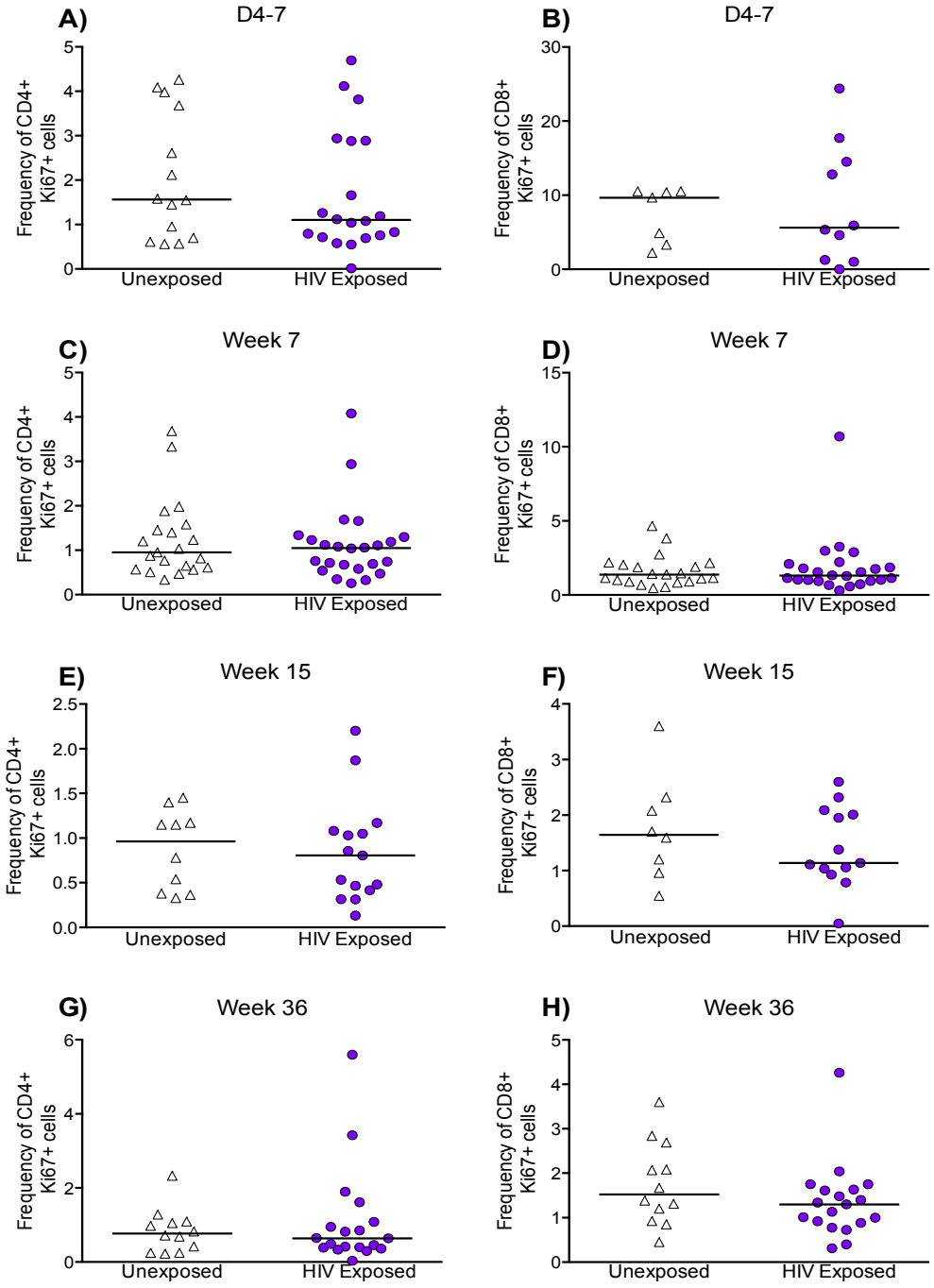


Figure A4.2: Effects of HIV Exposure on CD4+ and CD8+ T-cell Proliferation. Purple circles represent HIV exposed infants. Open triangles represent unexposed infants. Panels A, C, E and G represent CD4+ T-cell activation and panels B, D, F and H represent CD8+ T-cell activation. Comparisons between HIV exposed and unexposed infants were done with a Mann-Whitney test.

