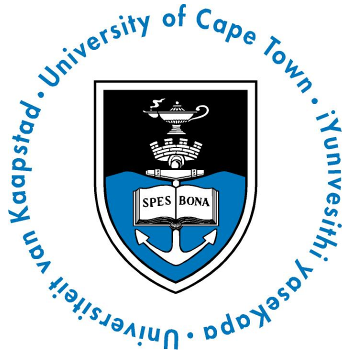

UNIVERSITY OF CAPE TOWN

DIVISION OF IMMUNOLOGY

CELLULAR IMMUNE ONTOGENY AND BIRTH TRANSCRIPTOME IN HIV-EXPOSED UNINFECTED INFANTS

A thesis submitted for the Degree of Doctor of Philosophy in Clinical Immunology

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Preamble

The following thesis is structured as follows: Chapter 1 is a literature review that covers three broad topics: (1) Immune ontogeny in early life, (2) The impact of HIV exposure on infant health and immunity and (3) EPI vaccines during infancy. This is followed by four main results chapters (Chapters 2-5). Each result chapter is written in the style of a publication with a brief introduction giving a rationale for the study, a methodology, results and discussion sections. Chapter 5 includes already published work (see Declarations) with some minor modifications. The final chapter is a summary and conclusions chapter.

Abstract

Background. In some regions of Sub-Saharan Africa, up to 30% of newborns are born to mothers infected with human immunodeficiency virus (HIV). Maternal antiretroviral treatment (ART) has reduced vertical transmission to lower than 1%. Despite the success of prevention of mother-to-child transmission (PMTCT) programmes, a large number of children born to these mothers are exposed to HIV and antiretrovirals (ARVs) *in utero* yet remain uninfected. These individuals, known as children who are HIV-exposed and uninfected (cHEU), succumb to higher rates of disease morbidity compared to children who are HIV-unexposed (cHU) which suggests altered immunity in the cHEU. Differences in the numbers and function of cells of the innate and adaptive immune system have been documented in cHEU though not consistently. While vaccine-induced antibody responses are robust in cHEU, data on potential cell mediated perturbations to vaccine antigens remains conflicting. This is in part due to inherent inter-cohort variation and differences in ART therapy strategies, feeding practices between cohorts and the assays used measure cell-mediated responses. We leveraged two independent cohorts from Nigeria and South Africa of mother-infant pairs receiving antenatal and postnatal care all under Option B+ PMTCT. All HEU infants received pre-exposure prophylaxis for 6 weeks and the majority were exclusively breast-fed until 6 months of age. We applied the same assays in both cohorts to test the hypotheses that HEU have altered T cell immunity compared to HU controls and distinct transcriptomic signatures at birth. These were tested in three distinct aims:

1. To identify transcriptional signatures at baseline that delineate cHEU from cHU

2. To compare the expression of surface marker broadly defining activated or regulatory phenotypes —and the expression of intracellular markers of T cell function between cHEU and cHU over the first 9 months of life.

3. To characterise how differences in the immunising strains of Bacille Calmette-Guérin (BCG), the first vaccine received in these infants, impacts T cell immunity to both mycobacterial and non-mycobacterial antigens in cHEU and cHU.

Methods. Two birth cohorts from Jos, Nigeria and Cape Town (CT), South Africa were recruited into this study as part of a larger parent study that aims to identify biological determinants of protection from mother-to-child transmission of HIV (Innate, Adaptive and Mucosal Immune Responses in Infants/INFANT study: HREC 285/2012). Infant blood was collected at several time points from birth to 36 weeks of life for immunological assays. Whole blood, collected at birth was preserved in PAXgene fluid for downstream messenger ribonucleic acid (mRNA) transcript analyses. Other whole blood samples were fixed and cryopreserved either directly *ex vivo* or after re-stimulation within 1 hour of phlebotomy with BCG, Tetanus Toxoid (TT), *Bordetella pertussis* (BP) antigens and Phytohemagglutinin (PHA). Multi-parameter flow cytometry was used to measure batched whole blood samples for (*i*) markers of T cell regulation and activation directly *ex vivo*, markers of T cell gut homing and proliferation—a proxy for HIV susceptibility, and (*ii*) vaccine-induced Th1 cytokine expression (IFN-γ, TNF-α, IL-2) and memory maturation. Cytokine responses were profiled for polyfunctionality by SPICE analysis and complemented by the COMPASS algorithm. Transcriptional profiling of whole blood at birth was done by RNA sequencing and differentially expressed genes were reported for absolute fold change of normalized counts >1.5 with FDR set at 0.05 using the DESeq2

package in R. Gene-set enrichment analysis (GSEA) was used to identify enriched or repressed gene pathways for absolute normalised effect sizes >1.5 with FDR set at 0.05. Longitudinal analyses used a mixed effects ANOVA with time and HIV exposure as explanatory variables. Cross-sectional analyses comparing HIV exposure groups used Wilcoxon Ranked Sum Test, with $p<0.05$ considered significant after multiple correction adjustment by Holm's step-down method.

Results. Aim 1: A small set of DEGs were found between HEUs and HU groups at birth, 3 of which were upregulated and 12 that were downregulated. Among the upregulated genes, two are homologues of the arrestins ARRDC4 (2.3 fold, adjusted *p-adj*<0.001) and TXNIP (1.4 fold, *p-adj*<0.001). Gene-set enrichment analysis however, showed no significant enrichment or suppression of gene pathways in HEUs.

Aim 2: HIV/ARV exposure did not have an interaction effect with age (all time points) in explaining the frequencies of T cell markers *ex vivo* in a mixed-effects model. In cross-sectional unadjusted analyses however, trends towards increased median frequencies of markers of activation in the HEU group compared to HU controls were observed for specific ages: at birth (%CD8+HLA-DR+: 0.12 vs. 0.01, p=0.05), at week 7 (%CD8+CD25+: 0.13 vs. 0.04, p=0.01 and %CD8+HLA-DR+: 0.84 vs. 0.07, p=0.01) and at week 36 (%CD8+CD25+: 0.52 vs. 0.03, $p<0.001$ and %CD8+HLA-DR+: 0.81 vs. 0.17, p=0.003). When adjusting for multiple comparisons, only CD25 expression remained significant on CD8+ T cells at week 36 (*p-adj*=0.04). The magnitudes of cytokine responses by T cells to vaccine antigens did not differ between HEU and HU infants however transient differences in the polyfunctional profile of cells was observed at week 1 for mycobacterial-specific Th1 profiles in CT infants (p=0.002) by SPICE analysis. There were later differences at week 7 for BP-specific Th1 profiles in Jos infants (p=0.01) and at week 36 for BP-specific Th1 profiles in CT infants (p=0.03). The more robust COMPASS algorithm only detected a trend towards increased polyfunctional scores to BP responses in CT infants at week 36 (p=0.03).

Aim 3: BCG immunising strain impacted the magnitudes and quality of responses to mycobacterial and non-mycobacterial vaccine antigens irrespective of HIV exposure status. Most significantly, at week 7, BCG-Denmark induced higher mycobacterial-specific frequencies of CD4 Th1 cytokines compared to Bulgarian (p<0.001) and Russian strains (and (p<0.001). BCG-Denmark induced greater triple cytokine profiles to mycobacterial antigen compared to Bulgarian (p<0.001) and Russian (p<0.001) strains in SPICE analyses and the resulted were confirmed by COMPASS algorithm polyfunctional scores. Furthermore, BCG-Denmark significantly enhanced antigenicity to TT and BP vaccines.

Conclusion. Transient differences exist in the frequencies of CD25 expressing CD8 T cells between HEU and HU groups, however other readouts of immunity suggest that in the context of effective PMTCT and exclusive breast feeding practices, HEU infants are indistinguishable from their HIV unexposed peers.

Dedication

To my parents Cheddi and Eliaichi Kiravu who sacrificed so much for me.

Declaration

I Agano Kiravu hereby declare that the work on which this 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, is to be submitted for another degree in this or any other University. I authorize the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

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Declaration– Inclusion of publication

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Kiravu A, Osawe S, Happel A-U, Nundalall T, Wendoh J, Beer S, Dontsa N, Alinde OB, Mohammed S, Datong P, Cameron DW, Rosenthal K, Abimiku A, Jaspan HB, and Gray CM. "Bacille Calmette-Guérin Vaccine Strain Modulates the Ontogeny of Both Mycobacterial-Specific and Heterologous T Cell Immunity to Vaccination in Infants" In: *Frontiers in Immunology* 10 (2019), pp. 2307

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List of acronyms and abbreviations

aP acellular-Pertusis.

ARRDC4 Arrestin Domain-Containing Protein 4.

ART Antiretroviral treatment.

ARV antiretrovirals.

BP *Bordetella pertussis*.

CBMC Cord blood mononuclear cells.

cHEU Children who are HIV-exposed and uninfected.

cHU Children who are HIV-unexposed.

CT Cape Town, South Africa.

DMSO Dimethylsulfoxide.

DTaP Diptheria-Tetanus-acellular Pertussis.

DTP Diptheria-Tetanus-Pertussis.

EBF exlusive breast feeding.

EPI Expanded Programme on Immunization.

FHA filamantous hemagglutinin.

GM-CSF Granulocyte-macrophage colony stimulating factor.

Hib *Haemophilus influenzae* type b.

IPV inactivated polio vaccine.

MCV Meningococcal vaccine.

NK Natural killer cells.

PAMPs PAMPs.

PBS Phosphate buffered saline.

PCR Polymerase chain reaction.

PHA Phytohemagglutinin.

PMTCT Prevention of mother to child transmission.

PPD purified protein derivative.

TB Tuberculosis.

Treg regulatory T cell.

TT Tetanus Toxoid.

TXNIP Thioredoxin Interacting Protein.

UK United Kingdom.

WHO WOrld Health Organisation.

WHO-UNICEF World Health Organisation-United Nations Childrens Fund.

wP whole-cell Pertussis.

Chapter 1

Literature review

1.1 Ontogeny of the human immune system

The concept of immune ontogeny—the evolution of the immune system from an immature state to that of a mature or adult-like state—is fundamental to understanding immunity in early life [1]. Much of what is known about immune ontogeny has been derived from studies using animal models, given the limitations of sampling human fetuses and neonates. The development of the immune system of can be broadly divided into two phases each with their own unique challenges. The pre-natal phase of immune development requires the maintenance of fetal-maternal tolerance, whilst simultaneously adapting to *in utero* pathogenic challenge whereas post-natal phase requires the rapid adaptation to a host of environmental and pathogenic challenges [1]. Pre-natal immunity begins with the protection of embryonic cells via innate immune processes such as the recognition of foreign pathogen associated molecular patterns (PAMPs) in a process described as cell autonomous immunity (CAI) [2]. Immune subsets that display adult-like characteristics are detectable in human fetal tissues as early as 5 weeks of gestation [3], however these cells in the fetus are functionally distinct from adults [4, 5, 6]. Several key observations demonstrate that post-natal immunity is age-dependent; firstly innate responses to

PAMPs stimuli mature from birth through the neonatal period to about 1 year of age [7, 8, 4, 9], secondly T and B cells at birth and during the neonatal period are of a naive-like phenotype [10, 11] suggesting limited antigen exposure *in utero* and thirdly a change the shift from Th2-like polarisation in early infancy towards a more balanced Th1/Th2 phenotype [12, 13].

1.1.1 Factors that drive immune ontogeny in early life

The development of immunity in early life is determined by the sum total of both abiotic and biotic factors. Some of the biological factors that shape immune ontogeny during infancy is discussed here. Amongst others they include host genetics, environmental microbes, the initial colonising microbiome, breastfeeding practices and maternal infections during pregnancy. These factors act to influence immune tolerance, allergic immunity and immune responses to pathogens and vaccines in early life [1].

Environment

Population differences in susceptibility to disease are well-known. These differences reflect intrinsic factors such as host genetics of innate immune sensors [14, 15] as well as extrinsic factors such as environment which impact on immune programming particularly in early life [1, 16]. Distinct cytokine expression patterns in response to toll-like receptor (TLR) stimuli have been observed in newborns of different racial backgrounds [17, 18] which support a role for host genetics in innate immunity. A recent study on the ontogeny of innate responses between Asian and Caucasian infants living in the same environment, found that the initial divergent patterns in cytokine expression at birth eventually converged by 2 years of age [18] suggesting that intrinsic factors can be over-ridden by environmental cues. When innate responses were compared in 2 year old infants from four different continents, a unique hypo-responsiveness to TLR4 and and TLR7/8 stimuli among South African was found

compared to European, North and South American populations [19] underscoring the role of environment in driving responses to microbial stimuli. Adaptive responses are also driven by environment as shown in Bangladeshi and American infants followed up from birth where significant divergence was observed at 2 years of age. Elevated IL-8 and cytotoxic T cell responses to polyclonal stimulus were the defining feature of Bangladeshi infants at 2 years of age, whereas at birth, T cell function was indistinguishable between the two cohorts [20]. The environments in the tropics are considered as being uniquely microbial rich, and the inhabitants of these areas suffer a disproportionate burden of infectious diseases compared lattitudes further away from the equator [21]. Compounding this, are factors related to limited resource settings where poor nutrition, hygiene and lifestyle affect infant health. The 'hygiene hypothesis' [22] proposes that continual exposure to microbes in early life, educate the immune system towards a Th1-biased phenotype and away from a Th2 phenotype that favours allergic immune responses [23]. Consistent with this is hypothesis is the epidemiological data showing an emergence of auto immune disorders and allergies in infant populations from urbanised Western settings—assumed to be less exposed to microbial stimuli [24]. Helminth infections are endemic in the tropics [25, 26, 27] and these infections are associated with Th2 skewing [28] and other immunomodulatory effects including impaired responses to vaccination, increased susceptibility to infections and a suppression of allergy responses [29, 30, 31, 32, 33, 34]. This may explain in part why BCG—a potent inducer of Th1 immunity—has poor efficacy in the tropics [35], where BCG-induced Th1 responses are lower compared to populations further away from the tropics [36, 32]. Given that helminth infections are common for pregnant women in helminth-endemic areas [37], it has been suggested that *in utero* sensitisation to helminth antigens pre-disposes newborns to other infectious burdens [38, 30, 39], alters their vaccine responses [30, 40] and perturbs immune subsets [41]. In Uganda, maternal hookworm infection was associated with increased IL-10 cytokine responses to Tetanus Toxoid vaccine in their infants in

a large observational study of 1500 mother-infant pairs [40]. Some studies have demonstrated that treating helminth infections improves immune responses to non-helminth [42, 43], leading to suggestions that helminth treatment during pregnancy may restore vaccine responsiveness in infants [37]. In a randomised controlled trial however, maternal anti-helminth treatment during pregnancy did not improve infant BCG immune responses but did result in reduced IL-5 and IL-13 responses for Tetanus Toxoid vaccine [44]. In early life, the immune system is Th2 biased, which is thought to contribute to the increased vulnerability to pathogens that typically require Th1 responses for clearance. Without any interventions, environments that delay the ontogeny of Th1/Th2 balance by skewing the immune system to a Th2 phenotype may render infants vulnerable to infection.

Microbiome

The developing fetus was assumed to be sterile, free from antigen exposures that could imprint on immune development. This dogma has since changed, and the amniotic fluid that surrounds the fetus is recognised as being non-sterile [45] with one study reporting that the placenta harbours a distinct microbiome [46]. The first post-natal exposures come from the transfer of microbial communities from the mother to child during the birthing process [47]. The most significant determinant of the colonising microbial profile in neonates is the initial maternal innoculum—determined by the mode of delivery [48]. Microbial communities established after C-section delivery resemble those of the maternal skin [49] and hospital related-bacteria [50]—considered unfavourable to the health of the infant [51, 52]. Vaginal delivery in contrast results in colonisation of beneficial *Bifidobacterium* species [47, 53], which are associated with reduced incidences of respiratory tract infections [54, 55]. In addition, feeding practices (breast-milk vs. formula, cessation of breast-feeding, introduction of solids etc.), the use of antibiotics (infant and/or maternal) determine the kinds of microbial communities that eventually colonise much of the neonatal gastrointestinal tract

[47, 56]. There is growing evidence that the profile of early microbiome and any disruptions thereof, impacts immunity to allergens, pathogens and vaccines [57, 58, 59, 60]. The pre-vaccination composition of gut microbial species was a significant determinant of Rotavirus vaccine antibody responders in a study of Ghanaian infants [60]. In a study of Bangladeshi infants, higher relative abundances of *Bifidobacterium longum* species was associated with elevated T cell responses to BCG, Tetanus-specific and Oral Polio vaccines [61]. Other *Bifidobacterium* species are positively associated with fecal Polio IgA antibody levels [62]. The cross-talk between gut microbes and the gut immune system determines the Th1/Th2 balance in early life [63] with specific microbial taxa and their metabolites impacting cytokine responsiveness to microbial stimulations [58]. A specific carbohydrate molecule absent in a mutant commensal *Bacteroides fragilis* species was sufficient to skew systemic responses to a Th2 phenotype in a mouse model [63], highlighting the importance of specific microbial communities in shaping early Th1/Th2 balance. Feeding patterns in the first year of life impact the microbial ecology of the infant gut. Breastmilk—in addition to its nutritional and passive immunity benefits—promotes the colonisation of healthy gut microbes to the infant [47, 64, 65]. A Swedish study found that the cessation of breast-feeding was significant in determining whether infant gut microbiome resembled that of an adult at 12-months of age [47]. In animal models significant changes in immune profiles have been observed depending on the feeding pattern. The dynamics of serum cytokines differ between formula-fed (FF) and breast-fed Rhesus macaques, with higher pro-inflammatory cytokines are detected in macaques at 4 months of age [64]. This coincides with a preferential accumulation of *Lactobacillus* species and a reduction of *Bifidobacterium* species compared to formula-fed infants [64]. Another study in Rhesus macaques found that breast-fed infants had significant elevated relative abundances of *Provotella*, *Clostridium* and *Lactobacillus* species in their guts compared to formula-fed infants [65]. Importantly, this study showed that CD4 T helper cell memory populations cells in peripheral

circulation developed quicker and were significantly elevated up to 12 months of age in the breast-fed group. These studies demonstrate how diet shapes the infant microbiome with consequences for mucosal immune ontogeny in the first year of life.

In summary, the colonisation of the newborns by specific microbial communities by birth mode, feeding practices represent an immunological imprint that determines how the immune system responds to antigen stimuli.

Chronic maternal infections

Early life immunity is impacted by maternal infections during pregnancy—even in babies that do not experience vertical transmission of the etiologic agent. Evidence is accumulating that the immune ontogeny of these "uninfected yet unaffected" [66] babies differ significantly from those born to healthy mothers. Incidences of helminth infections amongst pregnant women in some populations can reach as much as 30% [67, 68]. As previously described maternal helminth infections have immunomodulatory effects in the infant. Other chronic infections during pregnancy—particularly for tropical regions—with consequences for neonatal and infant immunity include *Trypanosoma cruzi*—the etiological agent of Chaga's disease [69], *Plasmodium falciparum* which causes malaria and HIV [66]. Infection with *Plasmodium falciparum* sensitises fetal T and B cells [70, 71, 72] with effects on neonatal innate responses [73, 74, 71] however, these do not translate to alterations to cell-mediated responses to Tetanus vaccine for example [75, 76]. Infection with *Trypanosoma cruzi* during pregnancy on the other hand, alters the ontogeny of antibody IgG responses against hepatitis B in infants with vertical transmission compared to infants born to uninfected mothers [77]. Furthermore, maternal *Trypanosoma cruzi* infection during pregnancy was associated with an enhancement of purified protein derivative (PPD) Th1 responses in infants that did not experience vertical transmission [77]. HIV infection remains high amongst pregnant women in Sub-Sahran Africa [78]. Despite a reduction in transmission rates due to antiretroviral treatment during pregnancy, infants born to

HIV infected mothers experience an increased burden of infectious disease morbidity that suggest a perturbed immune profile and/or immune ontogeny [79]. Studies on the ontogeny of immunity among children who are exposed *in utero* to human immunodeficiency virus/antiretrovirals have only recently been considered and the ontogeny of T cell phenotypes and function these infants is the focus of this thesis.

1.2 Immune differences between children who are HIV-exposed yet uninfected and HIV unexposed

Prevention of mother to child transmission programs (PMTCT) has drastically reduced vertical transmission rates of human immunodeficiency virus (HIV) from approximately 30% during the pre-antiretroviral treatment (ART) era [80] to lower than 1% in some countries [81]. Infants who have escaped HIV transmission *in utero* or postnatally are termed children who are HIV-exposed and uninfected (cHEU) infants [82]. The numbers of cHEU are increasing yearly and account for as many as 30% new births in Sub-Saharan Africa [78]. Although HIV-uninfected, these infants are disproportionately affected by infectious disease morbidity and mortality compared to children who are HIV-unexposed (cHU) [83, 84, 85, 86]. Notwithstanding the social disadvantages to being born in an HIV household, a number of biological factors are implicated in the poorer health outcomes in these infants [79]. This section will review the evidence for disproportionate infectious morbidity burden among cHEU and the potential immune perturbations in the infant that may account for this.

1.2.1 Infectious disease burden

Prior to the widespread availability of ART programmes for pregnant women, a large number of infants born to HIV-infected mothers died within the first year of

life [87, 78]. In the era of PMTCT, cHEU are still disproportionately affected by infectious disease compared to children who are HIV unexposed (cHU) [88]. Common infections observed in cHEU include those caused by bacterial, fungal and viral agents Epalzae631, mussi2010lower. Group B Streptococcus (GBS) infections in newborns and young infants can cause complications like sepsis and meningitis if left untreated [89, 90]. Increased incidences of GBS among HEU neonates and young infants have been found in both European [91] and African cohorts [92]. In a Belgian study assessing GBS incidence and clinical presentations among newborns at a referral hospital between 2001 – 2008, found that the risk of all GBS infections were higher in HEU compared to HU controls (RR = 19.6, 95% CI; 7.5 – 51.7), in particular late-onset GBS infection (RR = 125.2, 95% CI; 26.3 – 620.2) [91]. Importantly these observations were in cohorts where PMTCT programmes were in place in accordance to local guidelines [93]. In South African infants annual estimates for the risk of invasive GBS infections between 2004 and 2008 were 2.25-fold higher among HEUs compared to HUs [92]—a period in which PMTCT programmes were not widely adopted. Another South African study found that risk of death due to Invasive pneumococcal disease (IPD) in infants younger than six months was highest among HEU compared to HU controls (adjusted RR = 1.76, 95% CI; 1.09 – 2.85) [84].

Lower espiratory tract infections are also commonly reported for HEUs [94, 95, 96, 83]. A survey of cHEU in France between 2002 and 2010 showed that 40% of infections among this group that led to hospitalisations or death were a result of viral or bacterial broncho-pulmonary infections [97]. Crucially, that survey of nearly 7000 neonates excluded those were that were breastfed. Exclusive breastfeeding in the first months of life has been shown to reduce infectious morbidity in HEUs [98]. Current WHO guidelines recommend HIV-infected mothers practice exclusive breastfeeding until at least six months of age [99]. Two earlier reports, prior to this adoption of WHO recommendations, found that the incidences of lower respiratory tract

infections (LTRI) are higher among cHEU infants compared to cHU particularly within the first six months of life [100, 83]. A birth cohort assessing the incidence, severity and risk factors for pneumonia in the first year of life showed that the incidence rate ratio for pneumonia among cHEU was 1·38 (95% CI; 0·91 – 2·03) and a third of pneumonia cases in these infants required hospitalisation [100]. In that study, HIV exposure was a significant risk factor for severe pneumonia (RR = 4.04, 95% CI; 1.51 – 10.80) whereas the introduction of any formula feeding prior to six months was not [100]. In a Botswana study, the risk ratio among cHEU presenting pneumonia at a tertiary hospital was 1.8 (95% CI; 1.27 – 2.64, p=0.001) with in-hospital mortality 4.31 (95% CI; 1.44 – 12.87, p=0.01) [83]. In that study, 47% of cHEU in failed pneumonia treatment compared to 24% of cHU. Of note, was that breastfeeding rates were low among HIV-infected mothers, therefore the elevated risk of pneumonia may be related to inadequate passive immunity provided by breast milk. In a retrospective study of Kenyan infants, followed monthly over the first year of life, breastfeeding was found to be associated with a 47% lower risk of pneumonia among HIV-exposed infants compared to infants that were never breastfed (hazard ratio = 0.53, 95% CI; 0.3 – 0.73) [101]. Surprisingly, this reduction in risk was independent of maternal CD4 and viral load in women that were not on antiretroviral treatment. Thus, the practice of breastfeeding—which HIV infected mothers have historically opted against for fear of transmitting HIV to their babies—is a significant determinant in reducing the risk of lower respiratory tract infections. *Bordetella pertussis* (BP) is the cause of whooping cough in children [102] and has been shown to disproportionately affect cHEU in both incidence and severity in some studies [103, 104]. In both South African and Zambian cohorts, the incidence of BP infection and rates of hospitalisation as a result of BP infection were slightly higher in cHEU [103, 104], while one study showed that HIV exposure was not a risk factor BP infection in a South African setting [105]. In a TB endemic setting, similar rates of Mtb infection (as measured by IFN-γ release assays in blood) were observed between HIV infected

children and HEU—despite being BCG vaccinated and having received preventative Isonaizid therapy [106]. This suggests that HEU children are equally vulnerable to Mtb infection as HIV-infected children. Increased rates of Mtb infection among HEUs may be driven by exposure to household TB contacts due to maternal HIV-TB co-infection. In Uganda, cHEU under 5 years of age had higher odds of Mtb infection compared to cHU (OR = 21.2, 95% CI: 2.2 – 204.7, p=0.008) [107]. These HEU children in addition were underweight highlighting the need for adequate nutrition for this vulnerable population. Infectious burden among cHEU is not limited to bacterial infections. HIV-exposed children are at a higher risk for cytomegalovirus (CMV) infection during infancy compared to their unexposed counterparts [108]. Even in pregnant women on ART, increased incidences of congenital CMV infection are reported in their HIV-exposed uninfected newborns [109, 110, 111]. In summary, cHEU experience more bacterial-related and to a lesser extent viral infections than cHU. Many of the bacterial infections are lung-related, more severe and often fail treatment compared to cHU particularly for infants who are not breastfed. Combined, these studies suggest that altered immunity exists in cHEU that is likely ineffective in mounting protective responses that would limit or prevent childhood infections. This remains a relatively underexplored area of investigation.

1.2.2 Differences in immune cell numbers and function

Several innate and adaptive immune abnormalities have been documented in cHEU [79]. The innate immune system is the first line of defence against foreign pathogens and a number of studies have shown differences in both innate cell frequencies and function between cHEU and cHU groups [79]. Absolute neutrophil and monocyte counts in the peripheral blood have been shown to be lower in cHEU [112]. In a study by Bunders et al., [112], total lymphoctye counts in HIV exposed babies were positively associated with CD4 counts in their HIV infected mothers. This association with

maternal neutrophil counts have been shown to be consistently lower than cHU up to 8 months of age [112], which suggests an increased migration of these cells from the periphery to sites of inflammation among cHEU. Myeloid-derived dendritic cells (MDDC) have been shown to be elevated at birth in HEU compared to HU controls, but comparable from 3 – 12 months of age [113]. In addition to differences in cell numbers, innate cells respond differently between cHEU and cHU groups to a number of innate cell stimuli [114, 115] suggesting altered function. For example, monocytes and dendritic cells from cHEU produce elevated pro-inflammatory cytokines IL-6 and TNF-α in response to stimulation with bacterial pathogen associated molecular patterns (PAMPS) up to at least 6 weeks of age [114]. Cord blood mononuclear cells (CBMC) from HEU newborns stimulated with *Staphylococcus aureus* Cowan (SAC) produce significantly less IL-12 than SAC-stimulated CBMC from HU newborns [115]. Natural killer cells (NK) are a cytotoxic subset of the innate immune system capable of recognising virally infected cells [116]. A Kenyan study found that despite their lower frequencies at birth, NK cells from cHEU were functionally superior to cHU in cytotoxic killing assays *in vitro*, however this enhanced function was lost by 6 months of age [117]. In summary, innate immunity in the HEU is characterised by differences in cell numbers compared to HU and an increase in innate cells function during early infancy, however, this enhanced ability appears to be lost later on.

CD4 and CD8 T cells form part of the cellular immune response to pathogens exerting a number of functions after the engagement of the T cell receptor (TCR) to antigen on human leukocyte antigen (HLA) molecules. A number of studies report that HEU infants and children have lower CD4 and CD8 T cell counts compared to their unexposed peers [118, 119, 112, 120]. A study of HEUs stratified by antiretroviral exposure status (n=1820; maternal ART use during pregnancy and/ or infant prophylaxis vs. n=351; no ARV) [121] found a trend towards decreased CD4 and CD8 T cell counts in the ART-exposed group in the first two months of life which became significant in the subsequent 6–24 month period [121]. This suggests

that exposure to ART *in utero* or perinatally has possible implications the numbers of functional T cells in early life. These differences in T cell numbers persist to pre-adolescence [120], though not observed consistently [118]. Lower T cell counts in the blood of cHEU may be related to reduced thymic output or reduced progenitor function. A study by Nielsen et al., [119] found reduced CD4+ naive and memory subset counts in babies born to HIV-infected mothers on ART (the majority: 18/20 with CD4 counts >200/μL) suggesting that treatment during pregnancy does not normalise CD4 T cell counts in HIV exposed babies. Overall, there is evidence that a number of hematological parameters are reduced in infants born to HIV infected mothers—even on suppressive ART. This reduction in immune cell counts is partly associated with maternal CD4 counts and is more severe in the case of maternal lymphophenia. The clinical significance of these reduced cell counts on the health of the HEU is as yet unclear.

Whilst immune cell numbers may be important, the effector functions of these cells in cHEU may be more relevant [79]. Recently, a growing number of studies on cellular mediated immunity in cHEU are focused on responses to vaccination which is covered in the next section.

1.2.3 Antibody responses

Some of the more common morbidities observed in cHEU are against vaccine preventable diseases [84, 106, 104, 103] suggesting that this group fail to mount effective immunity following vaccination and therefore are at increased risk for acquiring infection. In addition to the nutritional benefits provided by breast milk, breast-fed infants are provided with passive immunity which lower the risk of infectious morbidity and mortality [122, 98]. HEU neonates have lower levels of passive immunity against vaccine-preventable diseases compared to HU neonates [123] which underscores the need for effective immunisation in this group. In a South African study

measuring antibody levels to vaccine preventable diseases (*Haemophilus influenzae* type b, Pneumococcus, *Bordetella pertussis*, Tetanus and Hepatitis) found that birth antibody titres were lower among HEU compared to HU groups, but routine vaccination resulted in comparable antibody levels [123]. In general, antibody responses to most routine vaccines are robust among cHEU who have received the full course of booster vaccinations and the vast majority maintain sero-protective levels to at least two years of age [114, 124, 123, 125].

1.2.4 Cell-meditated responses

Cell-mediated immunity (CMI) refers to the ability of immune cells to respond to antigenic stimulation by proliferating, exerting cytotoxic effects or producing effector molecules such as chemokines and cytokines. There is no broad consensus in the literature on whether cell-mediated immune reponses are perturbed in cHEUs. Prior to PMTCT programmes being widely adopted, comparisons of HEU and HU groups were heavily confounded by factors related to the health of HIV-infected mothers, including high viral loads and low CD4 counts. The impact of maternal and/or neonatal ART use on cell-mediated responses can only be assessed by observational studies, however limited sample sizes mean that only a few studies have directly assessed this. As more countries adopt standard PMTCT programmes like Option B+, HEU cohorts are likely to become more homogenous with respect to the use of ARV regimens and the overall benefits of antenatal and post-natal care provided by PMTCT programmes to the health of mother and child. Nevertheless, the interpretation of even recent CMI studies is difficult given other confounders related to experimental methodology, the stimulating antigen, geographical and age-related differences of the cohorts studied (Table 1.1). As pre-exposure prophylaxis during pregnancy expands in South Africa, more data on the effects of ARVs, in the absence of HIV, on foetuses and infants may accumulate.

Cellular responses to mycobacterial antigens

Much of the data on cell mediated immunity in cHEU have come from studies evaluating responses to BCG or mycobacterial antigens [126, 40, 127, 128, 129, 130, 131, 132]. This is because HIV endemic regions in sub-Saharan Africa suffer the added burden of TB disease [133], meaning that many cHEUs in these regions are repeatedly exposed to *Mycobacterium tuberculosis* (Mtb), and therefore at risk for TB disease [134, 107]. Although correlates of protection against TB disease remain undefined, understanding vaccine immunity against Mtb as well as immunity to Mtb itself in cHEU, may provide insight as to whether they maybe disproportionately at risk for TB disease. Typically, the read-out from these studies is either intracellular cytokines or soluble immune mediators released into culture media after short- or long-term incubation with antigen. In studies comparing cellular responses between HEU and HU groups following long-term (>24 hrs) culture with mycobacterial antigen, differences or lack thereof exist depending on the read-out. For example, the frequencies of total blasting T cells—as measured from light scatter properties by flow cytometry—are not impacted by HIV exposure status [129], whereas the frequencies of mycobacterial-specific proliferating cells—as measured by Ki67—following long-term culture, two studies found no differences between HEU and HU groups [40, 126] with one study showing that differences were age-dependent (greater proliferation among HEUs at 16 weeks but not 6 weeks) [128]. In a study characterising T cell function in HEUs whose mothers received ART during pregnancy and had CD4 counts >200 cells/mm^3, Kidzeru et al.,[128] found reduced proportions proliferating T cells producing any 3 combinations of IFN-γ, IL-2, Th17 or IL-13 in response to *in vitro* BCG stimulation at 6 and 14 weeks of age. Of note was that rates of lower respiratory tract infections in this study were twice that of the HU control group suggesting a possible link between altered immune responses and susceptibility to infection. Other studies have found no differences in polyfunctional T cells (simultaneously producing 3 or more cytokines)[127, 131], which are implicated in protective immunity [135,

136, 137]. A distinguishing feature of the lymphoproliferation assay by Kidzeru et al., [128] was the addition of PMA/ionomycin to BCG-stimulated culture in the final 4 hours of the assay, where others have used either Staphylococcus Entero-toxin B (SEB) [126] or no mitogenic re-stimulation [129, 130]. Thus, the results of long-term *in vitro* assays are confounded by the use of superantigen or mitogen in addition to the primary antigen stimulus. For levels of soluble mediators after long-term culture, TNF-α and GM-CSF were found to be elevated only in BCG-stimulated cultures of HEUs whose mothers who showed Mtb sensitisation by Quantiferon Gold testing [130], suggesting that maternal mycobacterial exposure may preferentially prime BCG responses in the HEU infant. Maternal BCG immunisation however does not appear to enhance infant responses to Mtb, as shown by a large study of 1500 Ugandan infants, where maternal BCG scar (indicating prior immunisation) was associated with lower infant IL-5 and IL-13 responses to Mtb secreted crude culture filtrate proteins (cCFP) [40]. In the same study, cCFP responses in the infant did not differ by maternal HIV status among HIV-uninfected infants [40].

Fewer studies have evaluated CMI to mycobacterial antigens between HEU and HU using short-term *in vitro* assays [127, 131]. The whole blood assay is a standard for short-term CMI measurements, where whole blood is incubated directly with antigen [138]. Unlike PBMC based assays, no isolation of cells is required, a logistical advantage for field studies where often the laboratory is not easily accessible. In addition, whole blood more accurately reflects physiological conditions *in vivo*, with appropriate growth, co-factors and cells for antigen processing and presentation [139]. The results from whole blood intra-cellular cytokine staining (ICS) assays suggest that HEUs are immune competent. For example Ugandan infants who were BCG-vaccinated at birth had total intra-cellular cytokine frequencies responding PPD at 3 months post vaccination [127] and a South African study showed higher frequencies of BCG-specific CD8 T cells positive for IFN-γ among HEUs compared to controls only at the 6-month time point [131]. A criticism of these two ICS studies is

that no correction for multiple comparisons was performed which may have negated the observed differences between HEU and HU groups. Furthermore, Mansoor et al., [131] did not report on potential maternal confounding variables such as CD4 counts and viral loads, however, as mentioned by the authors routine ART was not available at the time of study. In summary the impact of HIV exposure on infant cellular immunity to BCG is confounded by infant age, the cellular assays used and their readouts as well as maternal confounders.

Cellular responses to mitogens

One of the more consistent findings of cell mediated responses in the HEU is the impairment of a number of effector cytokines after *in vitro* stimulation with superantigen or mitogen (Table 1.1). After six-day stimulation with staphylococcus enterotoxin B (SEB), Kidzeru et al., [128] showed that the capacity to produce multiple cytokines at a single-cell level was diminished compared to HUs, with significant differences in proliferating CD8 cells expressing both IFN-γ and IL-13. This impaired cytokine production to SEB is supported by studies measuring total CD4 cells, where lower frequencies of cells expressing IFN-γ, IL-4, TGF-β and IL-10 after 72 hours of stimulation [140] and IL-2 and TNF-α after 12 hour stimulation are observed in HEU compared to controls [127]. Lower cytokine responses in HEU cell cultures stimulated with the lectin PHA have also been observed for IFN-γ, TNF-α and IL-2 [130, 132] suggesting that impaired cytokine production may involve other immune subsets beyond T cells. Interestingly, PHA stimulation shows comparable frequencies of proliferating T cells between HEU and HUs [129, 130] whereas SEB enhances T cell proliferation among HEUs albeit after secondary stimulation with PMA/ionomycin [128].

Cellular responses to other vaccine antigens

CMI studies to non-mycobacterial vaccine antigens that directly compare HEU and HU groups have been limited. Two studies have measured cellular responses to Tetanus Toxoid [40, 127] and one to Pertusssis antigens [128]. Altered cytokine responses to Tetanus in the HEU have been observed for long term *in vitro* stimulation assays [40] but not in short-term assays [127]. A large Ugandan study measuring cellular responses to Tetanus Toxoid in 12-month old infants, found reduced levels of IFN-γ, IL-5 and IL-13 in HEUs compared to controls following long-term *in vitro* stimulation, which remained significant after controlling for a number of maternal confounders including hookworm infection and maternal immunisation [40]. This suggests that even after a full course of vaccinations, significant defects in Th1 and Th2-mediated immunity to Tetanus exist in the HEU. A limitation to the aforementioned study, was the cross-sectional study design, where only one-year old infants were studied. For T cell cytokines measured by intracellular cytokine staining by flow cytometery after short-term Tetanus Toxoid stimulation, no differences in IFN-γ, IL-2 or TNF-α were seen for a similar Ugandan cohort between 3 and 12 months of age, even when accounting for the ART-use and CD4 counts [127]. To summarise, it appears there is no evidence that T cell function to Tetanus antigen is perturbed in the HEU, however the effector function of other immune cells maybe reduced as shown in culture assays. To date, only one study has directly compared HEU and controls for cellular responses to *Bordetella pertussis* (BP) antigens, finding lower frequencies of proliferating polyfunctional T cells in the HEU group [128].

Cellular responses to polyclonal and cytokine stimuli

As a measure of general immune competence by T cells, *in vitro* studies have used anti-CD3 in conjunction with anti-CD28 monoclonal antibodies to trigger the T cell receptor (TCR) and begin an intra-cellular signalling cascade that induces functional responses, by-passing classical antigen presentation [141]. Maternal viral load and

ART treatment during pregnancy have been shown to correlate anti-CD3-meditated function in the HIV exposed neonate [142, 143]. Stimulation with anti-CD3 resulted in reduced pro-inflammatory cytokines and increased regulatory cytokines in babies born to mothers with uncontrolled HIV replication compared to those born to women with undetectable viral loads in a Brazilian study [142]. These results were supported by another observational study where detectable viremia during pregnancy (defined as >80 copies/mL) was associated significantly elevated levels of IL-10, but lower levels of TNF-α and IFN-γ in culture supernatants from neonatal anti-CD3 stimulated cells compared to cells from mothers with undetectable viral loads during pregnancy [143]. Another study which evaluated the upregulation of chemokine receptors on mononuclear cells derived from cord blood in response to anti-CD3 stimulation found no differences between HEU and HU groups however, exposure to GM-CSF in cell culture preferentially upregulated CCR8 in HEU neonates [144].

1.2.5 Gene expression in HIV-exposed individuals

Gene transcription data from HEU infants have been lacking, and such studies may give a broader insight into potential immune dysregulation in this population. The most common genetic studies in HEU have measured mitochondrial DNA content—a proxy for mitochondrial function—with conflicting results [146, 147, 81, 148, 149, 150] but suggest altered mitochondrial function as a result of direct ARV-exposure [151, 152, 153]. In adult cohorts of known repeated exposures to HIV without infection, 'HIV resistance' is associated with a downregulation of genes and pathways related to energy metabolism, NK cytotoxicity and T cell receptor signalling [154]. The interpretation here is that resistance to infection is a result of a downregulation of genes that HIV needs for successful infection. For babies who are HIV exposed *in utero*, different physiological determinants are at play which may result in gene expression patterns that are distinct from the HIV-exposed adult. Non-nucleoside reverse transcriptase inhibitors, such as efavirenz, has been shown to

Table 1.1: Cell-mediated immune findings between HEU and HU infant groups

Study	Assay antigen & stim time	Infant age, confounders	Assay read-out	Findings relative to HU
[126]	BCG 6-day, SEB final 4hrs	16 wks, maternal QFT status	T cell ICS, proliferation, cytokine in sup	None
[40]	cCFP, TT, 6-day, no mitogen re-stim	12 months, maternal hookworm	cytokine in sup	No differences to cCFP, lower IFN-γ, IL-5, IL-13 to TT
[127]	PPD, TT, SEB, 12-hr	3, 12 months, maternal HAART	T cell ICS, activation	Increased total cytokine to PPD at 3 months, lower at 12 months
[128]	BCG 6-day, SEB 5-day, PMA/ionomycin final 4hrs	6, 14 wks	T cell ICS, prolif	Increased proliferation to BCG, Fewer polyfunctional proliferating cells & SEB
[129]	BCG 2-day, PHA 6-day, no mitogen re-stim	Birth	T cell proliferation to PHA, cytokine in sup to BCG	No differences in proliferation, increased IFN-γ to BCG
[130]	BCG, PHA 2-day, no mitogen re-stim	7 months	T cell proliferation, cytokine in sup	No differences in proliferation, lower IFN-γ, TNF-α to BCG, lower IFN-γ to PHA
[131]	BCG 12-hr	3, 6, 12 months, maternal HAART unknown	T cell ICS, cytokine in sup	Increased CD8 IFN-γ at 6 months
[140]	SEB 72-hr, no mitogen re-stim	Birth and 2-days	T cell ICS, CD107a	Decreased CD4 IFN-γ, IL-4, IL-10 and TNF-α
[143]	anti-CD3/anti CD28, HIV peptides, 3-day, no mitogen re-stim	Birth, maternal viral load	T cell proliferation, cytokine in sup	None
[142]	anti-CD3/28, HIV peptides, 3-day, no mitogen re-stim	Birth, maternal HAART	Cytokine in sup	Increased TNF-α, IL1-β (born to ART-treated mothers
[144]	anti-CD3/28 6-day, cytokine stim, no mitogen re-stim	Birth	Chemokine receptor expression	None
[132]	PHA (unknown stim time)	Birth	Cytokine in sup	Lower IL-2
[113]	TLR agonists 24-hr	Birth	CD80, CD86, B7-H1, IFN-α	Increased mDC expression of CD80, CD86B7-H1
[145]	IL-12 cyk 20-hr	Birth	Target cell death by NK cells, CD107a	Increased killing by NK cells and increased CD107a

ART-antiretroviral therapy, BCG-Bacille Calmette-Guerin, cCFP-crude cultture filtrate proteins, Cyk sup-cytokines in culture supernatant, HAART-Highly active antiretroviral therapy, ICS-intra-cellular cytokine staining, mDC-myeloid dendritic cells, NK-Natural killer cells PHA=Phytohemagluttin SEB-Staphylococcus Entero-toxin B, sup-supernatant TLR-Toll-like receptor, TT-Tetanus Toxoid

induce mitchondrial toxicity and damage with release of mitchondrial DNA [155, 156]. Vivanti et. al, [157] compared gene expression profiles between neonates exposed to azidovudine or tenofovir-based combination therapies *in utero* from unexposed controls (n=8, 9, and 8, respectively) by microarray analysis of isolated CD3 cells isolated from cord blood. They found significant downregulation in the transcription of genes related to DNA repair and telomere maintenance. The gene expression profiles of CD34+ hematopoetic stem cells from 7 azidovudine-exposed and 6 control newborns and found more than 300 differentially expressed genes in the exposed group [158]. A recent study of Kenyan infants compared gene profiles in peripheral blood mononuclear cells (PBMCs) in 12 month and 24 month-old HEU infants and age-matched HU controls [159]. Almost two-thirds of differentially upregulated genes in HEUs were related to plasma membrane receptor proteins found on immune cells and some of these altered genes are associated with activation or the regulation of inflammation, suggesting a dysregulation in the immune activation pathway. Importantly, genes related to neutrophil function were repressed in HEUs, indicating a potential defect of the innate immune system that may predispose these infants to bacterial infections [159]. In this PhD, we propose to examine gene expression in HEU birth blood, the sample closest to *in utero* HIV/ARV exposure and a time point that mitigates confounders related to environmental and other post-natal exposures.

1.3 ARV regimens in HIV-exposed and uninfected neonates

The World Health Organisation (WHO) has specified which antiretroviral drug regimens should be used during pregnancy, irrespective of clinical or immune status, with disoproxil fumarate (TDF) making up the backbone of recommended regimens for maternal health benefits, prevention of mother-to-child HIV transmission (TDF plus emtricitabine: FTC & efavirenz: EFV) and prevention of HIV infection in uninfected women (TDF-FTC, Truvada®) [160]. These recommendations are extrapolated from

limited clinical safety data. The scale-up of WHO's recommended triple therapy (Option B+) to pregnant and breastfeeding women living with HIV regardless of maternal immune status has significantly reduced paediatric HIV infections [161]. As a result of these interventions, more foetuses and infants are exposed not only to intrauterine HIV, but also intrauterine ARV, neonatal ARV prophylaxis, and in some cases, to maternal HIV/ARVs via breastmilk [162]. These exposures comprise both an infectious and drug exposure, making it often difficult to disentangle the effects between the two. HIV/ARV exposure during pregnancy may lead to mitochondrial toxicity [81, 163, 153], decreased postnatal growth [86], and genotoxicity [158, 157], all of which could perturb energy metabolism and impact the short and long-term metabolic health of cHEU.

1.4 Infant vaccination

As many as 1.5 million infant deaths yearly are attributed to vaccine preventable diseases [164]. Vaccination remains the most cost-effective public health intervention available for reducing childhood morbidity and mortality [165, 166, 167, 168]. Many national vaccination programmes exist, the majority following recommendations by WHO's Expanded Programme on Immunisation (EPI) in their national immunisation programmes [169, 170]. The following section will review broadly some of the vaccines in the EPI currently in use in Sub-Saharan Africa, with a focus on the immune responses generated by the vaccines and known correlates of protection.

1.4.1 Expanded Programme on Immunisation (EPI)

The World Health Assembly initiated the EPI in 1974 with the aim of increasing vaccine coverage to all children globally to reduce infectious disease burden and mortality in early life [171]. Vaccines against Tuberculosis (BCG), Diphtheria, Tetanus, Pertussis, Measles and Poliomyelitis were part of the initial recommendation but this

has since expanded to include yellow fever, Hepatitis B (HBV) and *Haemophilus influenzae* type b in countries endemic to these diseases [170, 172]. Since the introduction of the EPI, infant death under two years of age has decreased by 50% on average in Sub-Saharan Africa [173]. WHO-UNICEF estimates for coverage in South Africa between 2010-2018 are as follows: BCG (84%, 98% in the Western Cape), DTP 1st dose (90%), DTP 3rd dose (87%), Measles 1st dose (MCV1: 89%) [174]. For Nigeria, the estimates for vaccine coverage are lower than national reports [169] with WHO-UNICEF estimates for the same period as follows: BCG (55%), DTP 1st dose (58%), DTP 3rd dose (49%), Measles 1st dose (MCV1: 66%) [174]. Clinical and policy research output for childhood vaccines in South Africa and Nigeria are the highest on the continent [175], and these two sites are a feature and focus of this PhD thesis.

BCG

Tuberculosis is caused by *Mycobacterium tuberculosis* (Mtb) and is the leading cause of death by a single etiological agent, with 1.5 million deaths and 10 million developing active disease annually [173]. BCG is currently the only licensed vaccine against TB and is effective against severe forms of disseminated Mtb infection in children but has variable efficacy against the most common form of the disease, adult pulmonary TB [176]. The uncertainty surrounding vaccine efficacy means that national vaccination strategies differ, with many offering different doses, routes of administration, timing of vaccination, and sub-strains of BCG [177]. Current practices in South Africa and Nigeria are single intradermal dose at birth or shortly thereafter as per WHO guidelines even in newborns whose HIV status is not confirmed at delivery [178, 179]. BCG is contraindicated for symptomatic HIV-infected infants due to the risk of BCG adverse events and disseminated BCG [180]. In high TB endemic settings, the benefits of early BCG vaccination outweigh the risks associated with delaying BCG until HIV status is known, especially when PMTCT programmes

are in place [178].

While some studies demonstrate that BCG induces antibodies against mycobacterial antigens [181, 182, 183, 184], their association with protective function is poor in animal and human studies [185, 137]. BCG induces a complex cell mediated immune response including Th1, Th2 and Th17 cytokines [186, 17, 187, 36]. Variability in responses to BCG are in part related to geography as observed when comparing infant responses in the tropics vs. further away from the equator. A study comparing BCG vaccinated UK and Malawian infants, highlighted major differences in the cytokine profiles induced by *in vitro* PPD stimulation [36]. At 3 and 12 months post vaccination higher Th1 cytokines (IFN-γ, IL-2 and TNF-α) were observed in the supernatants of PBMC-stimulated cultures from UK infants compared to Malawian infants. In contrast Malawian infants had higher Th2 (IL-13, IL-5, IL-9 and IL-4) and pro-inflammatory cytokines compared to UK infants. These differences highlight how potential variations in environmental antigen priming, maternal factors and possible exposure to infectious agents impact BCG immunogenicity [32, 188, 40, 126, 189]. For example helminth infections are more common in the tropics and these infections may dampen immune responses [190, 191, 40] and treating helminth infection in BCG vaccinated infants rescues pro-inflammatory responses while limiting regulatory responses [32].

The route of immunisation appears to impact on the strength of immune responses [192]. A study comparing percutaneous and intradermal vaccination of infants receiving BCG-Japan strain showed greater Th1 (IFN-γ, IL-2 and TNF-α) and lower Th2 (IL-4) compared to intradermal vaccination with the same strain [192]. There is no evidence that the route of administration (percutaneous vs. intradermal) impacts vaccine efficacy [193]. Delaying BCG vaccination does not impact proliferative (Ki67+) Th1 responses [194] but has shown to increase the total CD4 memory Th1 response [187]. South African infants receiving BCG at birth had lower polyfunctional (cells 3 or more cytokines) at one year compared to infants

vaccinated at 10 weeks [187]. Polyfunctional CD4 responses are of interest in rational TB vaccine design studies as they correlate with delay or protection from disease for some intra-cellular pathogens including HIV [136, 195, 137]. In mice, BCG-induced polyfunctional responses in the lung have been shown to correlate with reduced bacterial burden following aerosol Mtb challenge [135]. In humans, while polyfunctional responses have correlated with *in vitro* mycobacterial growth inhibition [196], these responses to not correlate with Mtb protection *in vivo* [187]. In a case-control study of South African infants who received BCG at birth found no differences in polyfunctional CD4 cells at 10 weeks (IFN-γ, IL-2, TNF-α, IL-17) between infants who were protected from TB disease to those who went on to develop TB disease [187]. Polyfunctional cells induced by vaccination may not be sufficient alone to protect against TB disease but remain targets for vaccine-induced immunity in many novel TB vaccine candidates [197].

Diphtheria-Tetanus-Pertussis

Diphtheria is caused by toxogenic bacilli of the species *Corynebacterium diphtheriae*, whose toxins affect multiple mucous membranes including the nasal airways and skin [198]. It is spread through air droplets via coughing and complications brought on by the toxin can lead to death. The most vulnerable are infants where case-fatality rates can be as high as 20% [198]. EPI has had significant impact in reducing the global incidence of Diphtheria and rates have fallen drastically from close to 100 000 cases in 1980, to just over 7000 cases in 2016 [199]. In South Africa and Nigeria between 2010 and 2018 there were 23 cases and 1870 cases, respectively [199]. Tetanus is caused by the bacterium *Clostridium tetani*, and its exotoxin Tetanospasmin is a potent neurotoxin whose clinical manifestations include include lock-jaw, stiffness of the neck and difficulty swallowing [198] with eventual respiratory failure. Low doses of the toxin, which enter via open wound can be fatal. Neonatal Tetanus cases were low in South Africa between 2010 and 2018; with 8 cases

reported, and none reported in 2018, whereas 1334 cases in Nigeria are reported in the same year [200]. Pertussis (commonly known as whooping cough) is a contagious respiratory tract infection caused by the bacterium *Bordetella pertussis*. Bacterial products include pertussis toxin (PT), tracheal cytotoxin, filamentous hemagglutinin (FHA), and pertactin which are antigenic [201] and result in disease. Estimates of Pertussis incidences vary significantly due to the lack of reliable surveillance data and infrastructure for reliable diagnostics [202, 203]. Africa nevertheless represents the largest proportion of Pertussis cases and death in infants younger than 5 [204]. The re-emergence of Pertussis cases in lower income countries underscores the need for accurate surveillance and improved vaccination coverage [202], particularly for HEU infants as they benefit less from maternal antibody transfer during the neonatal period [123].

Both cellular and antibody responses are induced by DTP. Passive immunity from mothers is usually lower in HEU infants compared to HU infants, however protective antibody titres are observed following 3 doses of DTaP [123]. Antibody levels to DTP wane during childhood, and often booster vaccinations are required in adolescents or adults [205]. Cell mediated responses to TT and BP include Th1 and Th2 cytokines, with IFN-γ responses peaking at 6 months of age and waning thereafter [206]. Th2 responses, specifically IL-13 and IL-5 also peak at 6 months of age but are sustained up to 18 months of age in infants who receive 3 doses [206]. Current practices for vaccination against the aforementioned diseases are Diphtheria-Tetanus-Pertussis (DTP) multi-component vaccines such as DTaP-IPV/Hib which includes inactivated polio, *Haemophilus influenzae* type b and Hepatitis B antigens. This vaccine is typically delayed until 6 weeks in infants as a number of factors impact immunity to this vaccine: firstly maternal antibodies may interfere dampen responses to other components of the vaccine [207, 208, 209] and secondly the Th2 bias of the early infant immune system may affect responses to vaccination [210]. Studies in mice showed that both adoptively transferred CD4 cells specific for FHA and passively transferred

BP-specific sera were equally protective to aerosol BP challenge, suggesting both Th1 and antibody-mediated roles in protecting against Pertussis infection [211]. Interestingly, CD4 cells expressing IFN-γ and IL-2 are able to mediate protection in the absence of a detectable serum antibody response [211]. There has been a shift from the use of whole-cell form of the Pertussis component (wP) of DTP to acellular form (aP) due to adverse local reactions caused by the former [212]. Whole-cell Pertussis induces a predominantly Th1 response [213] and long term protection in mice is correlated with a sustained Th1 profile when antibodies have waned [214]. In children, aP induces a Th1/Th2 mixed response [215, 216] with antibody titres waning quicker than wP [217].