Appendix 5

EFFECTS OF FEEDING MODE ON ACELLULAR PERTUSSIS SPECIFIC CD4 AND CD8 T-CELL SINGLE IFN-G AND IL-2 EXPRESSION

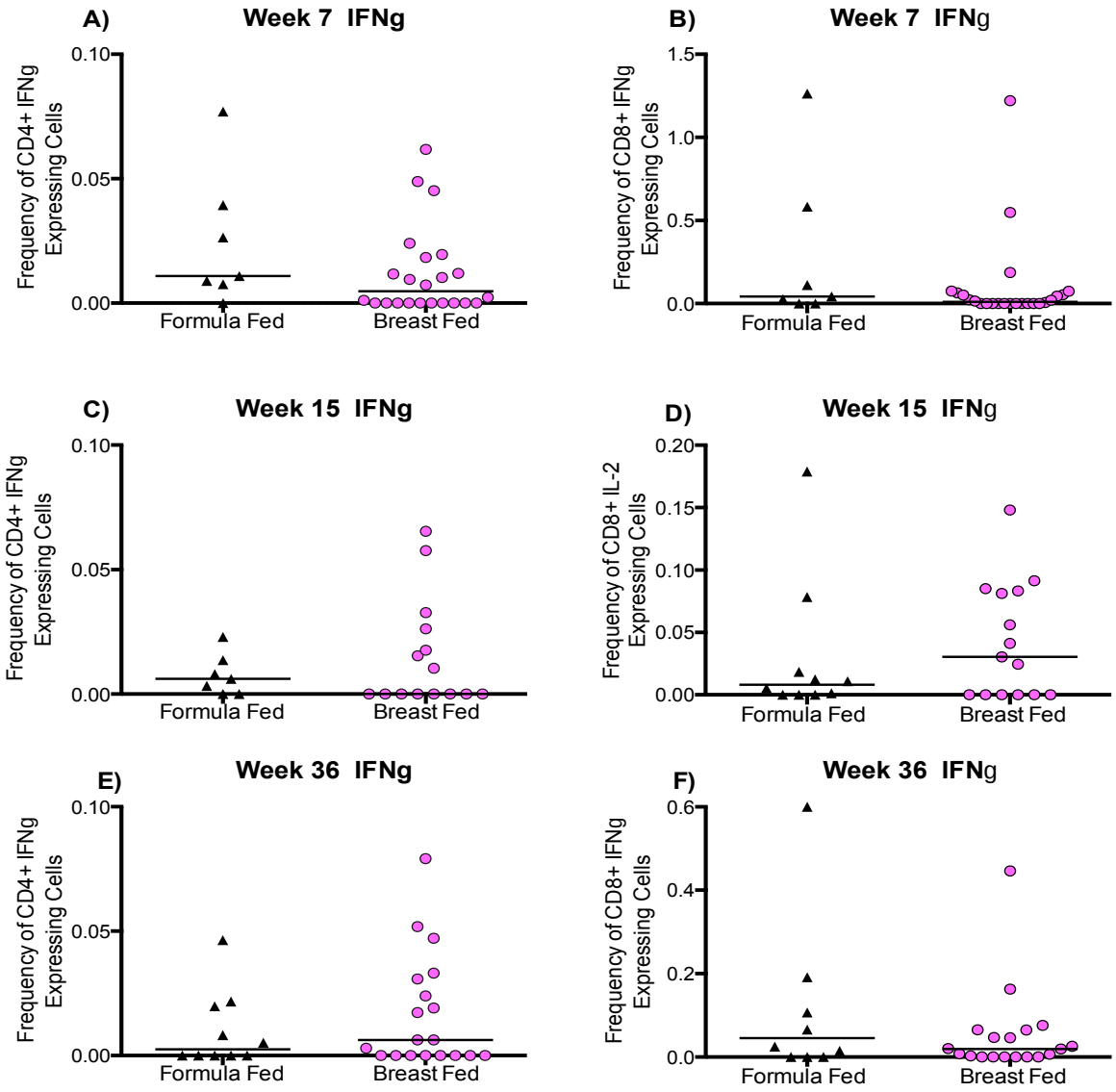


Figure A5.1: Net BP Specific CD4+ and CD8+ T-cell Expression of IFNγ at Week 7, 15 and 36. Black triangles represent formula fed infant responses while pink circles represent breast fed infant responses. Panels A,C and E demonstrate CD4+ T-cell responses and panels B,D and F represent CD8+ T-cell responses. Horizontal bars depict medians. Comparisons between breast fed and formula fed infants were done using a Mann-Whitney test.

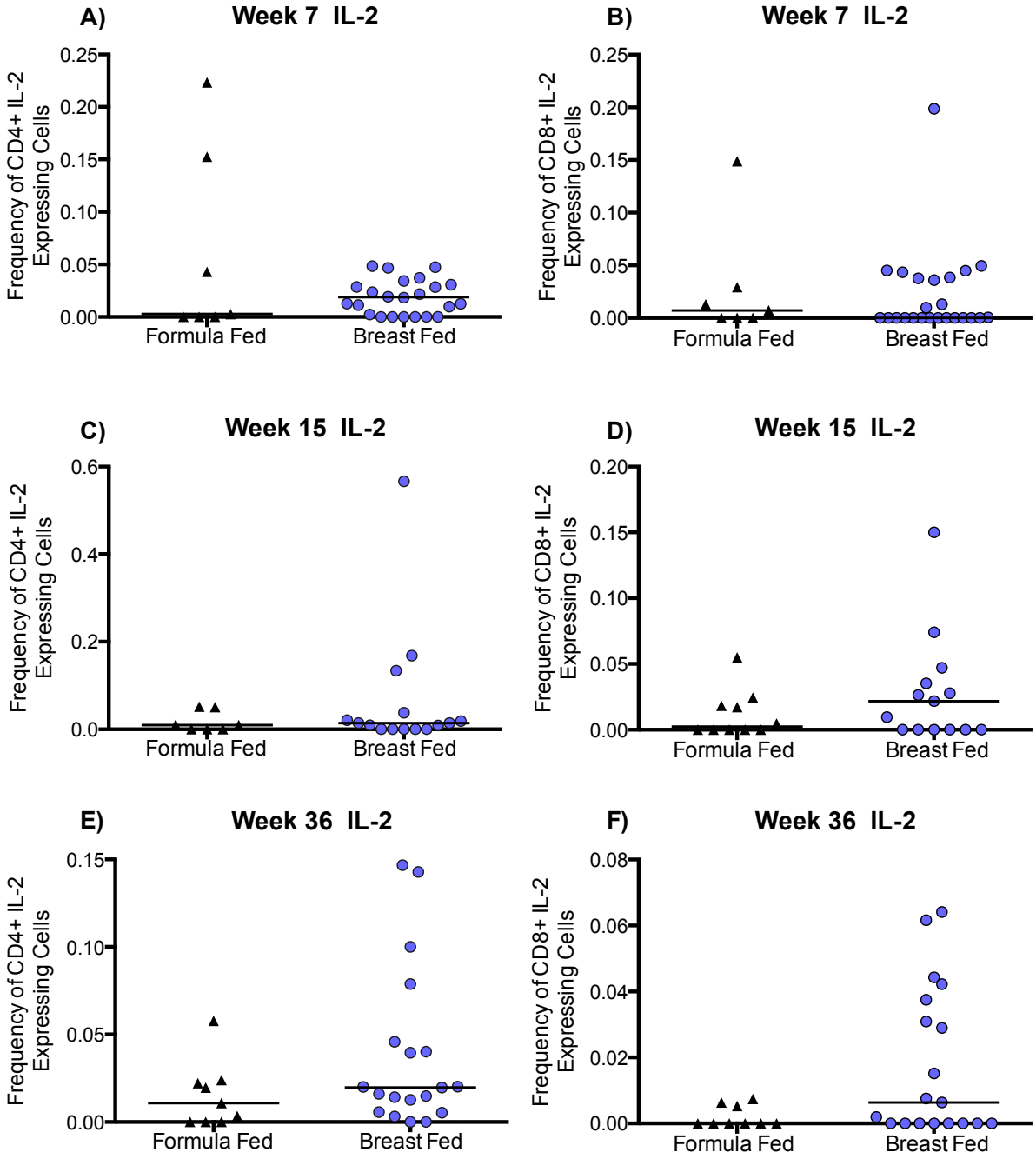


Figure A5.2: Net BP Specific CD4+ and CD8+ T-cell Expression of IL-2 at Week 7, 15 and 36. Black triangles represent formula fed infant responses while blue circles represent breast fed infant responses. Panels A,C and E demonstrate CD4+ T-cell responses and panels B,D and F represent CD8+ T-cell responses. Horizontal bars depict medians. Comparisons between breast fed and formula fed infants were done using a Mann-Whitney test.