1.5 Thesis aims

The over-arching aim of this thesis was to identify immune signals that differentiate children who are exposed to ART/HIV-exposed yet uninfected from those who are un-exposed. Infant whole blood samples were obtained from an existing multi-centre study (Innate, Adaptive and Mucosal Immune Responses in Infants/INFANT study: HREC 285/2012) that seeks to identify biological processes associated with protection of mother-to-child transmission of HIV. Bio-banked samples were available for two birth cohorts from Nigeria and South Africa infants sampled between birth and 9 months of age. This period coincides with both the administration of the majority of EPI vaccines and reported infectious morbidity in the HEU [218, 88]. We explored the hypotheses that HEU have altered T cell immunity compared to HU controls and distinct gene profiles at birth. These hypotheses were tested in three distinct aims:

1. To identify differentially expressed genes (DEG) at baseline that delineate cHEU from cHU.

2. To compare the ontogeny of T cell immunity between cHEU and cHU during the first 9 months of life.

3. To characterise how differences in the immunising strains of BCG, the first vaccine received in these infants, impacts T cell immunity to both mycobacterial and non-mycobacterial antigens in cHEU and cHU.

Chapter 2

Transcriptome of HEU newborns

2.1 Introduction

Rationale for measuring gene expression in the HEU

Gene transcription data from HEUs are lacking, and such studies may give a broader insight into potential pathways involved in immune dysregulation in this population. Because the transcriptome measures all actively transcribed genes in a sample in an unbiased manner, much the underlying biology from a sample can be captured in a single experiment without an *a priori* selection of which biological signals may differ between two groups of interest. In a recent study of 1 - 2 year-old Kenyan infants, a total of 262 differentially expressed genes between HEU and HU controls [159]. Among the differentially down-regulated genes were those related to neutrophil function in HEUs, suggesting a vulnerability of the innate immune system [159]. The infants in the Kenyan study were between 1 and 2 years of age, therefore postnatal exposures to environmental antigens are potential confounding variables in interpreting the data. Characterising the transcriptome in samples closest to *in utero* time point would better reflect transplacental exposures to virus, ART or the maternal cytokine milieu on the biology of the newborn immune system [219, 220, 144, 129, 149, 118, 221, 222, 113]. To this end, we characterised mRNA transcripts

in birth whole bood of HEU and HU newborns. An added advantage of using whole blood rather than PBMCs, was that more of the immune landscape could be covered as additional cellular subsets such as eosinophils and neutrophils are present in whole blood. Furthermore, this sample closely mimics *in vivo* physiological conditions. All infants were confirmed HIV negative by PCR at birth, and all HIV+ mothers received antiretroviral treatment during pregnancy as per national guidelines [223, 224].

2.2 Methods

2.2.1 RNA-Seq library construction and sequencing

A volume of 500µL of venous whole blood at birth was collected and aliquoted into PAXgene® fluid at the ratios recommended by the manufacturer (BD). Aliquots of whole blood in PAXgene® fluid were kept at room temperature for 24 hours before storing at -80°C as per manufacturer instructions. RNA was purified using the PAXgene Blood RNA Kit (Qiagen), and RNA concentration and quality was assessed using a NanoDrop and an Agilent Bioanalyzer, respectively. Alpha and beta globin mRNA was depleted from 1µg of RNA using the ThermoFisher GLOBINclear-Human Kit. Ten nanograms of globin-depleted RNA was used as input for cDNA synthesis using the Clontech SMART-Seq v4 Ultra Low Input RNA kit according to the manufacturer's instructions. Amplified cDNA was fragmented and appended with dual-indexed bar codes using the Illumina NexteraXT DNA Library Preparation kit. Libraries were validated by capillary electrophoresis on an Agilent 4200 TapeStation, pooled, and sequenced on an Illumina HiSeq3000 at 100SR at an average read depth of 15 million reads per sample. FASTQ files from the sequencer were aligned to the Ensembl reference genome using the STAR read aligner [225].

2.2.2 Differential gene analysis

We used the DESeq2 package to identify differentially expressed genes between groups. Briefly, the DESeq2 pipeline uses the high dimensional row count matrix from the sequencing reads it's data input. Quality control steps include normalisation of the read counts to account for systematic effects that are not related to biology [226]. DESeq2 models the raw counts for each gene using a negative binomial model, to estimate priors for log fold change and dispersion, and to calculate posterior estimates for these quantities. Variance stabilisation transformation from the fitted dispersion mean relations was applied using the regularised log (rlog) method in DESeq2 to normalise raw counts. We included cohort site and gender as co-variables in the DESeq2 model design to account for these effects on HIV exposure. Differentially expressed genes were considered based on based on FDR<0.05 and an absolute fold change (FC)>1.0 normalised counts.

2.2.3 Gene-set enrichment analysis

Gene set enrichment analysis was performed using the GSEA software version 3.0 [227]. Log transformed counts, generated using the rlog method from the DESeq2 package, were used as input for the GSEA analyses. Gene sets representing the activity of pathways were derived from the C2, C5 and Hallmark collections of the Molecular signature database (MSigDB)[228]. For all GSEA analyses, phenotype permutation was used. Gene-sets/modules were considered significantly up- or down regulated based on normalised effect sizes >1.5 between phenotype groups with an adjusted p-value (FDR) <0.05.

2.3 Results

2.3.1 Baseline characteristics

Paxgene samples were selected for sequencing based on RNA quality with RNA integrity values >7.5 (Appendix 7.3). It is unclear why Jos samples had poorer RIN scores compared to CT samples despite showing comparable RNA yields. A likely explanation may be related to fluctuations in shipping temperatures, thus only a total of 11 samples from Jos were included. All HIV+ women were on fixed dose combination antiretroviral treatment (efavirenz, emtricitabine and tenofovir disoproxil fumarate) during pregnancy. Clinical characteristics between cHEU and cHU control groups were similar except for the distribution of gender in the Jos cohort. To test the hypothesis that specific genes were up- or down-regulated in cHEU, the transcriptome of birth whole blood was analyzed in 61 samples using RNA Seq analyses (Table 2.1). To account for potential cohort and gender confounders, we included cohort and gender as covariates in our DESeq2 model design.

Table 2.1: Baseline characteristics for RNA Seq analyses

	CT		***P*-value**	**Jos**		***P*-value**
	HEU (n=33)	**HU** (n=17)		**HEU** (n=6)	**HU** (n=5)	
Gestational age (weeks)	39 [38-40]	39 [38-39]	0.22	39 [38-40]	40 [40-41]	0.18
Birth weight (g)	3270 [2880-3440]	2940 [2900-3230]	0.12	2825 [2538-3000]	3500 [3400-3600]	0.15
Length (cm)	48 [45-50]	49 [47-50]	0.68	45.5 [45-46.8]	50 [48-52]	0.14
n (Male/Female)	10/23	6/11	0.72	1/5	5/0	**0.006**

Numbers indicate median and (IQR) unless otherwise stated. *P*-values calculated using Wilcoxon Rank Sum test. Chi-squared test of homogeneity was used to compared distribution of infant genders between exposure groups.

2.3.2 Differential gene analysis identifies a minor set of upregulated genes in HEUs

We first performed a principal component analysis (PCA) on all 61 samples comprising HEU and HU infants from both cohorts. PCA did not cluster by HIV exposure, or

by cohort site (Figure 2.1), suggesting no distinct overall variation in genes between HEU and HU groups or between Jos and CT infant samples.

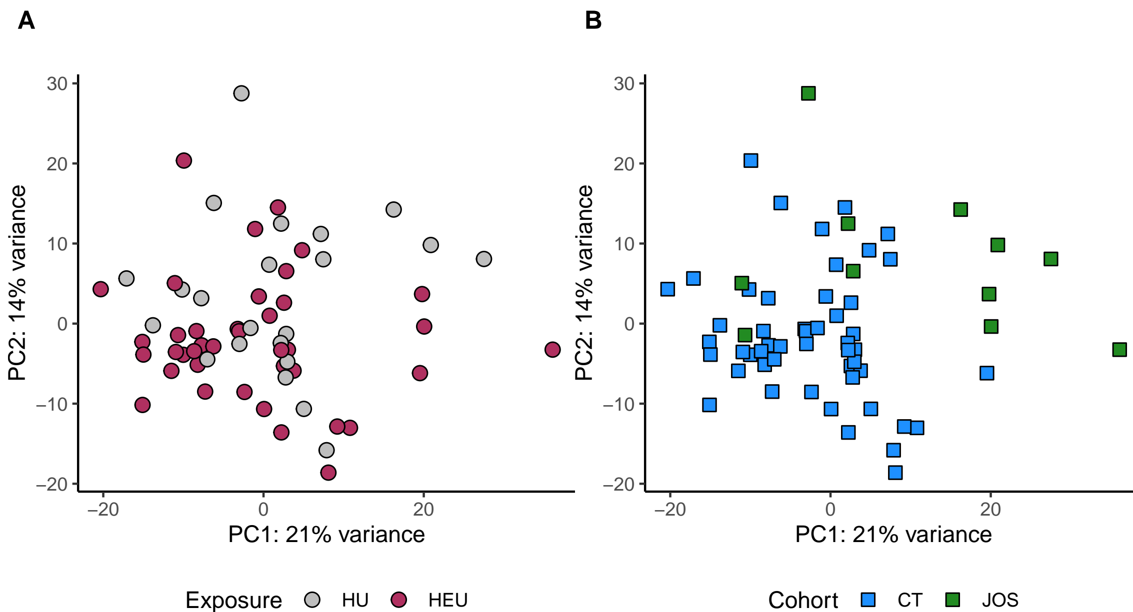

Figure 2.1: Principal component analysis of transcriptome data shows no clustering by HIV exposure status or cohort site. PCA plots are colour coded by HIV exposure (A) or Cohort site (B). HU = HIV-unexposed, HEU = HIV exposed and uninfected, CT = Cape Town

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After DESeq2 modelling, we filtered differentially expressed genes (DEGs) based on FDR<0.05 and an absolute fold change (FC) >1.0 for normalised counts. This resulted in a list of 15 DEGs, 3 of which were upregulated in HEUs, and 12 that were downregulated. Of the down-regulated genes 8/12 were Y-linked genes. Of the upregulated genes, 2 were homologues of the alpha arrestin family ARRDC4 (FC relative to HU: 2.3, *p*-adj<0.0001) and TXNIP (FC relative to HU: 1.4, *p*-adj<0.0001)(Table 2.2 and Figure 2.3).

Table 2.2: Results of DESeq2 analysis by HIV exposure status

Gene symbol	**Description**	**baseMean**	**Direction (wrt HU)**	**Fold change**	***p*-adj**
EIF1AY	eukaryotic translation initiation factor 1A Y-linked	3678.05	down	37.5	<0.0001
DDX3Y	DEAD-box helicase 3 Y-linked	109.02	down	25.4	<0.0001
ZFY	zinc finger protein Y-linked	54.16	down	27.6	<0.0001
PRKY	protein kinase Y-linked (pseudogene)	93.21	down	20.3	<0.0001
ARRDC4	arrestin domain containing 4	114.74	up	2.3	<0.0001
RPS4Y1	ribosomal protein S4 Y-linked 1	473.12	down	12.4	<0.0001
KDM5D	Lysine demethylase 5D	88.37	down	24	<0.0001
TXLNGY	taxilin gamma pseudogene, Y-linked	78.99	down	19.5	<0.0001
TXNIP	thioredoxin interacting protein	15627.76	up	1.4	0.001
TTTY15	Testis-Specific Transcript, Y-Linked 15	27.45	down	13.9	0.002
UTY	ubiquitously transcribed tetratricopeptide repeat containing, Y-linked	72.36	down	11.3	0.003
ANOS2P	anosmin 2, pseudogene	12.27	down	9.3	0.003
USP9Y	ubiquitin specific peptidase 9 Y-linked	24.04	down	10.6	0.006
LINC00278	long intergenic non-protein coding RNA 278	14.41	down	6.5	0.02
KLF10	Kruppel like factor 10	201.38	up	1.6	0.03

List of differentially expressed genes (> fold change 1.0 and p-adjusted values <0.05) relative to HU controls in whole blood samples from Cape Town and Jos infants at birth (N=61). baseMean refers to the average of the normalized count values, fold change is an estimate of gene expression in HEU relative to HU controls. Adjusted p values are shown after Benjamini Hochberg correction.

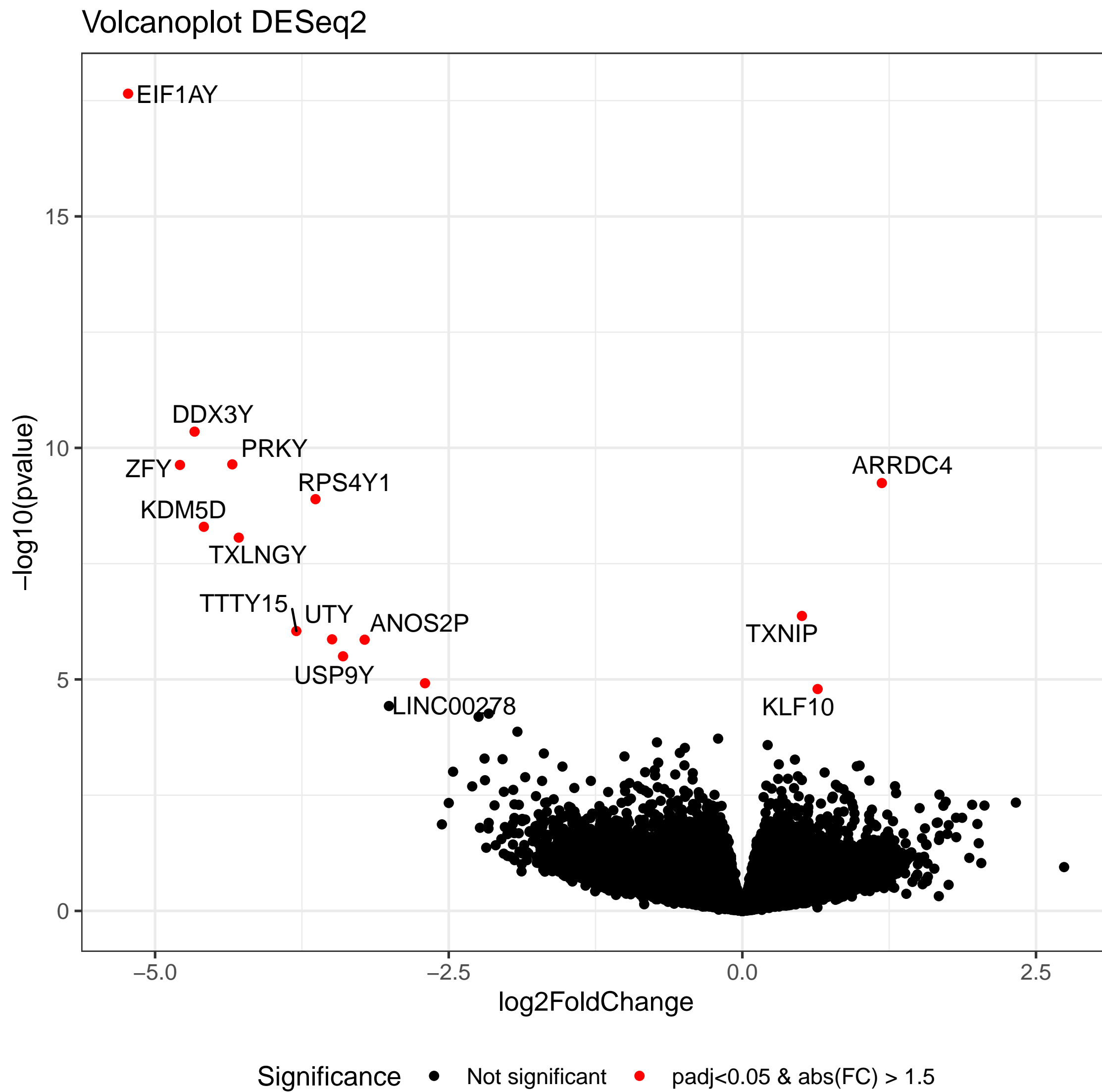

Figure 2.2: Volcano plot of differentially expressed genes between HEU and HU groups at birth. Significant differential expressed genes (DEG) are shown in red and gene names are labelled, and non significant genes are shown in black. Threshold for DEG relative to HU controls were set at >1.0 absolute fold change (abs FC) and p-adjusted <0.05 and are indicated in red and gene names are shown.

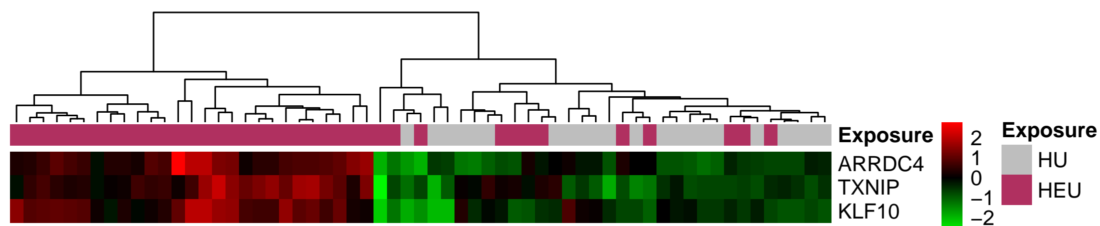

Figure 2.3: Three genes are increased in HEUs at birth. Heatmap shows the three genes with >1.0 fold increase in normalised counts relative to HEU and p-adjusted <0.05. Relative expression is based on z-scores of rlog transformed counts.

2.3.3 No distinct pathways are altered in HEUs

To complement differential expressed gene analyses, we applied gene-set enrichment analyses to identify whether the groups/modules of genes representing distinct biological pathways were enriched/downregulated in the HEU infant at birth. As shown in Tables 2.3 (C5: ontology genesets), 2.4 (C2: curated genesets) and 2.5 (Hallmark gene sets), no gene sets passed an FDR cut-off of less than 0.05. Importantly, while differential gene analyses found two genes involved in the glycolysis and/or ROS generation [229], none of the gene modules associated with glycolysis, reactive-oxygen species or oxidative phosphorylation pathways showed any significant differences between HEU and HU infants.

2.4 Discussion

We used RNA-sequencing technology as it has a number of advantages over microarray including wider dynamic range [230] and greater sensitivity and specificity [231, 232, 233]. We elected to use whole blood rather than isolated cell populations which provides a global snapshot of actively transcribed genes in HEU. Differential gene analysis revealed that TXNIP (Thioredoxin Interacting protein), ARRDC4 (Arrestin domain containing 4) and KLF10 (Kruppel-like factor 10) were over-expressed in the whole blood of HEU newborns compared to HU controls. Gene-set enrichment analyses however could not discriminate HEU from HU meaning that the small number of differentially expressed genes did not translate to differences in distinct biological pathways. We acknowledge the following caveats: (*i*) gene expression measurements on whole blood rather than pure cell populations mean that any small changes in gene modules/pathways are likely drowned out by the inherent noise of a mixed cell population, (*ii*) the sample size used in this study may have been limiting factor in detecting enriched gene modules directly *ex vivo* (*iii*) functional immune gene changes are less likely to be detected directly *ex vivo*. Nevertheless, differences

Table 2.3: Results of gene-set enrichment analysis for C2 database comparing HEU and HU groups at birth

PATHWAY/MODULE NAME	NES	NOM p-value	FDR
PID_TRAIL_PATHWAY	1.74	0.004	1.00
PID_EPHRINB_REV_PATHWAY	1.74	0.002	0.73
BRACHAT_RESPONSE_TO_CISPLATIN	1.71	<0.001	0.80
BIOCARTA_WNT_PATHWAY	1.69	0.004	0.70
REACTOME_MAPK_TARGETS_NUCLEAR_EVENTS_MEDIATED_BY_MAP_KINASES	1.67	0.002	0.75
BIOCARTA_SPPA_PATHWAY	1.66	0.002	0.78
REACTOME_ERK_MAPK_TARGETS	1.64	0.011	0.81
BARRIER_COLON_CANCER_RECURRENCE_DN	1.64	0.012	0.74
OUYANG_PROSTATE_CANCER_MARKERS	1.63	0.002	0.72

Normalised effect size (NES) are shown in descending order for top 10 gene modules with nominal p-values (NOM p-val). Only FDR q-values<0.05 were considered significant.

Table 2.4: Results of gene-set enrichment analysis for C5 database comparing HEU and HU groups at birth

NAME	NES	NOM p-val	FDR
POSITIVE_REGULATION_OF_OSTEOCLAST_DIFFERENTIATION	1.73	<0.001	1.00
POSITIVE_REGULATION_OF_MYELOID_LEUKOCYTE_DIFFERENTIATION	1.69	0.004	1.00
REGULATION_OF_LIPOPOLYSACCHARIDE_MEDIATED_SIGNALING_PATHWAY	1.68	0.002	0.82
NEGATIVE_REGULATION_OF_EXTRINSIC_APOPTOTIC_SIGNALING_PATHWAY_VIA_DDR	1.64	0.004	1.00
CARBOXY_TERMINAL_DOMAIN_PROTEIN_KINASE_COMPLEX	1.63	0.011	0.98
CYTOKINE_PRODUCTION_INVOLVED_IN_IMMUNE_RESPONSE	1.62	0.004	0.98
REGULATION_OF_UBIQUITIN_PROTEIN_LIGASE_ACTIVITY	1.61	0.006	0.95
NEGATIVE_REGULATION_OF_VIRAL_RELEASE_FROM_HOST_CELL	1.60	0.018	0.99
GDP_BINDING	1.60	0.006	0.92

Normalised effect size (NES) are shown in descending order for top 10 gene modules with nominal p-values (NOM p-val). FDR q-values <0.05 were considered significant.

Table 2.5: Results of gene-set enrichment analysis for Hallmark database comparing HEU and HU groups at birth

NAME	NES	NOM p-val	FDR q-val
IL6_JAK_STAT3_SIGNALING	1.19	0.19	1
IL2_STAT5_SIGNALING	1.16	0.14	1
TGF_BETA_SIGNALING	1.15	0.23	1
G2M_CHECKPOINT	1.12	0.34	1
E2F_TARGETS	1.09	0.38	1
MTORC1_SIGNALING	1.06	0.40	1
GLYCOLYSIS	1.05	0.36	1
HEDGEHOG_SIGNALING	1.02	0.44	1
HYPOXIA	0.99	0.47	1

Normalised effect size (NES) are shown in descending order for top 10 gene modules with nominal p-values (NOM p-val). FDR q-values <0.05 were considered significant.

in ARRDC4 and TXNIP mRNA in this heterogenous mixed cell samples are worth investigating further. Our finding that no significant gene pathways are altered in newborns from mothers on suppresive ART suggest that both biological and immune signals are indistinguishable from healthy un-exposed controls. We do not discount that age-related transcriptional differences in the HEU may become apparent later in life. A recent study of Kenyan HEU infants sampled at 12-month and 24 months found 188 upregulated and 74 down-regulated genes in the HEU [159]. Despite a relatively small sample size (19 HEU vs. 15 HU), these differentially expressed genes translated to significantly enriched immune gene pathways in the HEU. Among the downregulated genes in HEUs was the alpha arrestin ARRDC3 (log2foldchange = -1.5, p-adj<0.0001), which contradicted our finding of an upregulation in ARRDC4 (log2foldchange = 4.9, p-adj<0.0001)—a related alpha arrestin [234]. Alpha arrestins together with TXNIP regulators of glucose metabolism, playing key roles in glucose production in the liver and uptake from peripheral tissues [235, 236]. None of the functional enrichment terms found by Musimbi and colleagues translated to alterations in metabolic pathwways [159]. Differences in environmental exposures between HEU and age-matched HU controls may have confounded the interpretation

in the aforementioned study. For this study, we chose the birth time-point as being representative of direct *in utero* HIV/ART exposure. It is unclear what proportion of mothers were on antiretroviral treatment in the Kenyan study however, the authors did report that all infants received post exposure prophylaxis. We therefore report that in the context of suppressive ART during pregnancy, HEU newborns do not have significant alterations in gene transcripts in whole blood.

Chapter 3

T cell marker expression in HEU infants—activation, proliferation, regulation and markers of HIV susceptibility

3.1 Introduction

Rationale for measuring immune activation markers in the HEU

The over-arching hypothesis for this section is that maternal HIV infection increases markers of T cell activation and proliferation *ex vivo* in exposed infants. Furthermore, we hypothesise that over time, these differences in these markers between HEUs and HU infants become less apparent due to reduced HIV/ART exposure. The rationale for these hypotheses comes from two major observations: (*i*) maternal HIV infection is associated with increased soluble markers of inflammation both systemically and in the placenta [237] and (*ii*) patterns in the expression of plasma cytokines in HIV-infected mothers are mirrored in cord blood plasma of their uninfected newborns [142] suggesting a shared inflammatory milieu. This hyper-inflammatory environment

may not only activate immune subsets in the neonate [144], but also result in a compensatory regulatory response as seen in HEUs who express elevated levels of regulattory T cell markers [140]. In this PhD, the focus was on markers defining activated T cells, where upregulation of HLA-DR and Ki67 have been shown to be useful surrogates for disease state, disease susceptibility, antigen load and response to treatment [238, 239, 240, 241]. A flow cytometry panel was designed to define the following peripheral blood T cell populations: (*i*) activated cells defined by HLA-DR expression (alone or combination with proliferation marker Ki67), CD25 co-expressing CD127hi (from hereon referred to as CD25+ cells) or PD-1 expressing T cells; (*ii*) "regulatory-like T cells" defined by CD4 co-expressing CD25 and CD127lo (from hereon reffered to as "Treg-like"; (*iii*) HIV target cells defined by CD4 T cells co-expressing CCR5hi and gut-homing marker α4β7hi. The inclusion of the integrin α4β7 and the chemokine receptor CCR5 allowed the co-expression of activated gut-homing T cells which may also be potential targets for HIV infection [242, 243, 244, 245] and may predict HIV acquisition [245]. A major advantage of this study was that longitudinal sampling allowed us to model differences over time. Specifically, we tested three hypotheses with respect to HIV/ART exposure (*in utero* and/or postnatally via breastfeeding): (*i*) HEU infants have greater frequencies of markers of activation at birth compared to HU controls, and that these differences would decrease over-time; (*ii*) HEU infants have elevated frequencies of regulatory-like T cells compared to HU controls and (*iii*) HEU infants would have increased frequencies of HIV target cells compared to HU controls.

3.2 Methods

3.2.1 Whole blood fixation

For T cell activation measurements, whole blood was fixed with BD FACS^{TM}lysing solution directly *ex vivo* and within 6 hours of blood draw. Briefly, blood was drawn

into sodium heparin tubes and 500 μL of the anticoagulated blood was fixed before cryo-preservation in 10% DMSO and 90% FCS before storing in LN_2. For Jos samples, cells were cryo-preserved in 10% DMSO and 90% FCS, stored at -70°C and shipped within 6 months to the University of Cape Town (UCT) and then transferred to LN_2. Analysis of all samples was performed at the core laboratory at the UCT.

3.2.2 Cell staining, antibodies and flow cytometry

Batched stored samples were thawed quickly at 37°C and washed twice with 1X BD PermWash buffer then incubated in 1X BD PermWash for 10 minutes. The antibody cocktail mix diluted in 2% FCS in PBS was added and incubated at 4°C for 45 minutes. Cells were washed twice with in 2% FCS in PBS and re-suspended in 0.3 mL PBS for cell acquisition using a Beckton Dickinson LSRII flow cytometer (SORF model). The following monoclonal antibody-fluorochrome conjugates were used in the T cell activation/proliferation panel: α4β7-R-phycoerythrin (PE), CD8-V500, CD3-Alexa Fluor-700, CCR5-PE-Cy7, Ki67-Fluorescein isothiocyanate (FITC; BD), CD27 PE-Cy5, HLA-DR- Allophycocyanin-Cy7 (APC-Cy7), CD45-BV650 (Biolegend), CD4 PE-Cy5.5 (Invitrogen), CD127 PE-Texas Red-X (Beckman Coulter). A minimum of 50 000 CD3 events were collected on FACS DIVA v6 software. Post-acquisition compensation and analysis was performed in FlowJo version 9 (FlowJo, LLC). Figure 3.1 shows the gating strategy employed for *ex vivo* T cell marker quantification. Specific combinations of these markers identified subsets of interest: CD127 was used to define CD127lo and CD127hi subsets, which when co-expressing CD25, allowed us to discriminate T cells with Treg-like surface phenotype or CD25+ activated cells respectively [246, 247]. The co-expression of gut-homing marker α4β7+ and CCR5hi on CD4 cells were used as a proxy for HIV susceptibility or potential HIV target cells [242, 248, 243, 244, 245]. Other T cell subsets defined included activated (HLA-DR or PD-1) and/or proliferating (Ki67) cells.

3.2.3 Statistical analyses

Statistical analyses of flow cytometry data was performed in R [249]. Non-parametric comparisons between independent groups was performed using Wilcoxon Rank Sum test (Mann Whitney U test). For longitudinal data, a repeated measures analysis of variance (ANOVA) was used to assess the effect of time, HIV exposure or the interaction of these variables on the expression of T cell markers measured. A Friedman's test with the Wilcoxon-Nemenyi-McDonald-Thompson post-hoc test [250] was used to determine which time intervals were significantly different in the frequencies of markers measured.

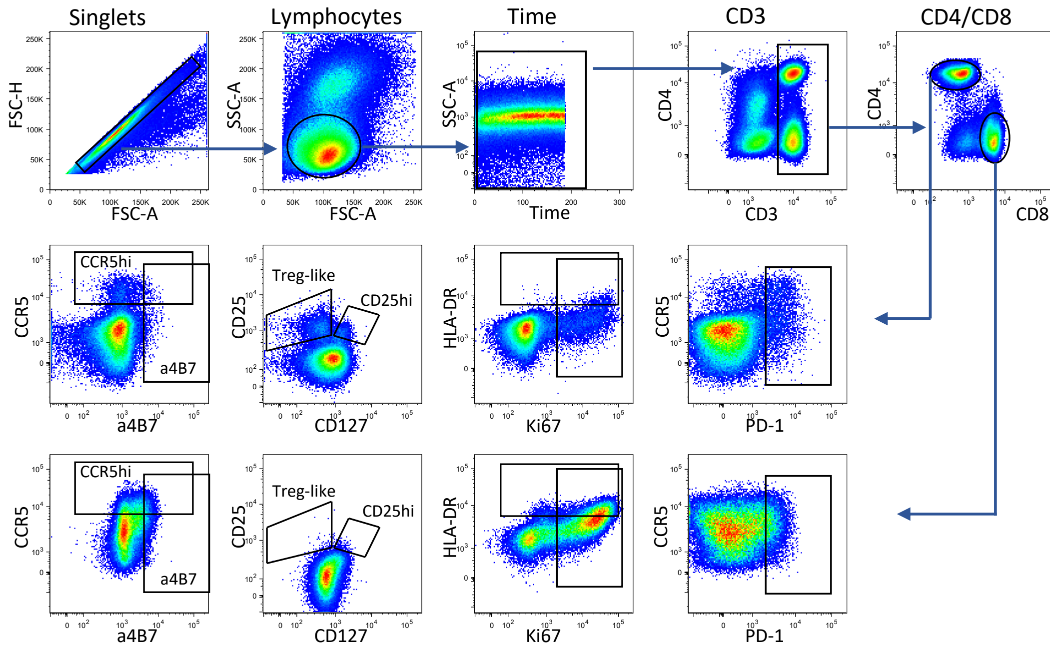

Figure 3.1: Flow cytometry gating strategy to define T cell markers. All markers were gated on parent CD4 and CD8 populations after gating on singlets, lymphocytes, time gates (to exclude acquisition artefacts) and CD3+ subsets. Activated and regulatory (Treg-like) subsets were defined based on the co-expression of CD25 with CD127hi or CD127lo expression respectively. In addition PD-1 expression was a proxy for activated cells. CCR5 was gated on brightest CCR5+ population (CCR5hi). Total HLA-DR and total Ki67 were gated on CD4 and CD8 cells. Potential HIV target cells were defined on the CD4 parent population co-expressing CCR5hi and the brightest α4β7 population (α4β7hi) [248]. Fluoresence minus one controls were run to establish optimal gates (Appendix 7.1).

Table 3.1: T cell markers measured *ex vivo* between HEU and HU infants

Marker	Function	Rationale for inclusion	References
CCR5	Adhesion and homing	Co-receptor for HIV viral entry	[244]
HLA-DR	Antigen presentation	Identifies activated T cells	[251]
CD25	IL-2 signalling	Identifies activated/regulatory T cells	[246]
Ki67	Nuclear protein	Identifies actively dividing T cells	[252]
CD127	IL-7 signalling	Down-regulated by regulatory T cells	[247]
α4β7	Adhesion/gut homing	Identifies potential HIV target cells	[244, 245]

3.3 Results

3.3.1 CD25 expression by CD8 T cells in HEUs is significantly increased at 36 weeks of life

A number of T cell markers (Table 3.1) were measured directly *ex vivo* and the frequency of expression was first compared between HEU and HU controls at each individual time point (Figures 3.3 and 3.4 & Table 3.4). This analysis was restricted to CT infants as the number of cHU controls available in the Jos cohort were insufficient for a comparison by HIV exposure groups (Appendix 7.2). Overall, no differences were seen in CD4 cells expressing the markers α4β7, CCR5hi, HLA-DR or Ki67 between HEU and HU controls at any time-point. For CD8 cells, there were trends towards higher expression of the activation marker HLA-DR in the HEU group compared to controls at week 0 (medians 0.12 vs. 0.01 respectively, p=0.05), week 7 (medians 0.84 vs. 0.01 respectively, p=0.01) and week 36 (medians 0.81 vs. 0.17 respectively, p=0.003), though these differences did not hold after multiple comparison correction. Of note, was the increased frequency CD8 cells expressing activation marker CD25 among HEUs compared to controls at week 7

(medians 0.31 vs. 0.04 respectively, p=0.01), and at week 36 (medians 0.52 vs. 0.03 respectively, $p<0.001$). Potential HIV target cells (CD4+CCR5hi+α4β7+) and T cells with a regulatory-like surface phenotype (CD4+CD25+CD127lo: 'Treg-like') were comparable between HIV exposure groups at all the time points measured in this study (Table 3.4). In summary, differences in activated phenotypes between HIV exposure groups was evident for CD8 cells, significantly, the expression CD25 at 36 weeks of age.

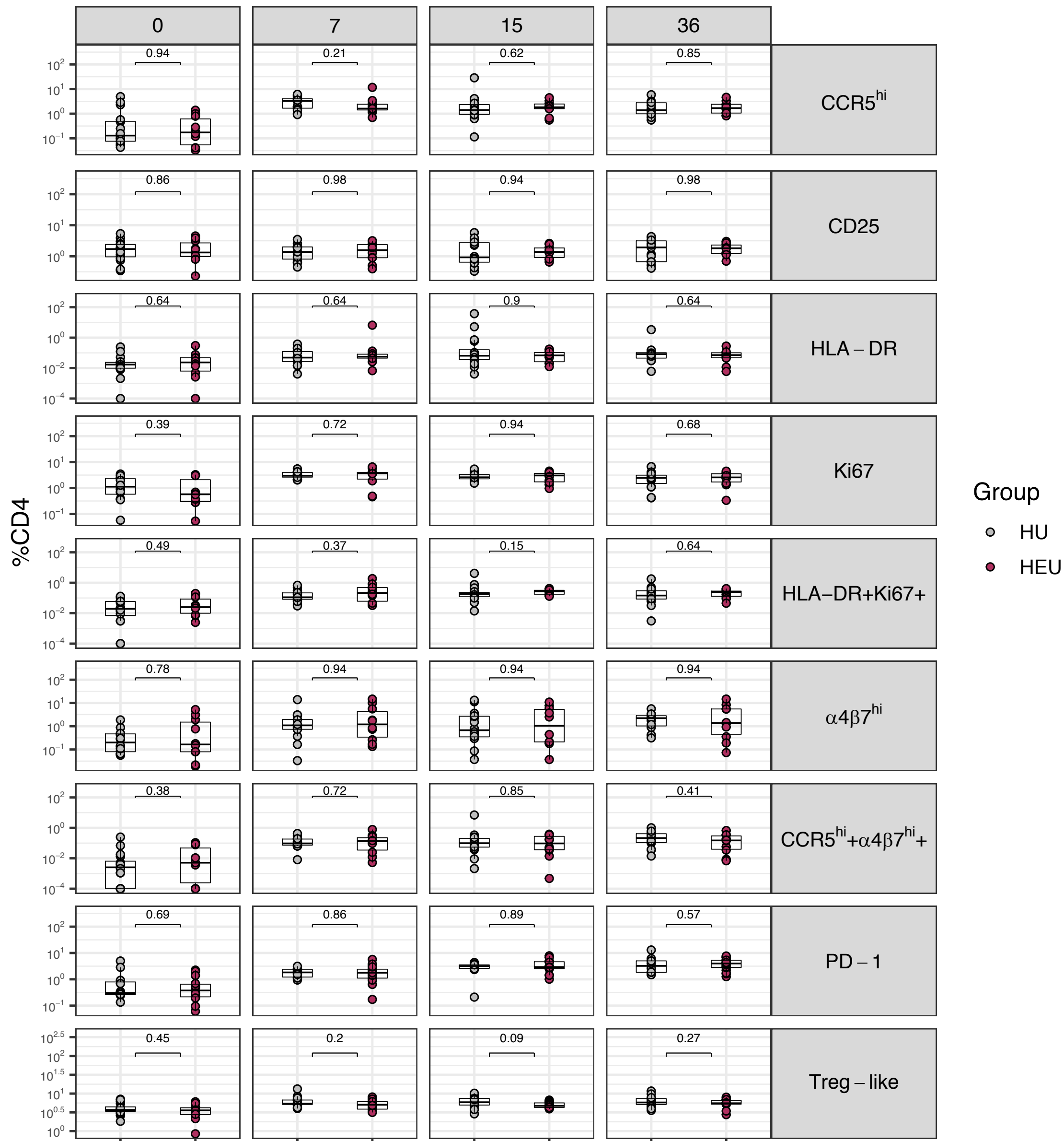

Figure 3.2: Comparison of CD4 markers of activation, proliferation and gut homing between HEU and HU controls. T cell markers (vertical strip text) that were measured *ex vivo* are compared between HIV exposure groups: HEU (maroon circles, n=10) and HU (grey circles, n=16) and are expressed as a percentage of the parent CD4 population. Horizontal strip text indicate age (weeks after birth). CCR5hi+α4β7hi represents potential HIV target cells, CD25 represents activated CD4 cells that co-expressed CD127hi and Treg-like cells were CD25+CD127lo. An unpaired Wilcoxon Ranked Sum test was used to compare expression between cHEU and cHU groups. *P*-values <0.001 were significant after adjusting for multiple comparisons using the Holm's step-down method.

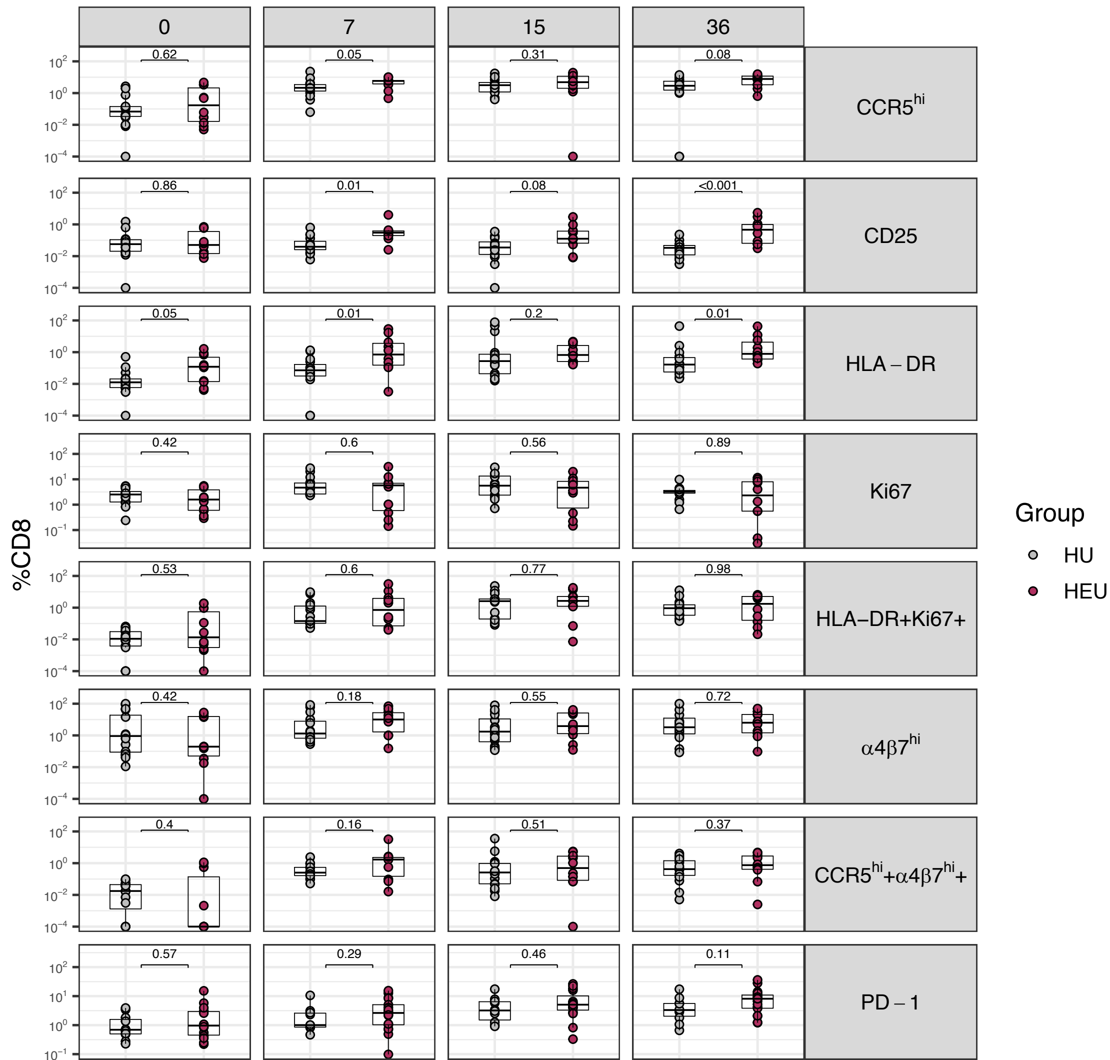

Figure 3.3: Comparison of CD8 markers of activation, proliferation and gut homing between HEU and HU controls. T cell markers (vertical strip text) that were measured *ex vivo* are compared between HIV exposure groups: HEU (maroon circles, n=10) and HU (grey circles, n=16) and are expressed as a percentage of the parent CD8 population. Horizontal strip text indicate age (weeks after birth). CCR5hi+α4β7hi represents potential HIV target cells, CD25 represents activated CD8 cells that co-expressed CD127hi. An unpaired Wilcoxon Ranked Sum test was used to compare expression between HEU and HU groups. *P*-values <0.001 were significant after adjusting for multiple comparisons using the Holm's step-down method.

Table 3.2: Frequencies of T cell markers *ex vivo* between HEU and HU control groups

Time	T cell subset	Marker	HEU median (IQR)	HU median (IQR)	*P*-value	*P*-adj
0	CD4	α4β7	0.16 (0.08-1.6)	0.21 (0.08-0.47)	0.78	1.00
		CCR5hi	0.17 (0.06-0.67)	0.13 (0.08-0.49)	0.94	1.00
		CD25	1.36 (0.98-2.78)	1.72 (0.96-2.41)	0.86	1.00
		HLA-DR+Ki67+	0.03 (0.01-0.09)	0.02 (0.01-0.06)	0.49	1.00
		HLA-DR	0.02 (0.01-0.05)	0.02 (0.01-0.02)	0.64	1.00
		Ki67	0.57 (0.3-2.45)	1.13 (0.59-2.29)	0.39	1.00
		CCR5hiα4β7hi[†]	0.01 (0-0.06)	0 (0-0.01)	0.38	1.00
		"Treg-like"[‡]	3.58 (2.77-4.2)	3.67 (3.38-4.45)	0.44	1.00
		PD-1	0.38 (0.22-0.66)	0.30 (0.26-0.80)	0.69	1.00
7	CD4	α4β7	1.28 (0.38-4.64)	1.09 (0.74-1.94)	0.93	1.00
		CCR5hi	1.61 (1.42-2.43)	3.29 (1.68-4.03)	0.21	1.00
		CD25	1.6 (0.9-2.39)	1.38 (0.82-2.01)	0.98	1.00
		HLA-DR+Ki67+	0.22 (0.07-0.48)	0.11 (0.08-0.22)	0.36	1.00
		HLA-DR	0.06 (0.05-0.08)	0.05 (0.03-0.13)	0.64	1.00
		Ki67	3.68 (2.31-4.03)	2.98 (2.64-4.03)	0.72	1.00
		CCR5hiα4β7hi[†]	0.14 (0.04-0.23)	0.1 (0.07-0.18)	0.72	1.00
		"Treg-like"[‡]	5.03 (3.87-6.1)	5.35 (5.14-6.75)	0.19	1.00
		PD-1	1.78 (1.11-2.42)	1.84 (1.23-2.41)	0.86	1.00
15	CD4	α4β7	1.47 (0.21-5.42)	0.68 (0.35-2.68)	0.98	1.00
		CCR5hi	1.83 (1.6-2.49)	1.4 (0.93-2.37)	0.6	1.00
		CD25	1.39 (0.94-1.86)	0.93 (0.64-2.74)	0.72	1.00
		HLA-DR+Ki67+	0.27 (0.18-0.32)	0.18 (0.12-0.23)	0.07	1.00
		HLA-DR	0.07 (0.03-0.11)	0.07 (0.04-0.23)	0.93	1.00
		Ki67	3.06 (1.74-3.66)	2.6 (2.27-3.32)	0.89	1.00
		CCR5hiα4β7hi[†]	0.11 (0.04-0.29)	0.1 (0.05-0.2)	0.93	1.00
		"Treg-like"[‡]	4.72 (4.29-5.66)	5.9 (4.94-7.44)	0.13	1.00
		PD-1	2.93 (2.56-4.64)	3.2 (2.60-3.54)	0.89	1.00
36	CD4	α4β7	1.37 (0.5-5.52)	2.22 (1.04-2.83)	0.98	1.00
		CCR5hi	1.69 (1.06-2.41)	1.38 (0.99-2.78)	0.62	1.00
		CD25	1.82 (1.23-2.31)	1.93 (0.67-3.14)	0.75	1.00
		HLA-DR+Ki67+	0.25 (0.13-0.28)	0.15 (0.09-0.3)	0.43	1.00
		HLA-DR	0.07 (0.05-0.1)	0.08 (0.05-0.1)	0.84	1.00
		Ki67	2.58 (1.75-3.57)	2.48 (1.56-3.14)	0.46	1.00
		CCR5hiα4β7hi[†]	0.15 (0.04-0.3)	0.21 (0.11-0.42)	0.56	1.00
		"Treg-like"[‡]	5.54 (5.22-6.61)	5.81 (5.15-7.31)	0.29	1.00
		PD-1	3.99 (2.80-5.32)	3.30 (1.84-5.02)	0.57	1.00
0	CD8	α4β7	0.2 (0.06-15.55)	0.91 (0.09-21.45)	0.41	1.00
		CCR5hi	0.27 (0.02-2.67)	0.08 (0.03-0.14)	0.62	1.00
		CD25	0.05 (0.01-0.46)	0.06 (0.02-0.11)	0.85	1.00
		HLA-DR+Ki67+	0.02 (0-0.73)	0.01 (0-0.03)	0.53	1.00
		HLA-DR	0.12 (0.01-0.56)	0.01 (0.01-0.02)	0.05	1.00
		Ki67	1.62 (0.6-4.1)	2.52 (1.28-3.34)	0.41	1.00
		CCR5hiα4β7hi	0 (0-0.42)	0.02 (0-0.04)	0.4	1.00
		PD-1	0.96 (0.45-2.94)	0.69 (0.50-1.59)	0.57	1.00
7	CD8	α4β7	10.27 (2.59-27.73)	1.32 (0.67-8.1)	0.17	1.00
		CCR5hi	5.83 (3.86-6.25)	2.14 (1.39-3.43)	0.06	1.00
		CD25	0.31 (0.2-0.39)	0.04 (0.03-0.09)	0.01	0.21
		HLA-DR+Ki67+	1.14 (0.09-3.89)	0.14 (0.1-1.28)	0.6	1.00
		HLA-DR	0.84 (0.16-3.72)	0.07 (0.03-0.18)	0.01	0.34
		Ki67	5.8 (0.62-6.97)	4.68 (2.64-7.15)	0.6	1.00
		CCR5hiα4β7hi	1.69 (0.21-2.32)	0.25 (0.16-0.54)	0.16	1.00
		PD-1	2.63 (1.03-5.06)	1.00 (0.85-2.59)	0.29	1.00
15	CD8	α4β7	4.01 (1.35-26.1)	1.8 (0.43-11.85)	0.49	1.00
		CCR5hi	4.89 (2.06-11.58)	3.15 (1.18-4.81)	0.39	1.00
		CD25	0.13 (0.07-0.38)	0.04 (0.01-0.08)	0.11	1.00
		HLA-DR+Ki67+	2.67 (1.22-5.1)	2.58 (0.19-3.55)	0.64	1.00
		HLA-DR	0.67 (0.26-2.8)	0.29 (0.04-0.74)	0.1	1.00
		Ki67	4.83 (1.08-8.29)	5.61 (2.38-13.45)	0.47	1.00
		CCR5hiα4β7hi	0.64 (0.09-2.79)	0.26 (0.05-0.96)	0.49	1.00
		PD-1	5.07 (3.34-11.24)	3.21 (1.58-6.42)	0.46	1.00
36	CD8	α4β7	6.43 (1.48-20.93)	3.27 (1.3-13.62)	0.66	1.00
		CCR5hi	7.82 (3.35-11.73)	2.89 (1.6-5.61)	0.06	1.00
		CD25	**0.52** (0.07-1)	**0.03** (0.01-0.05)	**<0.001**	**0.04**
		HLA-DR+Ki67+	2.29 (0.17-5.08)	0.92 (0.33-1.53)	0.75	1.00
		HLA-DR	**0.81** (0.37-4.68)	**0.17** (0.06-0.46)	**0.003**	0.10
		Ki67	2.68 (0.55-7.99)	3.26 (2.84-3.66)	0.88	1.00
		CCR5hiα4β7hi	0.75 (0.42-2.86)	0.42 (0.17-1.47)	0.25	1.00
		PD-1	8.11 (3.82-10.90)	3.31 (2.07-5.58)	0.11	1.00

Comparison of frequencies of T cell markers between HEU (n=11) and HU (n=16) in CT infants. Median percentage of parent population (CD4 or CD8) with (IQR) are shown for week 0–36 after birth. Wilcoxon Ranked Sum tests was used to compare HEU and HU groups. P-adjusted values <0.05 were considered significant after multiple comparison adjustment using Holm's step down method. [†]HIV target cells were defined as CCR5hiα4β7hi+ on CD4 cells.

3.3.2 HEUs differ from HU controls in the expression of activated CD8 T cells over the course of the first 36 weeks of life

We modelled the expression of these T cell markers in blood samples that were matched from birth to 36 weeks. Initially within group differences over time were analysed for each of the HIV exposure groups. As shown in Figure 3.2A, two CD4 subsets in particular followed similar kinetics in both HEU and HU groups—PD-1 and "Treg-like" CD4 cells which increased significantly from birth to week 36 (both $p<0.001$). Next, we applied a repeated measures analysis of variance (ANOVA) to account for correlated observations within subjects and to determine whether age and HIV exposure impacted the expression of these markers (Tables 3.2 & 3.3). Irrespective of HIV exposure, age (time) impacted on the frequencies of all CD4 markers measured except CD25 ($p=0.80$). When we stratified infants by HIV exposure groups, to examine which time intervals that explained the overall differences, we observed remarkable concordance between successive time points for HEU and HU groups (Table 7.2). For CD8 cells (Table 3.4), we observed significant overall differences (across all time points) by HIV exposure—in the expression of CD25 ($p<0.001$), HLA-DR ($p<0.001$) and PD-1 ($p<0.001$). Age as a variable did not have a significant interaction effect with HIV exposure group. Like CD4 cells, the frequencies of these markers followed similar increases/decreases between successive time points (Table 3.4).

The following conclusions were drawn from cross-sectional and longitudinal analyses: (*i*) the frequencies of CD4 and CD8 T cells expressing markers of activation/proliferation/HIV target cells between HEU and HU infants follow similar patterns of peaking and waning; (*ii*) CD8 T cells from HEUs are preferentially activated compared to HU controls—expressing significantly higher frequencies of PD-1, HLA-DR and CD25 markers, with the most significant difference seen at 36 weeks of age.