Appendix 6

EFFECTS OF FEEDING MODE ON CD4 AND CD8 T-CELL PROLIFERATION

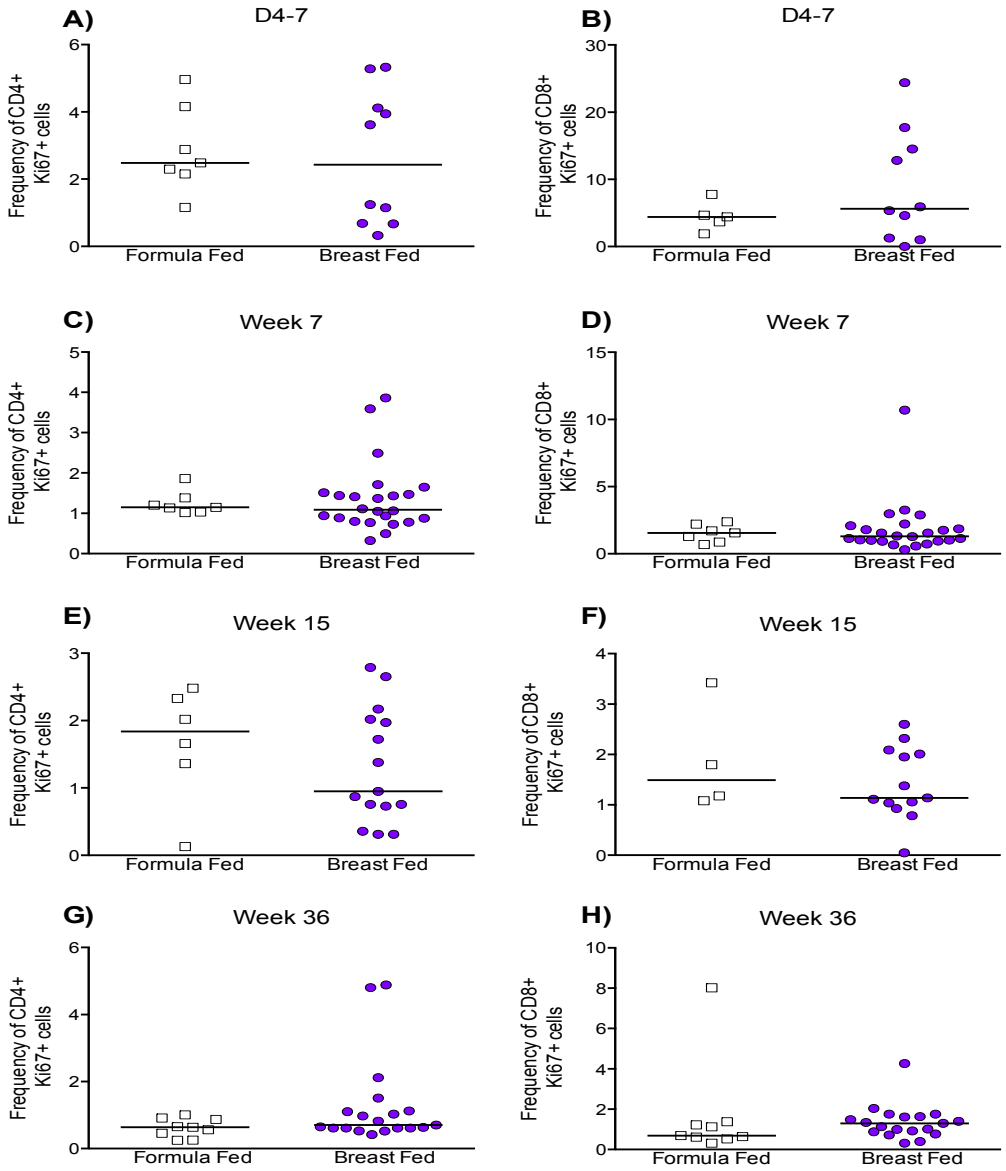


Figure A6.1: CD4+ and CD8+ T-cell proliferation. Open squares represent formula fed infant responses while purple circles represent breast fed infant responses. Panels A, C, E and G demonstrate CD4+ T-cell proliferation and panels B, D, F and H represent CD8+ T-cell proliferation. Horizontal bars depict medians. Comparisons between breast fed and formula fed infants were done using a Mann-Whitney test.