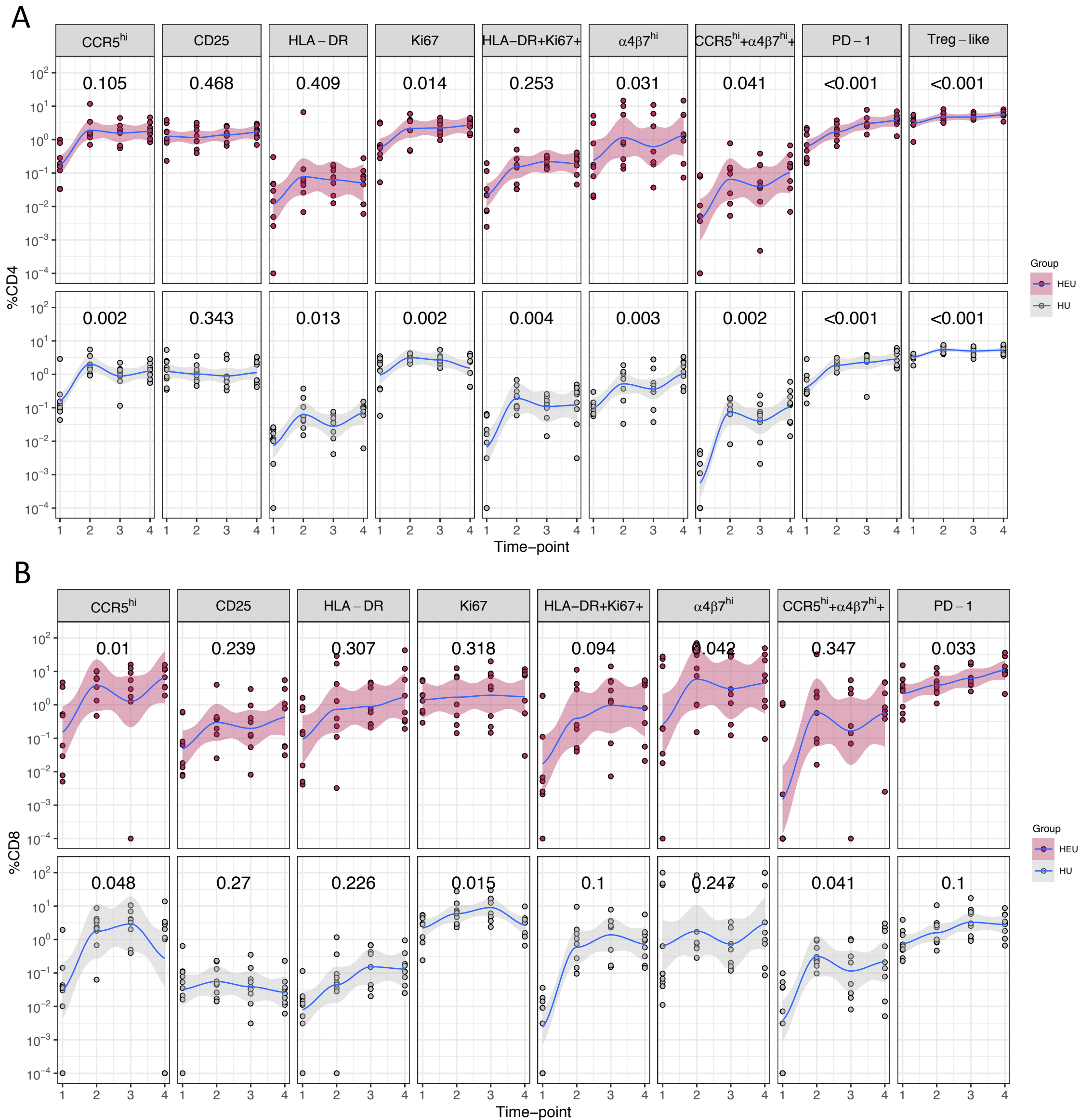

Figure 3.4: Kinetics of T cell markers by HIV exposure status. T cell markers (indicated in horizontal strip text) that were measured *ex vivo* are plotted longitudinally in matched samples. X-axes indicate the following time-points in weeks after birth: 1 (week 0), 2 (week 7), 3 (week 15), 4 (week 36). A local regression line (loess smoothing with 95% confidence intervals: shaded area) was fitted to the data for visualisation purposes. HIV exposure groups: HEU (maroon circles, n=10) and HU (grey circles, n=16) and are expressed as a percentage of the parent T cell population: A (CD4) or B (CD8). CCR5hi+α4β7hi represents potential HIV target cells, CD25 represents activated CD4 cells that co-expressed CD127hi and 'Treg-like' cells were CD25 cells co-expressing CD127lo. *P*-values shown are the result of a Friedman's test evaluating differences across all time-points, and $p<0.001$ were considered significant after multiple comparison adjustment.

Table 3.3: Repeated measures analysis of variance table for CD4 T cell markers

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Marker
Age	3	7.69	2.56	5.83	**0.001**	α4β7
Group	1	1.20	1.20	2.73	0.10	
Age:Group	3	0.21	0.07	0.16	0.92	
Residuals	60	26.36	0.44			
Age	3	12.08	4.03	29.00	**<0.001**	CCR5hi
Group	1	0.19	0.19	1.36	0.25	
Age:Group	3	0.18	0.06	0.43	0.73	
Residuals	60	8.34	0.14			
Age	3	0.11	0.04	0.34	0.80	CD25
Group	1	0.19	0.19	1.86	0.18	
Age:Group	3	0.09	0.03	0.29	0.83	
Residuals	60	6.25	0.10			
Age	3	16.51	5.50	14.65	**<0.001**	HLA-DR+Ki67+
Group	1	0.92	0.92	2.44	0.12	
Age:Group	3	0.82	0.27	0.73	0.54	
Residuals	60	22.54	0.38			
Age	3	8.30	2.77	6.60	**0.001**	HLA-DR
Group	1	0.26	0.26	0.63	0.43	
Age:Group	3	0.57	0.19	0.45	0.72	
Residuals	60	25.13	0.42			
Age	3	3.25	1.08	7.77	<**0.001**	Ki67
Group	1	0.05	0.05	0.37	0.55	
Age:Group	3	0.54	0.18	1.29	0.29	
Residuals	60	8.35	0.14			
Age	3	37.09	12.36	23.55	**<0.001**	CCR5hi+α4β7+
Group	1	0.65	0.65	1.24	0.27	
Age:Group	3	2.48	0.83	1.57	0.20	
Residuals	60	31.50	0.52			
Age	3	0.57	0.19	10.79	<**0.001**	'Treg-like'
Group	1	0.01	0.01	0.61	0.44	
Age:Group	3	0.02	0.01	0.30	0.82	
Residuals	60	1.06	0.02			
Age	3	6.54	2.18	23.77	<**0.001**	PD-1
Group	1	0.15	0.15	1.61	0.21	
Age:Group	3	0.11	0.04	0.41	0.75	
Residuals	60	5.50	0.09			

Results of a repeated measures analysis of variance (ANOVA) to assess the effect of age (weeks 0–36) or HIV exposure (Group), and the interaction between these two variables, on the frequency of expression of the listed T cell markers by CD4 cells in CT infants. Pr<0.05 were considered significant.

Table 3.4: Repeated measures analysis of variance table for CD8 T cell markers

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Marker
Age	3	9.27	3.09	2.19	0.10	α4β7
Group	1	0.88	0.88	0.62	0.43	
Age:Group	3	2.63	0.88	0.62	0.60	
Residuals	60	84.72	1.41			
Age	3	27.85	9.28	7.10	<**0.001**	CCR5hi
Group	1	4.47	4.47	3.42	0.07	
Age:Group	3	6.73	2.24	1.72	0.17	
Residuals	60	78.41	1.31			
Age	3	2.38	0.79	1.52	0.22	CD25
Group	1	8.39	8.39	16.12	<**0.001**	
Age:Group	3	2.29	0.76	1.47	0.23	
Residuals	60	31.21	0.52			
Age	3	57.32	19.11	20.69	<**0.001**	HLA-DR+Ki67+
Group	1	0.34	0.34	0.37	0.55	
Age:Group	3	3.01	1.00	1.09	0.36	
Residuals	60	55.42	0.92			
Age	3	16.07	5.36	6.91	<**0.001**	HLA-DR
Group	1	18.45	18.45	23.81	<**0.001**	
Age:Group	3	0.49	0.16	0.21	0.89	
Residuals	60	46.49	0.77			
Age	3	1.57	0.52	1.55	0.21	Ki67
Group	1	2.68	2.68	7.95	**0.01**	
Age:Group	3	0.78	0.26	0.77	0.52	
Residuals	60	20.26	0.34			
Age	3	55.90	18.63	13.89	<**0.001**	CCR5hi+α4β7+
Group	1	0.21	0.21	0.16	0.69	
Age:Group	3	1.66	0.55	0.41	0.74	
Residuals	60	80.52	1.34			
Age	3	4.25	1.42	8.70	<**0.001**	PD-1
Group	1	3.11	3.11	19.10	<**0.001**	
Age:Group	3	0.22	0.07	0.46	0.71	
Residuals	60	9.78	0.16			

Results of a repeated measures analysis of variance (ANOVA) to assess the effect of age (weeks 0–36) or HIV exposure (Group), and the interaction between these two variables, on the frequency of expression of the listed T cell markers by CD8 cells in CT infants. Pr<0.05 were considered significant.

3.4 Discussion

Most studies of T cell markers between HEU and HU controls have been cross-sectional studies (Table 3.5). For the most part, these studies reveal increased activation profiles in HEU. In the present study, infants were followed up from birth to 36 weeks of age enabling the simultaneous tracking of T cell markers over time and differences in the expression of these markers between HEU and HU controls. Overall, the kinetics of these T cell markers did not differ between exposure groups; following similar patterns in peaking and waning. In cross-sectional analyses however, HLA-DR expression by CD8 cells was increased in the HEU and CD25 significantly increased after correction for FDR.

Maternal HIV infection has been associated with hyper inflammatory systemic milieu [253, 237, 254], even in women on ART [255, 256]. Increased levels of soluble inflammatory mediators have been found in the placenta of HIV infected mothers [237] thereby providing potential stimulatory signals that may activate innate and adaptive immune subsets [144]. Furthermore, higher levels of these maternal inflammatory mediators correlate with increased risk of HIV transmission [237, 254]. While many studies have compared levels of soluble inflammatory mediators between HIV infected, cHEU and healthy controls, fewer studies have evaluated *ex vivo* T cell activation between infants that are HIV-exposed and healthy un-exposed infants. Gut homing CD4 cells, expressing the integrin $\alpha 4\beta 7$ and co-expressing CCR5, a co-receptor for HIV entry, were of interest as they are potential targets for HIV infection [244] and may predict HIV acquisition [245]. In adult cohorts who are highly exposed to HIV yet persistently sero-negative, HIV "resistance" is associated with reduced absolute numbers of HLA-DR+ and CCR5 bearing CD4 cells ([257]. An important study which tracked the frequencies of integrin $\alpha 4\beta 7$ expression in peripheral blood longitudinally, found that each 1% increase in integrin $\alpha 4\beta 7$ expression prior to acquisition was associated with a 17% increased risk of HIV acquisition [245] in adults. In HIV-exposed infants, different mechanisms may be at play resulting in a

potentially increased vulnerability to HIV infection. Animal models of mother to child transmission of simian immunodeficiency virus (SIV) suggest that target cell availability is the important determinant of reduced transmission rates observed in Sootey Mangabeys—the natural hosts of SIV—compared to Rhesus macaques) [258]. The frequency of target cells (CD4+CCR5+) in the gut and peripheral blood were significantly higher in frequently transmitting Rhesus macaques compared to rarely transmitting Sootey mangebeys [258]. The human infant gut has an abundance of potential HIV target cells [259], and CD4 cells from the cord blood of *in utero* HIV exposed newborns which have been activated *in vitro* with soluble inflammatory mediators, are more susceptible to HIV infection [144]. Crucially the induction of HIV infectable cells by soluble factors *in vitro* was evident in women on supressive ART with CD4 counts >200 cellsµL. The data presented here would be strengthened by measuring *in vivo* maternal pro-inflammatory mediators which correlate with increased HIV acquisition risk [237, 254]. BCG vaccination at birth was associated with an increase in potential HIV target cells compared to delayed vaccination in HIV-exposed infants in a randomised control trial [260]. However, compared to unvaccinated animals, BCG-induced increases in target cells in Rhesus macaque newborns did not translate to an increased SIV transmission via the oral mucosal route compared to unvaccinated new borns [261]. In this study—where all infants were BCG vaccinated at birth, we observed comparable increases in the frequencies of CD4+CCR5hi cells between HEU and HU infants with age. No distinct differences in the frequencies of these HIV target cells by HIV exposure group was found suggesting that HEU infants are no more vulnerable to HIV infection (based on frequencies of target cell) than their un-exposed peers.

In addition, we found no differences in "Treg-like" phenotypes between exposure groups were seen in this study, with the caveat that FoxP3, a transcription factor that programmes Tregs [262], was not included in the staining panel. Treg-like phenotypes were described in this study as CD4+CD127lo+CD25+, as CD127

inversely correlates with FoxP3 and suppressive function [247]. In highly HIV-exposed yet uninfected adult cohorts has been defined by increase HIV-exposed uninfected adults have increased circulating regulatory T cells that are thought to contribute to their protection from HIV infection [263] and cHEU have increased regulatory phenotypes that include CD4 expressing CD127lo and CD25hi [264] and CD8 cells expressing markers FoxP3, TGF-β and IL-10 [140, 220]. Our findings support those seen in a Kenyan cohort where cHEU and cHU had comparable frequencies of CD4+CD25+FoxP3+ cells at 3 and 12 months of age [127]. It is worth noting that while phenotypes of Treg-like cells may be comparable between exposure groups, functionally they may differ and studies comparing the suppressive ability of isolated Treg populations between cHEU and cHU infants are warranted.

The activation of T cells is critical component of adaptive immunity, where specific extra-cellular and intracelluar proteins are upregulated on immune-competent cells in response to antigen presentation. The levels of activation proteins alone or in combination have been found to be useful surrogates for disease state, disease susceptibility, antigen load and response to treatment [238, 239, 240, 241]. The frequencies of Mtb antigen-specific cells expressing HLA-DR, CD38 or Ki67 markers are able to discriminate active TB vs. latent TB disease states [239, 240] and the expression of these markers on these Mtb responsive cells decrease with successful TB treatment in those with active TB [240]. In bulk T cells however, higher baseline HLA-DR expression is associated with an increased risk for TB disease [238]. On the other extreme, significantly low levels of T cells expressing HLA-DR or CD25 may be a sign of malnutrition as illustrated in a recent study of neonates with severe vitamin D compared to healthy controls [265]. Our findings of consistently higher HLA-DR expression by CD8 cells among HEUs suggests that T cell activation is induced *in utero* and persists to 36 weeks of life. This is supported by a number of studies showing increased frequency of activated T cell phenotypes in the HEU infant from birth [127, 132, 266, 267]. We did not measure the activation marker CD38 in

this study, however other studies report increased CD38 among HEUs compared to HU controls both by median fluorescence intensities (MFI) and frequency measures [266, 118]. In one study by Rich and colleagues [132], no differences were found between infant age-matched HEU and control groups in the expression of HLA-DR alone, however levels of HLA-DR co-expressing CD38 T cells were up to 2-fold higher among HEUs till 6 months of age. Only one study contradicts these findings where HLA-DR+CD38+ co-expression among HEUs was lower compared to HUs at 6 months [268] however, the study in question was confounded by both sample size comparisons (n=38 HEU vs. n=4 HU) and population differences (HEU cohort from South Africa vs. HU cohort from North America). The most striking and significant difference in activation phenotypes between HEU and HU was observed for CD25 (co-expressing CD127 to indicate activation). CD25 or IL-2Rα is a receptor with high affinity for IL-2 cytokine signalling [269] and is upregulated upon antigen stimulation requiring constant IL-2 signals for memory differentiation of virus-specific CD8 cells [270]. To our knowledge, only one other study has compared CD25 expression as an activation marker between HEU and HU, albeit on bulk T cells with no CD127 co-expression measured [132]. In that study, no differences in CD25 expression were found between HEU and HU infants aged between than 6 months. Whether this increased activation is a direct consequence of exposure to inflammatory mediators in the HEU, virus or other environmental antigens and whether this is a marker for increased risk of infection was not directly addressed in this study. Our results nonetheless suggest that CD8 T cells in particular are activated in the HEU during the first 36 weeks of life.

To summarise, the strongest *ex vivo* T cell marker signals that differentiated cHEU infants from their unexposed counter parts in this study was increased expression of HLA-DR and CD25 on CD8 cells at 36 weeks.

Table 3.5: Studies comparing markers of T cell activation, proliferation or regulation between HEU and HU groups

Study	Cohort characteristics	Markers	Findings relative to HU
[268]	4 HU, 38 HEU (6 months), 67% maternal ARV during pregnancy	CD38, HLA-DR	Decreased CD38+HLA-DR+ by CD4 and CD8 cells in unadjusted analyses
[127]	10 HU, 19 HEU (3 months) 16 HU, 16 HEU (12 months), maternal ART for CD4 <350 cells/µL	CD38,PD-1,CD25,FoxP3, HLA-DR	Increased CD38+HLA-DR+ (3, 12 months) and PD-1 (3 months) by CD4 cells in unadjusted analyses
[144]	12 HU, 10 HEU (birth), 100% maternal ART, CD4 >200 cells/µL, undetectable maternal VL, 55% ever breast-fed	CXCR4,CXCR6, chemokine receptors	Increased CCR1 and CCR8 by CD4 cells in unadjusted analyses
[128]	46 HU, 46 HEU (6,14 weeks), 98% maternal ART, CD4 >200 cells/µL, equal EBF rates between HEU and HU at 6,14 weeks	Ki67	Increased Ki67+cytokine+ (14 weeks) by CD4 and CD8 cells in adjusted analyses
[267]	16 HU, 12 HEU (2 weeks) 10 HU, 11 HEU (10 weeks)	PD-1	Increased PD-1 by CD4 cells (10 weeks) in unadjusted analyses
[266]	45 HU, 45 HEU (birth) 23 HU, 23 HEU (12 months), 100% maternal ART, 57% <50 copies/mL at delivery	CD38	Increased CD38 MFI by CD4 and CD8 cells (birth, 12 months) in unadjusted analyses
[118]	13 HU, 23 HEU (birth), maternal ART	CD38	Increased CD38 bright/dim by CD4 and CD8 cells in multiple linear regression
[132]	36 HU, 28 HEU (0-2 months) and 16 HU, 24 HEU (2-6 months)	CD25/CD38/HLA-DR	Increased CD38+HLA-DR+ expression by CD4 cells (0-2-month) after FDR correction

ART=antiretroviral treatment, EBF=exclusive breastfeeding, VL= viral load, MFI=median fluorescence intensity

Chapter 4

T cell function in HEU infants—vaccine immunogenicity

4.1 Introduction

There is reliable evidence that cHEUs are capable of mounting seroprotective antibody titres following vaccination [123, 114], however, the ability of cHEUs to mount robust T cell responses of comparable magnitude and quality to HIV-unexposed infants unclear [79], particularly in the Option B+ era. While T cell correlates of vaccine protection in humans are not well defined for the vaccine antigens measured in this study, we propose to measure the magnitude and quality of Th1 cytokines as a proxy for T cell function—comparing cHEU with cHU control groups over the first 9 months of life. Th1 cytokines are induced by BCG [186], Tetanus Toxoid [206] and both acellular and whole cell forms of Pertussis vaccines [206, 213]. The read-outs for the majority of studies evaluating cell-meditated functional responses in cHEUs are derived from long-term *in vitro* culture assays, but these studies have yielded inconsistent findings [126, 40, 128, 129, 130]. Some studies report no impairment in the production of cytokines following antigen stimulation [40, 126], while others report either significant impairment [128, 271] or paradoxically, enhanced cytokine

production in cHEUs [129]. These long-term assays have a number of limitations which confound the interpretation of their data. Firstly, these assays require that the cells proliferate and are viable (often after several days in culture); secondly, the cellular source of secreted factors into culture media is unknown, unless intracellular cytokine staining is used. Furthermore, to detect intracellular cytokines after long-term culture requires re-stimulation of cells with super antigen in the final hours of the assay, thus confounding the interpretation of antigen specificity. The short-term whole blood assay proposed for use here, allows for the identification of re-call responses in physiological conditions that are similar to those *in vivo* [138]. The major differences in this proposed present study from other published work include the following: (*i*) leverage of two independent birth cohorts to test the hypothesis that HIV exposure alters vaccine immunity; (*ii*) application of several analytical techniques to measure the magnitude and quality of vaccine-induced immunity—combined with robust statistical methods to account for type 1 error; (*iii*) measurement of three vaccine antigens in the same assay, two of which (Tetanus Toxoid and *Bordetella pertussis* antigens) have not routinely been measured in cell-mediated studies in HEUs and (*iv*) the study involved breastfed infants of mothers on suppressive ART.

4.2 Methods

4.2.1 Cohort description

Mother-infant pairs were recruited at the Midwifery Obstetric Unit (MOU) at Site B in Khayelitsha, Cape Town (CT), South Africa and the Plateau State Specialist Hospital in Jos, Nigeria from November 2014 to November 2016 (Table 4.1). Infants were followed from birth, at day 4–7 and at weeks 7, 15 and 36 of life. Pretest counselling and HIV testing was done at the time of antenatal care registration. Both mothers with and without HIV infection were eligible for the study. Mothers with

HIV and their infants were provided with ARV according to the current country-specific guidelines [224, 223]. All mothers in the study were of consenting age and provided written informed consent. Exclusive breastfeeding (EBF) was advised to all mothers from the time of delivery to 6 months. Breastfeeding rates to 6 months of age between HEU and HU groups for the entire study cohort were 81% and 88% respectively (Table 7.1). Infants born before 36 weeks and with birth weights lower than 2.4 kilograms were ineligible for the study. Further exclusion criteria included pregnancy or delivery complications as previously described [194]. All HEU babies were confirmed as negative by PCR at birth and at 6 weeks of age according to PMTCT guidelines [224, 223]. A total of 186 HEU and 97 HU infants across the two cohort sites were included in the analysis for this chapter.

4.2.2 Immunisation

Routine vaccines were given to all infants according to the WHO's EPI (Table 4.1). Infants from CT received intradermal Danish BCG strain (1331, Statens Serum Institute, Denmark) from April 2013 to January 2016 and thereafter Russian strain (BCG-I Moscow, Serum Institute of India, India). Both strains were given at 2×10^5 CFU/dose at birth. Infants from Jos received the Bulgarian strain (BCG-SL 222 Sofia, BB-NCIPD Ltd, Bulgaria) at 0.3×10^5 CFU/dose and due to logistical reasons were vaccinated between 4-7 days after birth. Diptheria-Tetanus-acellular Pertussis (DTaP) or whole-cell Pertussis (DTwP) vaccines were administered at 6, 10 and 14 weeks in Cape Town and Nigerian respectively. Other routine vaccines and their schedules are listed in Table 4.1 below.

Table 4.1: Infant vaccination schedule and whole blood assay testing

Age	South Africa	Nigeria	Antigens
0	BCG†/OPV/HBV		BCG
1		BCG‡/OPV/HBV/PCV	BCG
6	DTaP/Hib/OPV/HBV/RV/PCV	DTwP/Hib/IPV/HBV	
7			BCG/TT/BP
10	DTaP, Hib/HBV	DTwP/Hib/HBV/OPV	
14	DTaP/Hib/Rota/PCV/HBV	DTwP/Hib/IPV/PCV	
15			BCG/TT/BP
36	Measles/PCV	Measles/YFV	BCG/TT/BP

Vaccine schedule by infant age (in weeks) and cohort site. BCG (Bacille-Calmette Guerin), †Denmark/Russia strains, ‡Bulgaria strain, DTaP (Diptheria-Tetanus-acellular Pertusiss, DTwP (Diptheria-Tetanus-whole cell Pertusiss), OPV (Oral Polio vaccine), Hib (Haemophilus influenzae type b), HBV (Hepatitis B vaccine), IPV (Inactivated polio vaccine), PCV (Pneumococcal conjugate vaccine), YFV (Yellow fever vaccine). WBA test antigens: BCG (Bacille-Calmette Guerin), TT (Tetanus Toxoid antigen), BP (Bordetella Pertussis antigens)

4.2.3 Whole blood assay and whole blood fixing

For T cell vaccine measurements, a 12-hour whole blood assay was used [138]. Briefly, 250 µL of anticoagulated blood was incubated with vaccine antigens or PHA at 37°C within one hour of blood draw. For BCG stimulation, 12 x 10^5 CFU/mL of *Mycobacterium bovis* was reconstituted in RPMI media from either the vaccine vial (BCG-Denmark strain 1331: SSI or BCG-SL 222 Sofia strain: BB-NCIPD) or live BCG culture (BCG-Denmark strain 1331: AJVaccines, Denmark)(see Table 7.3). Other stimuli included Tetanus Toxoid (TT) antigens (Sanofi Pasteur) at 5 IU/mL and 0.01% v/v of *Bordetella pertussis* (BP) antigens (BD Difco). Phytohemagglutinin (PHA) (50 µg/mL) or RPMI with 10% fetal calf serum (FCS) were used as positive and negative controls respectively. After 5 hours, Brefeldin-A (Sigma Aldrich) was added to a final concentration of 10 µg/mL and incubated for an additional 7 hours. Thereafter, red blood cells were lysed followed by washing and staining with

LIVE/DEAD® fixable Violet stain (ViViD, ThermoFisher). For the CT samples, cells were cryo-preserved in 10% DMSO and 90 FCS and stored immediately in liquid nitrogen (LN_2). For Jos samples, cells were cryo-preserved in 10% DMSO and 90% FCS, stored at -70°C and shipped within 6 months to the University of Cape Town (UCT) and then transferred to LN_2. Analysis of all samples was performed at the core laboratory at the UCT.

4.2.4 Cell staining, antibodies and flow cytometry

Batched stored samples were thawed quickly at 37°C and washed twice with 1× BD PermWash buffer. Cells were then incubated in 1× BD PermWash for 10 minutes before incubation with the antibody cocktail mix in 2% FCS in PBS at 4°C for 45 minutes. After incubation, cells were washed twice with in 2% FCS in PBS and the resuspended in 2% FCS in PBS for cell acquisition using a Beckton Dickinson LSRII flow cytometer (SORF model). The following monoclonal antibody-fluorochrome conjugates were used in the T cell vaccine panel: IL-2-R-phycoerythrin (PE), CD8-V500, IFN-Alexa Fluor-700, TNF-α-PE-Cy7, Ki67-Fluorescein isothiocyanate (FITC; BD), CD27 PE-Cy5, HLA-DR- Allophycocyanin-Cy7 (APC-Cy7), CD3-BV650 (all from Biolegend), CD4 PE-Cy5.5 (Invitrogen), CD45RA PE-Texas Red-X (Beckman Coulter). A minimum of 50 000 ViViD negative (viable) CD3 events were collected for T cell vaccine measurements using FACS DIVA v6 software. Post-acquisition compensation and analysis was performed in FlowJo version 9 (FlowJo, LLC). Figure 4.1 shows the gating strategy employed for T cell vaccine responses. Total cytokine frequencies were from the total T cell population and based on boolean combinations of the 3 gated cytokine populations.

4.2.5 Statistical analyses

Statistical analyses of flow cytometry data was performed in R [249], SPICE software [272]. Non-parametric comparisons between two independent groups was

performed using Wilcoxon Rank Sum test (Mann Whitney U test). A repeated measures analysis of variance (ANOVA) or Kruskal Wallis tests were used for longitudinal data. The Holm's step down method was used for multiple comparison correction. For differences by exposure groups for qualitative measures of vaccine immunity we used SPICE software. Only antigen responders were selected (Appendix 7.7) with the following threshold set: each sample's stimulated cytokine frequency had to be greater than its unstimulated cytokine frequency and at least 2× unstimulated cytokine frequency. These were then background subtracted in the SPICE workflow before group comparisons were made.

4.3 Results

4.3.1 Detection of post-vaccination antigen responses

To determine which time-points the whole blood assay was able to detect vaccine-induced responses (above background), we compared total cytokine producing CD4 frequencies between unstimulated and vaccine antigen-stimulated samples for the entire data set (CT and Jos infants) across all time-points measured. Only participants with matching unstimulated - stimulated pairs were used in these analyses (Figure 4.2). Figure 4.1 shows the gating strategy used to define T cell cytokine responses. We defined time points with detectable antigen responses if the following criteria were met at each time point: (*i*) the median frequency of cytokine+ cells was greater than the median frequency of cytokine+ cells in background samples (unstimulated) and (*ii*) differences between unstimulated and simulated magnitudes were statistically significant after FDR adjustment. Cytokine frequencies in antigen-stimulated samples were indistinguishable from unstimulated samples at pre-vaccination time points (Table 4.2 and Figure 4.2). For BCG, detectable responses were at 7, 15- and 36-weeks post BCG vaccination with median CD4 cytokine frequencies in unstimulated and BCG-stimulated samples as follows: at week 7 (0.07 and 0.50, respectively

$p<0.001$), at week 15 (0.07 and 0.28, respectively $p<0.001$) and at week 36 (0.06 and 0.16, respectively $p<0.001$). Detectable TT responses were observed at 15- and 36-weeks post BCG median CD4 cytokine frequencies in unstimulated and TT-stimulated samples as follows: at week 7/ 1 week DTaP/wP (0.06 and 0.08 respectively, $p=0.005$), at week 15/ 5 weeks post 2nd DTaP/wP (0.08 and 0.14, $p<0.001$) and at week 36/ 24 weeks post 3rd DTaP/wP (0.06 and 0.13, $p<0.0001$). BP responses were significantly above background for 7, 15- and 36-weeks post BCG where median frequencies of CD4 cytokine cells between unstim and BP antigens were: week 7/ 1 week DTaP/wP (0.06 and 0.10 respectively, $p<0.001$), week 15/ 5 weeks post 2nd DTaP/wP (0.08 and 0.11, $p<0.001$), week 36/ 24 weeks post 3rd DTaP/wP (0.06 and 0.1, $p<0.001$). In summary, BCG responses were detected at 7 weeks post BCG and thereafter, TT responses were detectable at 15 weeks post BCG and thereafter whilst BP responses were detected at 7 weeks post BCG and thereafter.

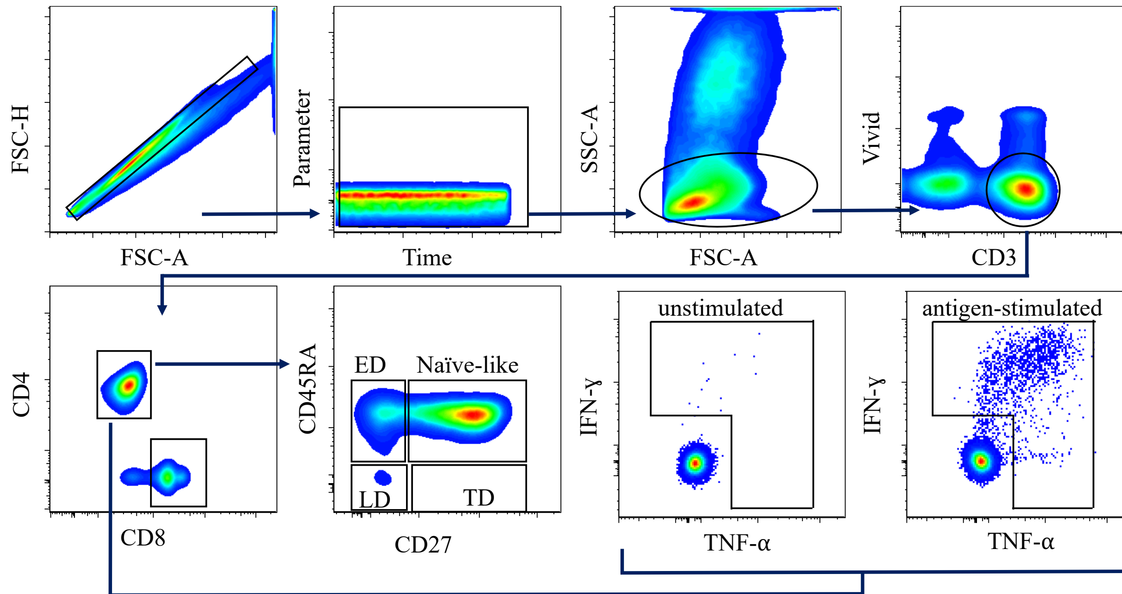

Figure 4.1: Flow cytometry gating strategy to define T cell cytokine responses to antigen stimulation. Singlets and lymphocytes were defined by forward (FSC-A) and side scatter (SSC-A) properties. Flow acquisition artefacts were gated out for each parameter in time gates. Cytokine responses were measured from the parent CD4+ population. Cytokine memory subsets were quantified from parent CD4 population to boolean combinations of cytokine+ cells. Memory subsets were defined by CD45RA and CD27 such that CD45RA+CD27+ represent naïve-like, CD45RA-CD27+ early differentiated (ED), CD45RA-CD27- late differentiated (LD) and CD45RA+CD27- terminally differentiated (TD) memory phenotypes.

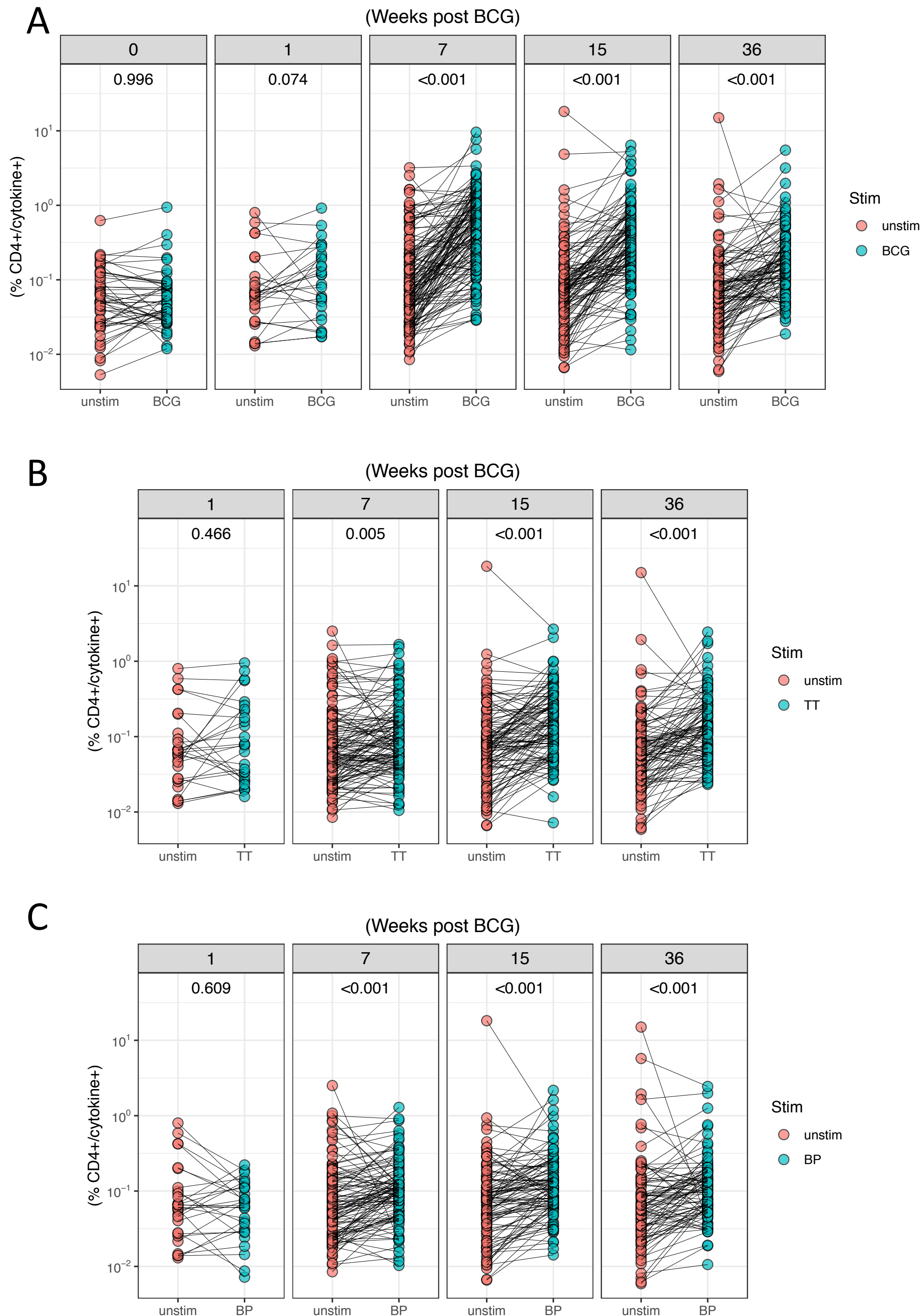

Figure 4.2: Whole blood assay detection of vaccine-specific responses. The magnitude of CD4 cytokine+ frequencies are compared between unstimulated (red points) and vaccine antigens **(A)** BCG, **(B)** Tetanus Toxoid (TT) and **(C)** *Bordetella pertussis* (BP) antigen-stimulated samples (turquoise points). Strip-panel text indicate weeks post BCG vaccination. For TT and BP antigens 1 wk BCG = -5 wk pre-DTaP/wP, 7 wk BCG = 1 wk post 1st DTaP/wP, 15 wk BCG = 5 weeks post 2nd DTaP/wP, 36 wk BCG = 24 weeks post 3rd DTaP/wP (Table 4.1). A paired Wilcoxon-rank Sum test was used to test differences between unstimulated and antigen stimulated conditions and unadjusted p-values comparing the groups are shown. $P<0.001$ were considered significant after FDR correction.

Table 4.2: Pre-post vaccination frequencies of CD4+cytokine+ cells

					% of CD4 cytokine+		
Antigen	**BCG wk**	**DTaP/wP wk**	**Stim**	**n**	**median**	**IQR1**	**IQR2**
BCG	0		unstim	51	0.05	0.01	0.09
			BCG	51	0.05	0.02	0.08
	1		unstim	28	0.06	0.03	0.09
			BCG	28	0.10	0.02	0.18
	7		unstim	143	0.07	0.01	0.16
			BCG	143	**0.51**	0.07	0.95
	15		unstim	118	0.07	0.02	0.13
			BCG	118	**0.28**	0.07	0.50
	36		unstim	102	0.06	0.01	0.11
			BCG	102	**0.16**	0.05	0.27
TT	1	-5 pre 1st dose	unstim	28	0.06	0.03	0.10
			TT	28	0.08	0.02	0.17
	7	1 post 1st dose	unstim	124	0.06	0.01	0.12
			TT	124	0.08	0.03	0.14
	15	**5 post 2nd dose**	unstim	113	0.08	0.02	0.13
			TT	113	**0.14**	0.03	0.24
	36	**24 post 3rd dose**	unstim	96	0.06	0.02	0.10
			TT	96	**0.13**	0.03	0.23
BP	1	-5 pre 1st dose	unstim	29	0.06	0.03	0.10
			BP	29	0.07	0.03	0.11
	7	**1 post 1st dose**	unstim	107	0.06	0.00	0.11
			BP	107	**0.10**	0.06	0.15
	15	**5 post 2nd dose**	unstim	105	0.08	0.02	0.13
			BP	105	**0.11**	0.04	0.18
	36	**24 post 3rd dose**	unstim	89	0.06	0.01	0.11
			BP	89	**0.10**	0.04	0.16

Median frequencies of CD4 cells expressing total cytokines are shown for unstimulated and vaccine antigen-stimulated conditions with upper and lower interquartile ranges (IQR1 and IQR2 respectively). Data is combined for CT and Jos infants. BCG vaccination weeks (BCG wk) are indicated with corresponding Diptheria-Tetanus-acellular/whole-cell Pertussis vaccination week (DTaP/wP wk). Time-points where vaccine-antigen frequencies that were significantly greater than unstimulated samples after paired Wilcoxon Rank Sum tests and multiple comparison adjustment (p-adjusted<0.05) are indicated in bold.

4.3.2 Magnitude of T cell vaccine-antigen responses are similar between cHEU and cHU

We compared the CD4 cytokine frequencies of antigen responses between HEU and HU groups across all time-points measured to determine whether exposure had an impact of the magnitude of total cytokines, stratifying responses by cohort site. There were no differences in the magnitude of background (cytokine+ cells in unstimulated condition) by HIV exposure status. Importantly, the magnitudes of CD4 responses to vaccine antigens did not differ by HIV exposure status in both cohorts (Figure 4.3 A-C).

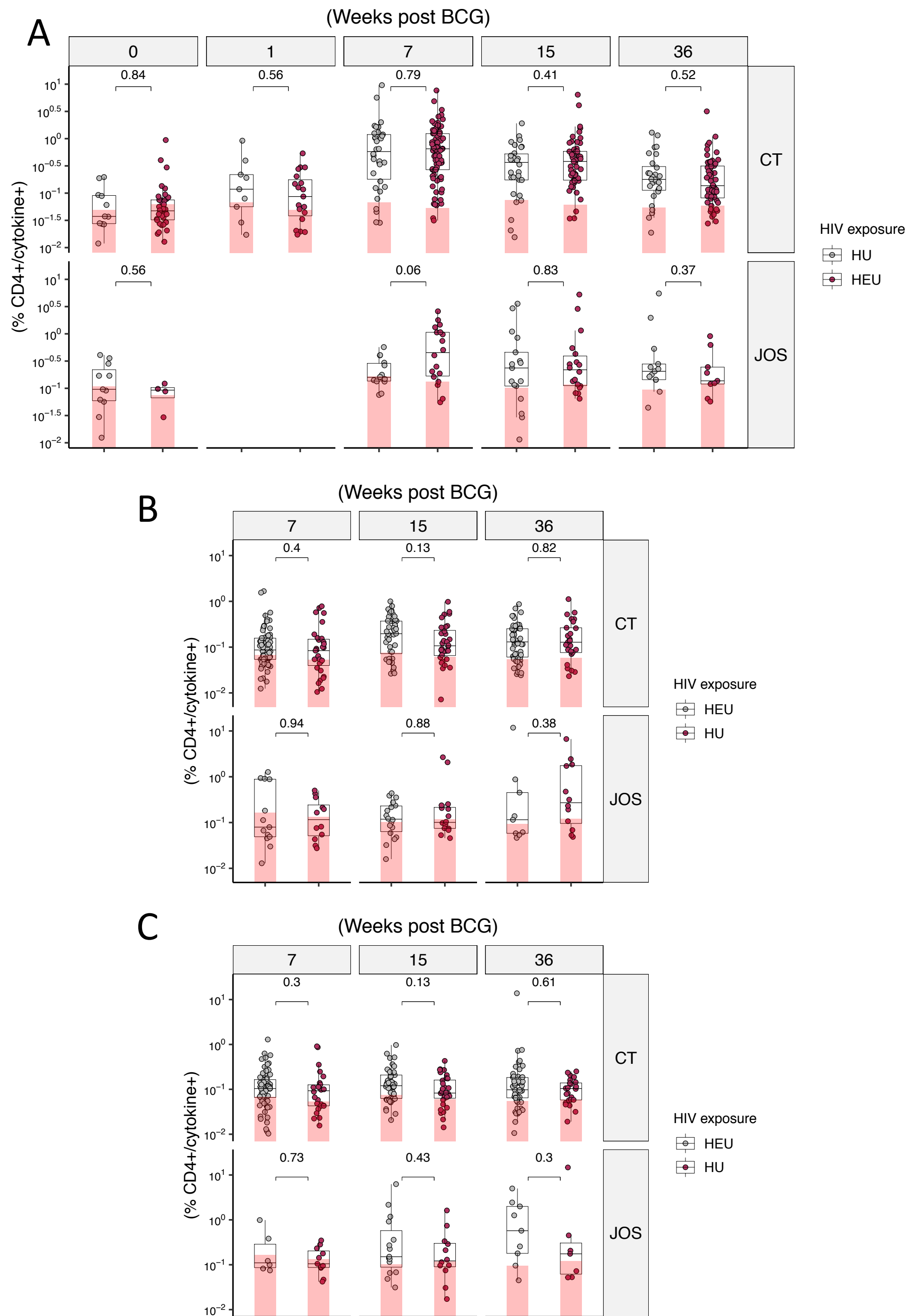

Figure 4.3: Magnitude of T cell vaccine-antigen responses are similar between cHEU and cHU. Cross-sectional comparison of total CD4 T cell cytokine responses (IFN-γ, TNF-α or IL-2) to *in vitro* antigen stimulation: **(A)** BCG, **(B)** Tetanus Toxoid (TT) and **(C)** *Bordetella pertussis* (BP) between HEU (maroon points) and HU (grey points) groups. Shaded bars show the median background per group and per time point. Strip text at the top indicates weeks post BCG and the strip text on right-hand side indicate the cohort. For TT and BP antigens 1wk BCG = 5 wk pre-DTaP/wP, 7 wk BCG = 1 wk DTaP/wP, 15 wk BCG = 9 wk DTaP/wP, 36 wk BCG = 30 wk DTaP/wP. Wilcoxon ranked sum tests were used to compare HEU and HU goups and p<0.01 were considered significant after adjusting for multiple comparisons using Holm's step-down method.

4.3.3 Subtle differences in cytokine profiles to vaccine antigens in cHEU: SPICE analysis results

Having established that response magnitudes were unaffected by HIV exposure, we next asked whether the profiles of Th1 cytokines induced by vaccine antigens *in vitro* (combinations of IFN-γ, TNF-α or IL-2) were altered compared to controls (Figures 4.4–4.8). We started with SPICE analysis (Simplified Presentation of Incredibly Complex Evaluations)[272]. For CT infants, BCG induced cytokine patterns that included dual and triple positive (polyfunctional) CD4 cells at weeks 7, 15 and 36 post BCG vaccination (Figure 4.4). These patterns differed between cHEU and cHU infants only at one week post BCG vaccination in CT infants (Figure 4.7A) where the majority of the cytokine response among HEUs was TNF-α compared to HUs (p=0.03). In contrast, HUs predominantly expressed IFN-γ relative to HEUs (p=0.08). These differences in mycobacterial-specific cytokines were evident despite comparable magnitudes in the total CD4 response (Figure 4.7B). Differences in the patterns of cytokine expression between HIV exposure groups were also evident for BP antigen among CT infants at week 36 (Figure 4.6) despite no differences in the magnitude of the total CD4 response (Figure 4.3). At week 36, HEUs had lower single positive TNF-α as a proportion of the total cytokine response compared to HUs (p=0.007: Figure 4.6). For Jos infants, the cytokine patterns induced by BCG were in stark contrast to that of CT infants, where less dual and polyfunctional CD4 cells were observed. However, cytokine profiles between HEU and HU were largely comparable at all time points post BCG (Figure 4.6). Similar to CT infants, BP antigen induced slight changes in overall cytokine patterns between HEU and HU groups in Jos. These differences were at week 36 (p=0.01) where HEU expressed a greater proportions of single positive IFN-γ (p=0.007) CD4 cells compared to the cHU respectively (Figure 4.6). Cytokine patterns induced by TT antigen did not differ by HIV exposure among Jos infants (Figure 4.5). CT infants showed marginal

differences in cytokine patterns between cHEU and cHU responding to TT antigen at week 36 (p=0.09; Figure 4.5), where cHU had greater proportions of dual positive TNF-α+IL-2+ (p=0.02) and less single positive IFN-γ (p=0.03) CD4 cells, though this did not pass signficance in adjusted analyses.

In summary cytokine patterns induced by BCG were significantly different by HIV exposure only at 1 week after BCG vaccination (only observed in CT infants). Moderate differences in cytokine patterns by HIV exposure status were for TT antigen (in CT infants at week 36) and BP (at weeks 7 and 36 for Jos and CT respectively) but these did not reach significance in adjusted analyses. Combined these results show that whilst modest qualitative differences in vaccine responses can be observed between cHEU and cHU, the most significant differences occurs early within the first week of life.

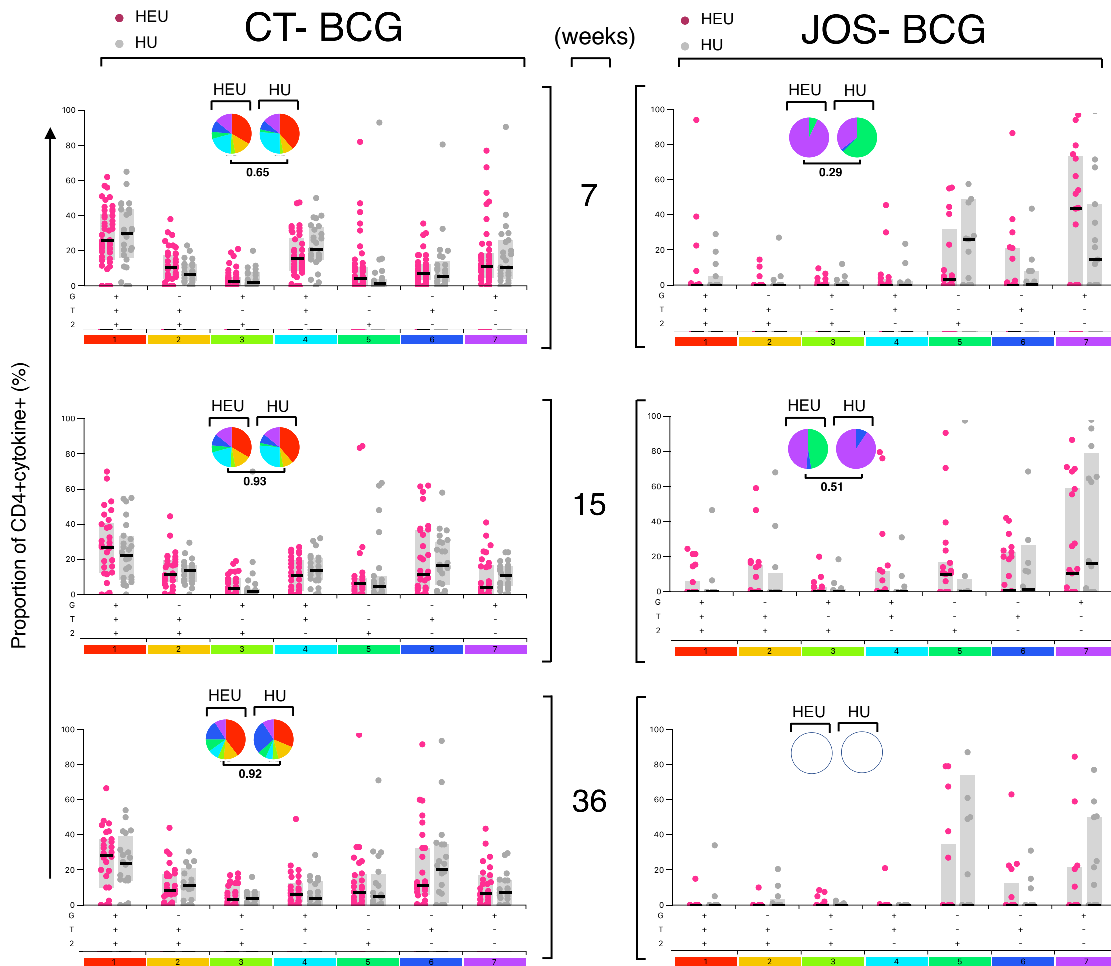

Figure 4.4: BCG-specific cytokine profiles by HIV exposure status. CT infant responses are shown on the left and JOS infant responses on the right. Scatter plots show the frequency of CD4 cells expressing each boolean cytokine combination (G = IFN-γ, 2 = IL-2, T = TNF-α) with each combination numbered 1-7 in colour blocks below each graph. Scatter dots are colour-coded by HIV exposure status (maroon dots: cHEU, grey dots: cHU). Shaded grey bars show inter-quartile ranges with black line showing medians. Pie charts show median proportions of each cytokine combination (1-7) as a fraction of the total response with each pie slice colour corresponding to the colour blocks below the scatter plots. Pie charts are compared using the SPICE permutations test with $p<0.01$ considered significant and each boolean cytokine combination was compared using a Wilcoxon Ranked Sum Test with $p<0.01$ were considered significant.

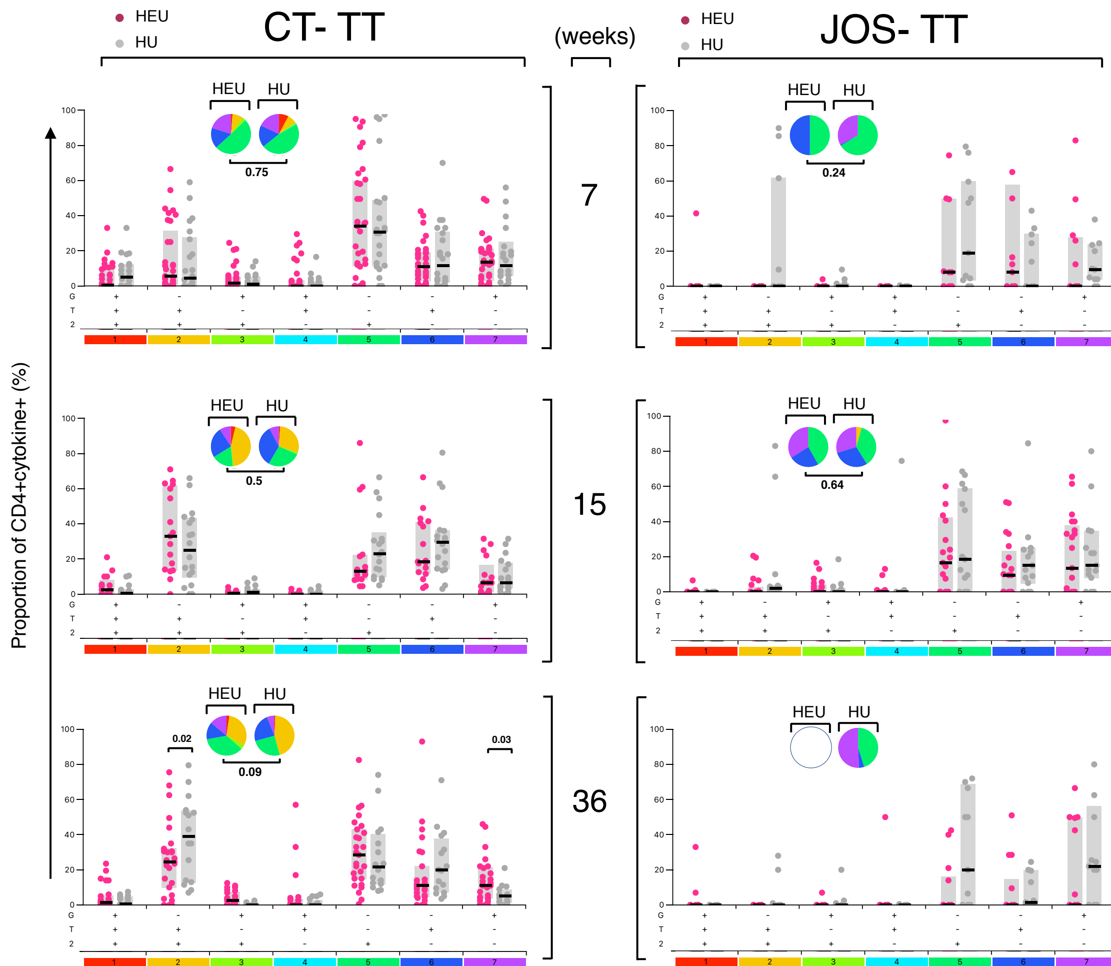

Figure 4.5: Tetanus Toxoid-specific (TT) cytokine profiles by HIV exposure status. CT infant responses are shown on the left and JOS infant responses on the right. Scatter plots show the frequency of CD4 cells expressing each boolean cytokine combination (G=IFN-γ, 2=IL-2, T=TNF-α) with each combination numbered 1-7 in colour blocks below each graph. Scatter dots are colour-coded by HIV exposure status (maroon dots: cHEU, grey dots: cHU). Shaded grey bars show inter-quartile ranges with black line showing medians. Pie charts show median proportions of each cytokine combination (1-7) as a fraction of the total response with each pie slice colour corresponding to the colour blocks below the scatter plots. Pie charts are compared using the SPICE permutations test with $p<0.01$ considered significant and each boolean cytokine combination was compared using a Wilcoxon Ranked Sum Test with $p<0.01$ were considered significant.

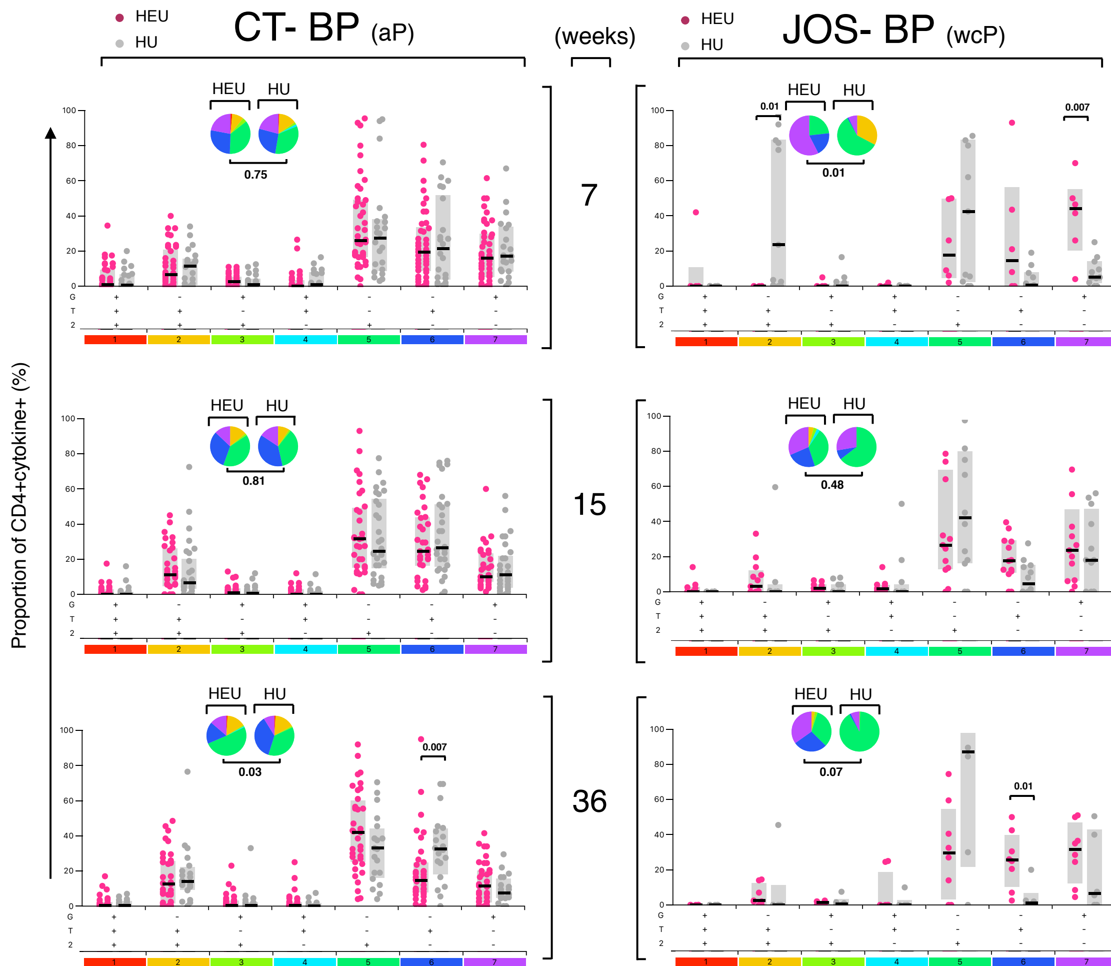

Figure 4.6: *Bordetella pertussis*-specific (BP) cytokine profiles by HIV exposure status. CT infants vaccinated with acellular Pertussis (aP) and are shown on the left whereas Jos infants were vaccinated with wP (whole-cell Pertussis) and are shown on the right. Scatter plots show the frequency of CD4 cells expressing each boolean cytokine combination (G=IFN-γ, 2=IL-2, T=TNF-α) with each combination numbered 1-7 in colour blocks below each graph. Scatter dots are colour-coded by HIV exposure status (maroon dots: cHEU, grey dots: cHU). Shaded grey bars show inter-quartile ranges with black line showing medians. Pie charts show median proportions of each cytokine combination (1-7) as a fraction of the total response with each pie slice colour corresponding to the colour blocks below the scatter plots. Pie charts are compared using the SPICE permutations test with $p<0.01$ considered significant and each boolean cytokine combination was compared using a Wilcoxon Ranked Sum Test with $p<0.01$ were considered significant.

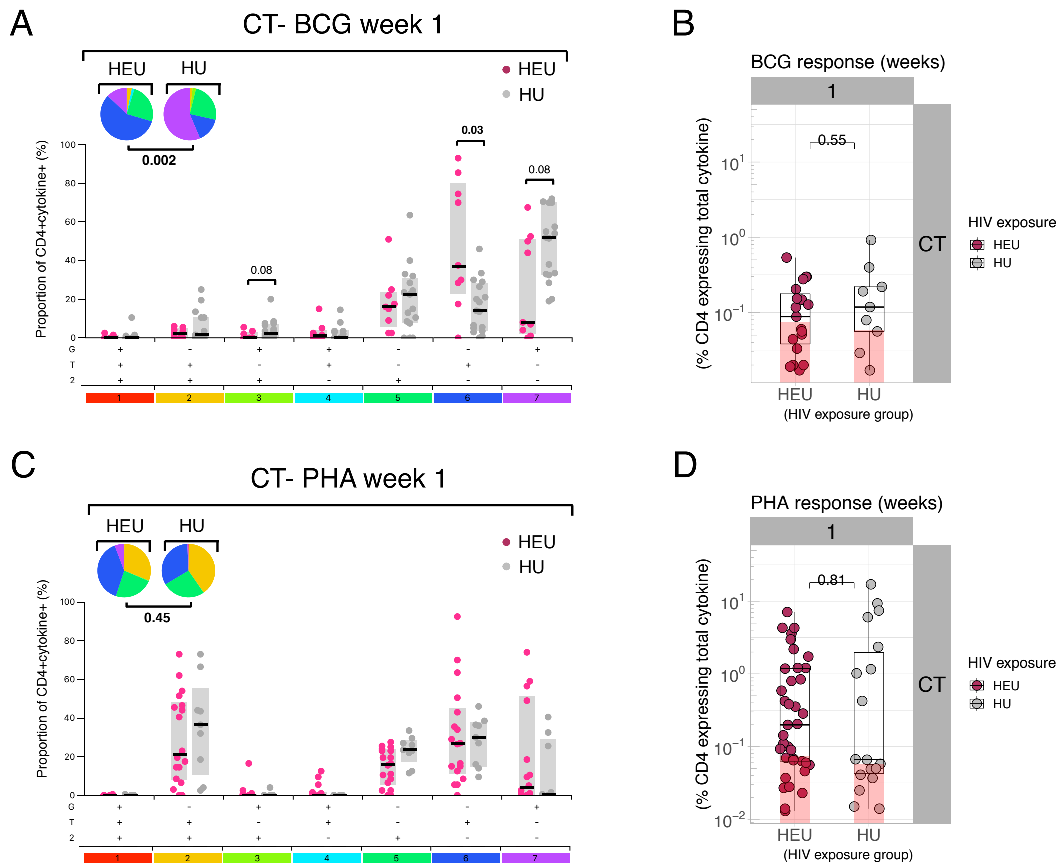

Figure 4.7: Early differences in single cytokine profiles to mycobacterial antigen among HEUs despite parity in total cytokine frequencies. (**A**) & (**C**) BCG-specific and PHA-specific cytokine profiles respectively for CT infants. SPICE dot plots show the frequency of CD4 cells expressing each cytokine boolean cytokine combination (G=IFN-γ, 2=IL-2, T=TNF-α) and are colour-coded by HIV exposure status (maroon circles: HEU, grey circles: HU). Shaded grey bars show interquartile ranges with line showing medians. Pie charts show median proportions of each cytokine combination as a fraction of the total response strain group by SPICE analysis. Pie charts are compared using the SPICE permutations test with $p<0.01$ considered significant. Cytokine combination subsets were compared using a Wilcoxon Ranked Sum Test with $p<0.01$ were considered significant. (**B**) & (**D**) Week 1 CD4 response magnitude to BCG and PHA by HIV exposure status respectively. Y axes show the frequency of CD4 cells producing total cytokine (IFN-γ, TNF-α or IL-2). Shaded bars show the median frequency in the unstimulated samples. Wilcoxon ranked sum tests were used to compare HEU and HU groups with $p<0.01$ were considered significant.

4.3.4 Trend towards increased polyfunctional and functional scores in cHEU: COMPASS analysis results

We complimented the SPICE analyses of the quality of vaccine responses by HIV exposure with a computational algorithm for an unbiased combinatorial polyfunctionality analysis of antigen-specific T-cell subsets (COMPASS) [273]. COMPASS models the probability of antigen-specific responses (posterior probabilities) for each possible combination of cytokine subsets, assigning small probabilities to subsets that are least likely to be present for a particular individual which can be viewed as a heatmap (Figure 4.8). COMPASS also assigns functional scores (FS) for each individual—a summary statistic that quantifies all possible functional subsets. COMPASS also assigns polyfunctional scores (PFS)—a similar summary statistic to FS but weighted to polyfunctional (2 or more cytokines). These scores are continuous variables that can be compared between phenotypes, time points etc. and are statistically more robust than SPICE analyses. As shown in Figure 4.9, two main observations were apparent: firstly, the majority of polyfunctional and functional scores to vaccine antigens were equal between HEUs and HUs, secondly, where there were differences, these tended to reflect higher—rather than lower polyfunctional/functional scores among HEUs. For example at week 15, HEUs in the Jos cohort had higher PFS to mycobacterial antigen compared to HUs (median 0.23 vs. 0.07 respectively, p=0.01) and concomitant FS (median 0.32 vs. 0.17 respectively, p=0.03). Similar trends in higher PFS among HEUs were observed for responses to BP antigen in CT infants at week 36 (median 0.1 vs. 0.04 respectively, p=0.03) and concomitant FS (median 0.18 vs. 0.08 respectively, p=0.03). These results however, did not pass multiple comparison correction. COMPASS analyses on the whole reflected findings in SPICE analyses which showed no alterations to TT antigen but subtle differences to BP antigen. In summary, COMPASS analyses suggest no convincing immune perturbations in the quality of responses to vaccine antigen.

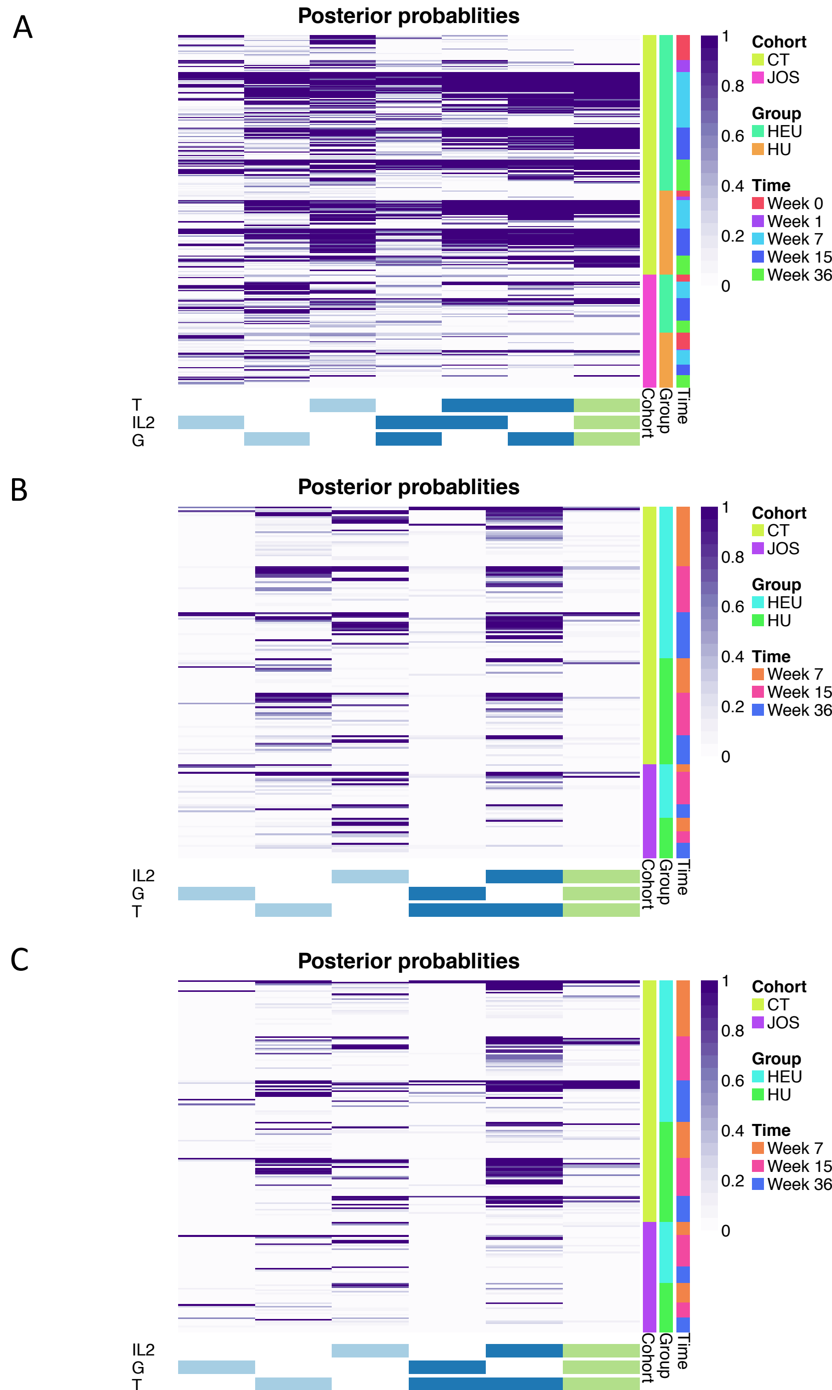

Figure 4.8: Heatmap of posterior probabilities of antigen responses estimated by COMPASS analysis. Heatmaps for **(A)** BCG, **(B)** TT = Tetanus Toxoid and **(C)** BP = *Bordetella pertussis* antigens. Rows represent individual infants and grouped by HIV exposure status and time post BCG vaccination (colour-coded annotations on right side of heat map). Columns represent cytokine subsets (combinations) ordered by degree of functionality (single to polyfunctional; right to left). The colour of each cell corresponds to the probability (range 0-1) of an antigen response for a particular cytokine cell subset per infant, with 1 indicating an antigen-specific response and white indicating background.

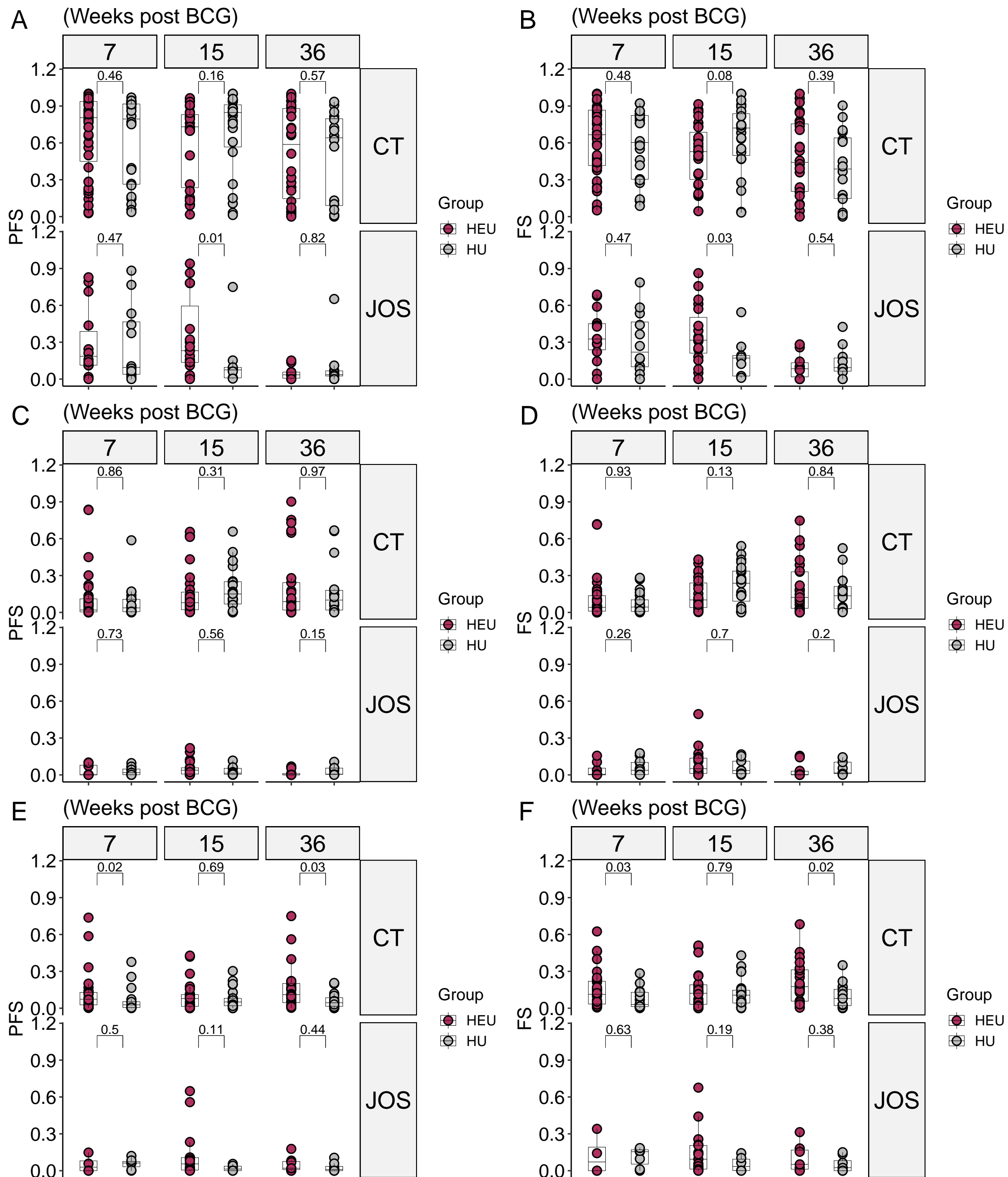

Figure 4.9: Trend towards increased polyfunctional and functional scores in HEUs. Scatter plots show COMPASS analyses for polyfunctional scores (PFS: column 1) and functional scores (FS: column 2) of antigen specific responses: **(A) & (B)** BCG, **(C) & (D)** TT = Tetanus Toxoid and **(E) & (F)** BP = *Bordetella pertussis* antigens. Maroon points = HIV-exposed uninfected (HEU) and grey points = HIV-unexposed (HU). *P*-adjusted values are shown after multiple correction adjustment using Holm's step-down method, with *p-adj*<0.05 considered significant.

4.4 Discussion

In this section, we found that the impact of HIV exposure on cell-mediated vaccine responses appeared to be limited to partial qualitative differences rather than differences in the response magnitude. While some studies have suggested quantitative differences between HEU and HU groups [130, 126, 127, 140], we found no such evidence. The unique aspect of our study was that this observation was replicated in two independent cohorts. The cohorts in this study are from derived from communities where Mtb infection is common [274, 275], therefore, the functional capacity of mycobacterial-specific T cells from HEUs was of particular interest, specifically the induction of polyfunctional T cells which correlate with enhanced Mtb protection in animal models [135]. Polyfunctional T cell responses to vaccination against Mtb are the focus of novel Tb vaccination strategies [197]. Others have reported differences in mycobacterial polyfunctional responses between HEU and HU infants—in proliferating T cells [128]. SPICE analyses in this study revealed differences in the proportions of single cytokines one week after BCG vaccination, whereas polyfunctionality assessed by COMPASS polyfunctional scores suggest that T cells from HEUs are equally immune competent as HUs in responding to mycobacterial antigen. The absence of any qualitative differences to mycobacterial antigen beyond the first month of life has also been shown in both Ugandan and South African cohorts, vaccinated with BCG at birth [131, 127].

Interestingly, we detected marginal qualitative differences for *Bordetella pertussis*, with higher polyfunctional scores among HEUs, suggesting immune competance to this vaccine. An important consideration here is that our cohorts were under Option B+ where all HIV+ women received ART during pregnancy and thereafter. In addition, exclusive breastfeeding rates (up to 6 months of age) were high compared to other studies [83, 100] which improves clinical outcomes in these infants [194]. In summary, we found no convincing evidence for perturbations in both the magnitude and quality of vaccine responses by HIV exposure. Instead, we observed far greater

differences in vaccine immunogencity by cohort site, particularly for BCG, where Jos infants tended to have lower overall cytokine production compared to CT infants and lower polyfunctional scores (Appendix 7.4 and 7.5). This appears to support the observation that responses to mycobacterial antigen from cohorts closer to the tropics tend to exhibit lower Th1 cytokine profiles than those further from the tropics [276, 36]. We do acknowledge that sample integrity (related to cold-chain) may have been a contributing factor in explaining the lower cytokine responses for the Jos cohort. Nonetheless, within-cohort comparisons of cytokine frequencies between HEU and HU groups suggest that exposure does not affect the magnitude of response.

Overall the data presented in this chapter suggests that T cell function is normalized after the first week of life and that HIV/ARV exposure may have no deleterious long-term impact on vaccine immunogenicity.

Chapter 5

Impact of BCG immunising strain on T cell function

5.1 Introduction

In Chapter 4, CD4 cytokine responses to vaccine antigens were compared between cHEU and cHU from two African cohorts. Substantial differences were noted in the frequencies of CD4+cytokine+ cells and polyfunctional scores (by COMPASS) for mycobacterial responses between cohorts (Appendices 7.4 & 7.5), irrespective of HIV exposure status which warranted further investigation. CT infants recruited in this study had received BCG-Denmark until January 2016 when BCG-Denmark became unavailable and the South African EPI program began using BCG-Russia. BCG-Russia is genetically identical to BCG-Bulgaria [277], the strain given to infants in Nigeria. In this observational study, we leveraged the change to EPI BCG immunising strain to test the hypothesis that immunogenicity to BCG-Denmark would differ to that of Russian and Bulgarian BCG immunised infants.

Many TB endemic countries provide Bacille Calmette-Guérin (BCG) vaccine to infants routinely soon after birth [192, 188, 278, 128]. BCG is efficacious against childhood TB, particularly extrapulmonary forms [279]. Numerous strains of BCG exist,

with various duplications and deletions in protein coding regions [280], which have been shown to affect the type of immunity elicited by the vaccine [192, 281]. Although no correlate of protection against *Mycobacterium tuberculosis* (Mtb) exists [186], Th1 CD4+ T cells are believed to be important [282, 283] and are therefore used to measure BCG vaccine immunogenicity [192, 128, 281, 187, 131, 194, 127]. BCG-Denmark strain has been shown to induce a greater magnitude and polyfunctional CD4+ Th1 cytokine production [281] and also has a range of heterologous effects [278, 284, 285, 286]. For example, in adults, BCG-Denmark increases monocyte cytokine production against unrelated antigen stimulation; where such "trained immunity" after primary infection or vaccination has been shown to confer protection against secondary infection, independent of the adaptive immunity [285]. In adults vaccinated with BCG-Denmark, increases Th1 and Th17 cells are observed in response to *in vitro* stimulation with heterologous antigen [287]. BCG-Denmark vaccinated infants have higher IFN-γ responses to Phytohaemagglutinin (PHA) and Tetanus Toxoid (TT) versus those vaccinated with BCG-Russia in cultured whole blood supernatants [286]. In low birth weight infants, BCG-Denmark vaccination has been associated with increased innate cytokine levels in whole blood stimulated with Toll-like receptor (TLR) ligands [288]. Whether vaccine strains other than BCG-Denmark have a similar effect on T cell responses to unrelated antigens in African infants has not been assessed. In this study, we compared CD4+ T cell immunity to BCG, Tetanus and Pertussis vaccines in two cohorts of newborn infants recruited from Jos, Nigeria and Khayelitsha, Cape Town, South Africa (Methods section 4.2.1: Chapter 4), and interrogated the effects of BCG strain. We show that BCG vaccine strain not only impacts significantly on CD4+ T cell polyfunction and memory maturation, but also on heterologous T cell responses to other vaccines—irrespective of HIV exposure status.

5.2 Methods

5.2.1 Cohort description

Mother-infant pairs were recruited at the Midwifery Obstetric Unit (MOU) at Site B in Khayelitsha, Cape Town (CT), South Africa and the Plateau State Specialist Hospital in Jos, Nigeria from November 2014 to November 2016 as described in detail in section 4.2.1.

5.2.2 Immunisation

Routine vaccines were given to all infants according to National Vaccine programme guidelines (Table 4.1). Infants from CT received intradermal Danish BCG strain (1331, Statens Serum Institute, Denmark) from April 2013 to January 2016 and thereafter Russian strain (BCG-I Moscow, Serum Institute of India, India). Both strains were given at $2{\times}10^5$ CFU/dose at birth. Infants from Jos received the Bulgarian strain (BCG-SL 222 Sofia, BB-NCIPD Ltd., Bulgaria) at $0.3{\times}10^5$ CFU/dose at 4–7 days after birth (Table 7.3).

5.2.3 Whole blood assay

A 12-hour whole blood assay was used to evaluate vaccine responses as previously described in Chapter 4, section 4.2.3. The *in vivo* vaccine dose and whole blood assay antigen concentrations are listed in Table 7.3.

5.2.4 Cell staining, antibodies and flow cytometry

For vaccine immunogencity measurements, cell staining and flow cytometery was carried out as described in Chapter 2, section 4.2.4. The following monoclonal antibody-fluorochrome conjugates were used in the T cell vaccine panel: IL-2-R-phycoerythrin (PE), CD8-V500, IFN-γ-Alexa Fluor-700, TNF-α-PE-Cy7, Ki67-Fluorescein isoth-

iocyanate (FITC) (all from BD), CD27 PE-Cy5, HLA-DR- Allophycocyanin-Cy7 (APC-Cy7), CD3-BV650 (Biolegend), CD4 PE-Cy5.5 (all from Invitrogen), CD45RA PE-Texas Red-X (Beckman Coulter). A minimum of 50 000 ViViD negative (viable) CD3 events were collected for T cell vaccine measurements using FACS DIVA v6 software. Post-acquisition compensation and analysis was performed in FlowJo version 9 (FlowJo, LLC).

5.2.5 Statistical analyses

Participant BCG-strain and HIV exposure groupings were unblinded after flow-gating analyses. All statistical analyses were performed in R [249]. Non-parametric comparisons between independent groups was performed using Wilcoxon rank sum test (Mann Whitney U test), with the Holm's step down method used for multiple comparison correction. The Kruskal Wallis test was used for longitudinal data. For cytokine profiling analyses in SPICE, we only analysed samples from participants classified as antigen responders (Figure 7.7) to avoid skewing related to samples that were likely to be background. Cytokine combinations were assessed using SPICE software [272] and in addition, we used a more unbiased statistical approach for measuring polyfunctionality with COMPASS (Combinatorial polyfunctionality analysis of antigen- specific T-cell subsets) [289] to estimate the posterior probabilities of antigen specific T cell subsets. COMPASS is a statistical model for multi-functional (e.g. multiple cytokine subsets) analysis of flow cytometry data sets. Polyfunctionality scores which summarize the functional profile of each subject were calculated from posterior responses probabilities as described [289].

5.3 Results

5.3.1 Cohort characteristics

A total of 186 samples from CT and 84 samples from Jos spanning the first nine months of life were included in this study (Table 5.1). There were no differences in gestational age at delivery, birth weight or baby length by vaccine strain received (i.e BCG-Denmark, BCG-Bulgaria or BCG-Russia).

Table 5.1: Infant birth characteristics by BCG immunising strain received at birth

	BCG strain			
	Bulgaria (N=84)	**Denmark (N=154)**	**Russia (N=32)**	***P*-values**
Age (weeks)	38 [38-39]	38 (37-39)	38 [38-39]	0.22
Birth weight (g)	3000 [2700-3300]	3025 [2778-3330]	2930 [2840-3230]	0.16
Length (cm)	47 [46-49]	48 [46-50]	47 [46-50]	0.1
Female (%)	41	58	57	0.29
HEU (%)	78	68	54	0.03

BCG-Denmark and BCG-Russia were infants from the Cape Town cohort, BCG-Bulgaria were infants from Jos cohort. Numbers represent median with interquartile ranges in brackets. Wilcoxon Rank Sum test was used to compare strains for continuous variables (B-D; Bulgarian vs. Danish, D-R; Danish vs. Russian). $P<0.05$ were considered significant. Chi Squared test for homogeneity was used for categorical variables

5.3.2 Magnitude of mycobacterial-specific CD4 T cell cytokine responses differ according to BCG strain

The rationale for comparing responses by BCG strain was due to the observation of lower response magnitudes among Jos infants (all having received BCG-Bulgaria) vs. CT infants (the majority of whom had received BCG-Denmark at birth: Table 4.1). Infant whole blood samples had been re-stimulated *in vitro* with the vaccine-matched antigen, except cells from CT infants vaccinated with BCG-Russia were stimulated with BCG-Denmark culture (SSI) *in vitro* (Appendix 7.3). We confirmed that the

in vitro stimulating antigen did not impact the magnitude of quality of the T cell cytokine magnitude and polyfunctional response (Figure 5.1).

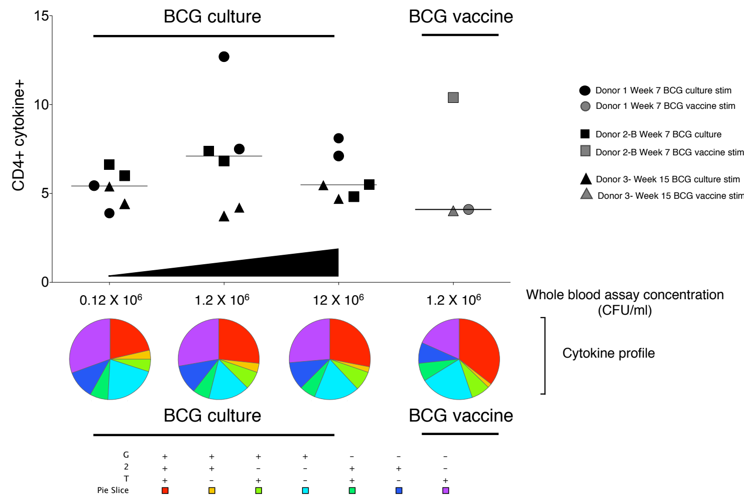

Figure 5.1: The *in vitro* simulating antigen does not impact response magnitude or quality. The unavailability of BCG SSI Denmark (SSI, Denmark) for laboratory assays necessitated the switch to BCG-Denmark culture (AJ Vaccines) for *in vitro* stimulation. Different *in vitro* concentrations of BCG culture were compared to the standard BCG vaccine *in vitro* concentration used in whole blood assay for CT infants. Donors representing different week 7 and week 15 post BCG time points were used in the same experiment. Total CD4 cytokine frequencies are shown in the dot plots and median proportions of each cytokine (IFN-γ, IL-2 and TNF-α) are represented by the pies.

Due to the unequal distribution of HEU vs. HU infants among BCG-Bulgaria group (Table 5.1), the magnitudes of CD4 cytokine responses were first stratified by HIV exposure group and response magnitudes were compared by BCG strain (Figure 5.2 & Table 5.2). Total CD4 cytokine response magnitudes were comparable between strains before vaccination in both HIV exposure groups (Figure 5.2B). At week 7 post BCG vaccination, however, the BCG-Denmark strain vaccinated infants had higher frequencies of CD4+ cytokine-producing cells over background (HEU median=0.77%, HU median=1.01%) compared to both Jos infants vaccinated with BCG-Bulgaria (HEU median=0.45%, p=0.09; HU median=0.16%, p<0.0001 respectively) and CT infants vaccinated with BCG-Russia (HEU median=0.19%, p<0.0001; HU median=0.05%, p<0.0001 respectively: Figure 5.2 and Table 5.2). Of note was that infants from the same cohort but receiving different BCG strains at birth showed significant differences in the magnitudes of responses. CT infants

vaccinated with BCG-Denmark had higher cytokine+ frequencies compared to those vaccinated BCG-Russia regardless of HIV exposure status at week 7 (5.2B). A trend in these within-cohort differences extended to weeks 15 and 36 however did not pass significance after adjustment for multiple comparisons (Table 5.2). Examining longitudinal data from matched samples across all time points further emphasised that infants vaccinated with BCG-Denmark, peaked at week 7 (Figure 5.2C), continued to express significantly higher CD4 cytokine responses compared to BCG-Russia vaccinated infants at week 15 (median=0.40 vs. 0.17, p=0.03) and week 36 (median=0.23% vs. 0.05%, p=0.006; Figure 5.2C). These results suggest that BCG immunising strain impacts on the magnitude of mycobacterial response irrespective of HIV exposure status.

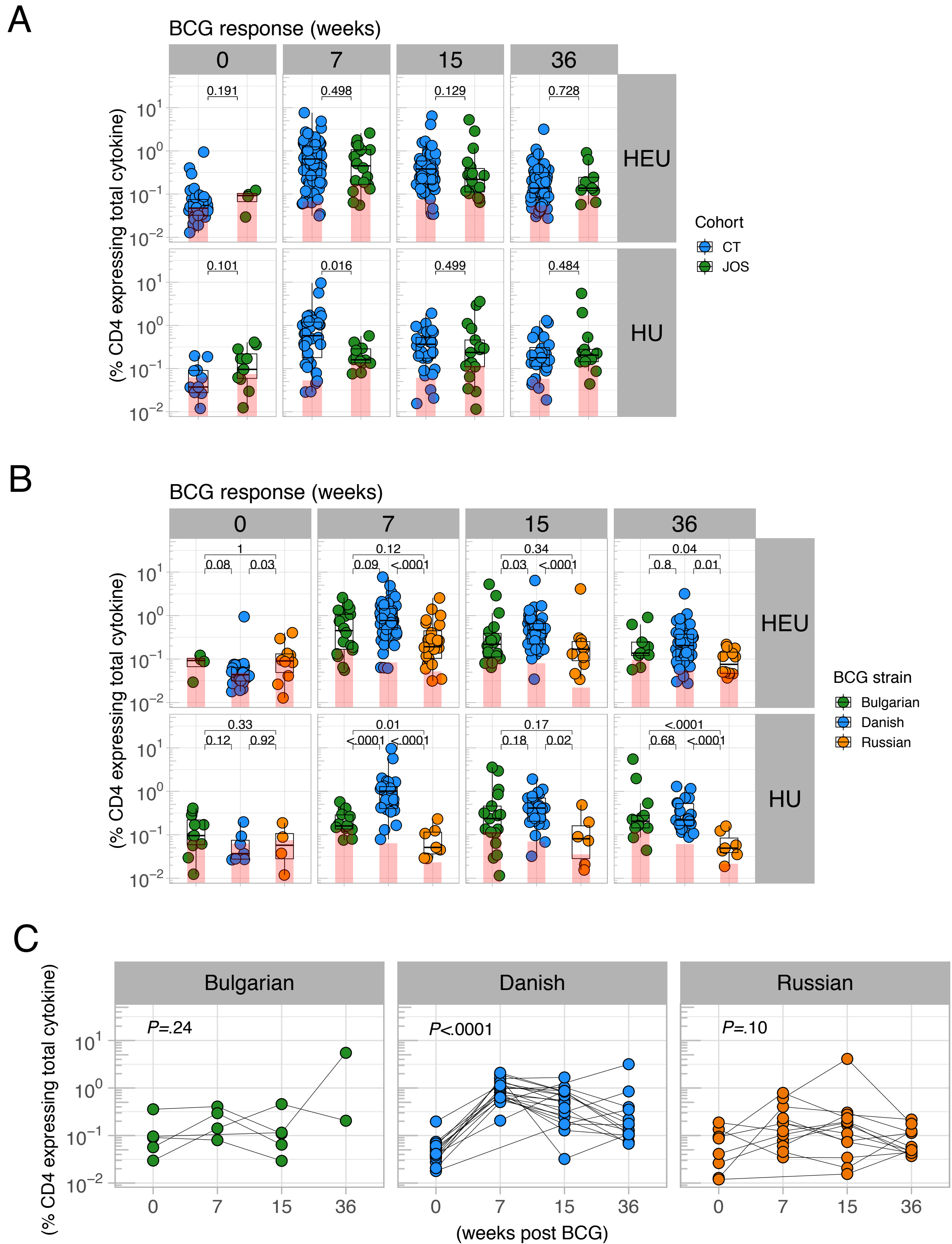

Figure 5.2: Magnitude of myco-bacterial CD4 cytokine responses differ by BCG strain. A & B: Cross-sectional analysis of mycobacterial-specific CD4 cytokine responses between cohorts (A): irrespective of BCG immunising strain, (B) between BCG immunising strain stratified by HIV exposure. Weeks indicate weeks post vaccination with week 0 being the pre-vaccination time point (birth for the CT cohort and a median of 4-7 days after birth for the Jos cohort). Y axes show the frequencies (%) of CD4+ cells producing total cytokine (any combination of IFN-γ, IL-2 or TNF-α). Shaded bars show the median value of cytokine+ cells in unstimulated samples. Boxes with mid-line show interquartile ranges and median. (C) Longitudinal responses to BCG in samples matched for at least 3 time points between week 0 and 36, stratified by BCG strain. Wilcoxon Rank Sum Test was used to compare cross-sectional data, with *p*-values <0.001 considered significant after adjustment for multiple comparisons. Kruskal Wallis test was applied to test for differences over time for longitudinal data.

Table 5.2: Magnitude of mycobacterial-specific CD4 cytokine responses by BCG strain

Time	Exposure	Median CD4 cyk (IQR)			*p*-values		
		Bulgarian	Danish	Russian	B-D	D-R	B-R
0	HEU	0.09 (0.07-0.1)	0.04 (0.03-0.06)	0.09 (0.05-0.13)	0.08	0.03	1
7		0.45 (0.17-1.07)	**0.77** (0.48-1.46)	**0.19** (0.1-0.46)	0.09	**<.001**	0.12
15		0.22 (0.11-0.4)	**0.47** (0.22-0.65)	**0.17** (0.09-0.25)	0.03	**<.001**	1
36		0.14 (0.12-0.24)	0.2 (0.09-0.37)	0.08 (0.05-0.15)	0.8	0.01	0.04
0	HU	0.1 (0.06-0.23)	0.04 (0.03-0.08)	0.06 (0.03-0.11)	0.12	0.92	0.33
7		**0.16** (0.14-0.29)	**1.01** (0.4-1.28)	**0.05** (0.04-0.12)	**<0.001**	**<.001**	0.01
15		0.24 (0.11-0.46)	0.41 (0.2-0.7)	0.08 (0.03-0.17)	0.18	0.02	0.17
36		**0.2** (0.14-0.28)	**0.22** (0.16-0.52)	**0.05** (0.04-0.09)	0.68	**<.001**	**<.001**

Numbers represent median frequencies of CD4+ cells producing total cytokine (any combination of IFN-γ, IL-2 or TNF-α) with interquartile ranges in brackets. Time is expressed as weeks post BCG vaccination with week 0 the pre-vaccination time point (birth for BCG-Denmark and BCG-Russia, and Week 1 after birth for BCG Bulgaria). Wilcoxon Rank Sum Test was used to compare strains (B-D; Bulgarian vs. Danish, D-R; Danish vs. Russian, B-R; Bulgarian vs. Russian) with P<0.001 significant after multiple comparison using Holm's step down method.

5.3.3 Polyfunctional mycobacterial responses differ between strains

When examining the combinations of cytokines (IFN-γ, IL-2 and TNF-α) induced by *in vitro* mycobacterial antigen stimulation by BCG strain, irrespective of HIV exposure status, we observed that fewer polyfunctional (triple cytokine positive) cells were induced among infants receiving BCG-Bulgaria. The majority of the total response in BCG-Bulgaria immunised infants was single positive for IFN-γ (Figure 5.3A & B). Conversely, infants receiving BCG-Denmark had a higher proportions of CD4 cells expressing all three cytokines. This polyfunctional profile was sustained from 7 to 36 weeks constituting more than a quarter of the total cytokine response (median IFN-γ+IL-2+TNF-α+ at week 7 = 28%, week 15 = 26%, week 36 = 27%). The proportions of these triple positive cells were significantly lower for BCG-Bulgaria and BCG-Russia immunised infants (median at week 7 = 0% and 6%, respectively, p<0.001, week 15 = 0% and 7% respectively, p<0.001, week 36 = 0% and 7%, respectively, p<0.001). Furthermore, the proportion of dual-expressing IFN-γ+ and IL-2+ CD4+ T cells was higher among BCG-Denmark recipients compared to BCG-Russia recipients (week 7 = 3% vs. 0% respectively,

p<0.001) while the proportion of single TNF-α+ cells was lower (median at week 7 = 7% vs. 42%, p<0.001). When infants were further stratified by HIV exposure status, the differences of cytokine profiles induced by BCG strains was still evident BCG as shown in the example of week 7 responses (Figure 5.3C). To summarise, SPICE analyses revealed polyfunctional and dual functional cytokine profiles induced by myco-bacterial antigen stimulation that were significantly over-expressed by BCG-Denmark immunised infants compared to both BCG-Bulgaria and BCG-Russia irrespective of HIV exposure.

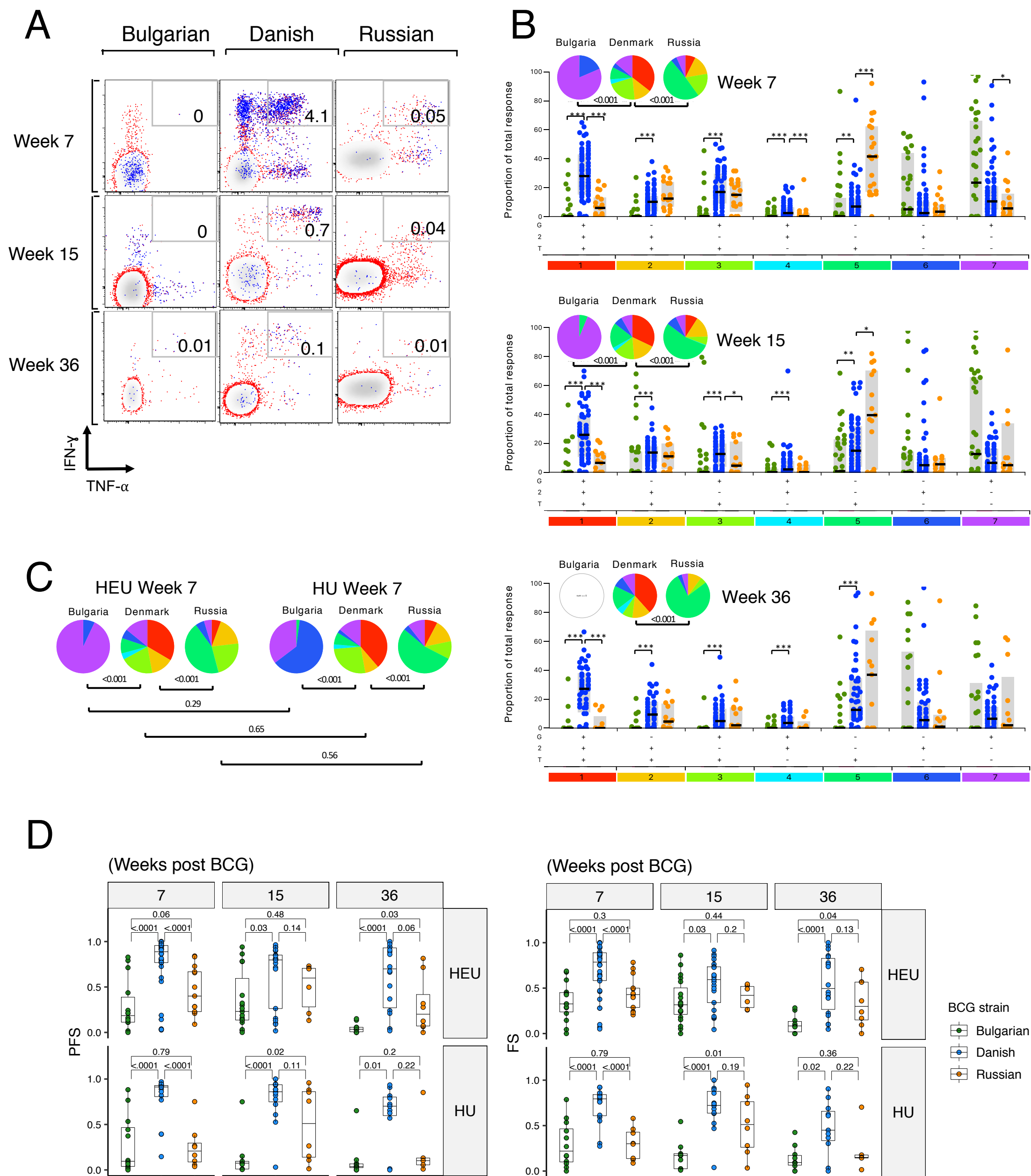

Figure 5.3: Cytokine profiles of mycobacterial-specific responses differ between BCG strains irrespective of HIV exposure status. (A) Representative flow cytometry plots showing mycobacterial-specific antigen responses stratified by BCG immunising strain. Axes show IFN-γ vs. TNF-α expression with IL-2+ cells overlaid and represented by blue dots. (B) Jitter points show the median proportion of each cytokine combination (G=IFN-γ, 2=IL-2, T=TNF-α) per infant as a fraction of total cytokine+ cells per infant responding to BCG irrespective of HIV exposure and are colour-coded by BCG immunising strain (green = Bulgaria, blue = Denmark, orange = Russia) status. Shaded bars show interquartile ranges with line showing median. Wilcoxon Rank Sum test was used to compare BCG strains vs. BCG-Denmark, $p<0.01$ were considered significant after adjustment for multiple comparisons ***$p<0.001$, **$p<0.01$, *$p<0.05$. Pie charts are a summary of the median proportions of cytokine combinations (G = IFN-γ, 2 = IL-2, T = TNF-α) as a fraction of the total response in each BCG strain group irrespective of HIV exposure status by SPICE analysis. Pie charts are compared using the SPICE permutations test with $p<.01$ considered significant. (C) Pie charts summarising week 7 cytokine profiles by HIV exposure status and BCG immunising strain. (D) Week 7 polyfunctional and functional scores to BCG by BCG immunising strain. Scores are stratified by HIV exposure status.

SPICE analyses (Figure 5.3) was complimented with COMPASS [289], a statistical tool that allows for the analysis of antigen-specific subsets in an unbiased manner. The COMPASS model calculates posterior probabilities for each observed cytokine subset, selecting those most likely to be antigen specific. In addition, the COMPASS algorithm evaluates a polyfunctional score (PFS) for each participant which is, a single statistic summarising a participant's entire functionality profile [289]. PFS are defined as the proportions of antigen-specific subsets detected amongst all possible subsets and weighted towards those with higher functionality (2 or more cytokines) [289]. We first grouped infants by BCG immunising strain irrespective of HIV exposure status and a heatmap of posterior probabilities for cytokine subsets were plotted and annotated by time and BCG strain (Figure 5.4A).

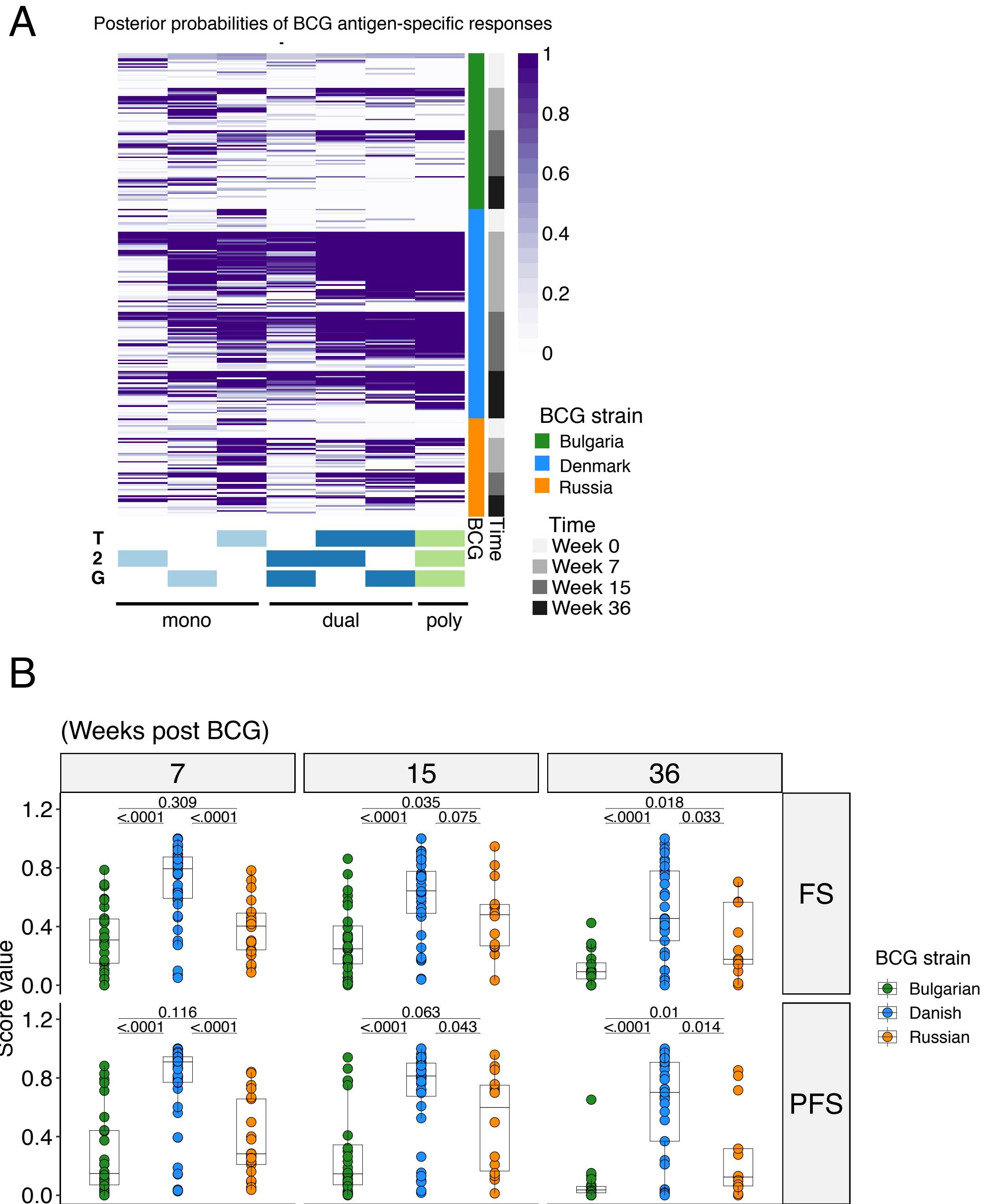

Figure 5.4: Polyfunctional and functional scores to BCG by COMPASS analysis differ between BCG strains. (A) Heatmap of posterior probabilities of mycobacterial-specific responses estimated by COMPASS analysis. Rows represent individual infants and grouped by BCG immunising strain and time post BCG vaccination (colour-coded annotations on right side of heat map). Columns represent cytokine subsets (combinations) ordered by degree of functionality (single to polyfunctional; right to left). The colour of each cell corresponds to the probability (range 0-1) of a mycobacterial-specific response for a particular cytokine cell subset per infant, with 1 indicating an antigen-specific response and white indicating background. (B) Cross-sectional analysis of Polyfunctional scores (as calculated in COMPASS model) stratified by BCG immunising strain [Green (BCG-Bulgaria), blue (BCG-Denmark), orange (BCG-Russia)]. (C) Correlation between Polyfunctional scores and cytokine response magnitude. Spearman's ranked correlation coefficient is reported for n=109 infants in all BCG strain groups. Mann Whitney U test was used to test for differences by BCG strain. $P<.001$ were considered significant after multiple adjustment correction using Holm's step-down method.

In the heatmap we observed that BCG-Denmark infants had more antigen-specific

polyfunctional and dual functional responses compared to either BCG-Russia and BCG-Bulgaria recipients. When comparing polyfunctional scores by strain (Figure 5.4B), as expected these were not different among the BCG strains at the pre-vaccination time point (week 0), however BCG-Denmark immunised infants had PFS that were statistically significant at week 7 compared to the other strains (median BCG-Denmark PFS = 0.9 vs. BCG-Bulgaria = 0.15 (p<.001), BCG-Russia = 0.29 (p<.001)). This trend remained until week 36 were BCG-Denmark immunised infants continued to display higher median PFS than the other strains. Furthermore, polyfunctional scores were significantly correlated with the magnitude of mycobacterial responses suggesting that the higher total cytokine induced by BCG, the more likely the participant had a polyfunctional response (Figure 5.4C). This more unbiased approach to measuring Th1 polyfunctionality is compatible with our SPICE analyses (Figure 5.3B) and shows that BCG-Denmark elicits a more functional immune response in the newborn infant. We also evaluated polyfunctional scores by BCG immunising strain, but stratified by HIV exposure status (Figure 5.3D). At week 7 post BCG, both HEU and HU polyfunctional scores to BCG were equally affected by the immunising strain. Collectively, these data show that BCG-Denmark is more immunogenic than BCG-Bulgaria and BCG-Russia strains, characterised by a higher magnitude of response and a more polyfunctional cytokine profile.

5.3.4 BCG-Denmark induces a more differentiated memory phenotype compared to other strains

To better understand whether BCG-Denmark may result in a more mature antigen-stimulated CD4+ T cell memory differentiation, which along with greater polyfunctionality provides important insight into protective responses against infections [290, 136], the memory phenotypes of the total cytokine BCG responding cells (i.e. cytokine positive) defined as naïve (CD27+CD45RA+), early differentiated

(ED, CD27+CD45RA-), late differentiated (LD, CD27-CD45RA-) and terminally differentiated (TD, CD27-CD45RA+) CD4+ T cells. Our analysis of these subsets was restricted to only those infant cells that responded to BCG (above 2-fold background). A large population of cytokine positive cells with a naïve phenotype (CD27+CD45RA+) was evident, which we hereon refer to as naïve-like (Figure 5.5). Although there was a large range in the frequency of cytokine expressing memory cell subsets, we consistently observed that BCG-Denmark induced a lower frequency of these naïve-like cells across all time points post BCG vaccination. BCG-Denmark induced a predominantly ED profile, particularly at week 15 where BCG-Denmark induced the lowest frequencies of CD4+ cytokine positive cells expressing naïve-like markers (median = 22%) compared to BCG-Bulgaria and BCG-Russia (median = 40% and 36%; p=0.02 and p=0.04, respectively). Conversely, the CD4+ cytokine positive cells expressing ED markers were highest among BCG-Denmark vaccinated infants (median = 53%) compared to BCG-Bulgaria and BCG-Russia (37% and 33%; p=0.03 and p=0.04, respectively). Collectively, these data suggest that BCG-Denmark pushes cells into more differentiated memory state, which may explain the higher magnitude and polyfunctionality of response. Conversely, vaccination with BCG-Russia results in the probable accumulation of naive-like CD4+ T cell responses.

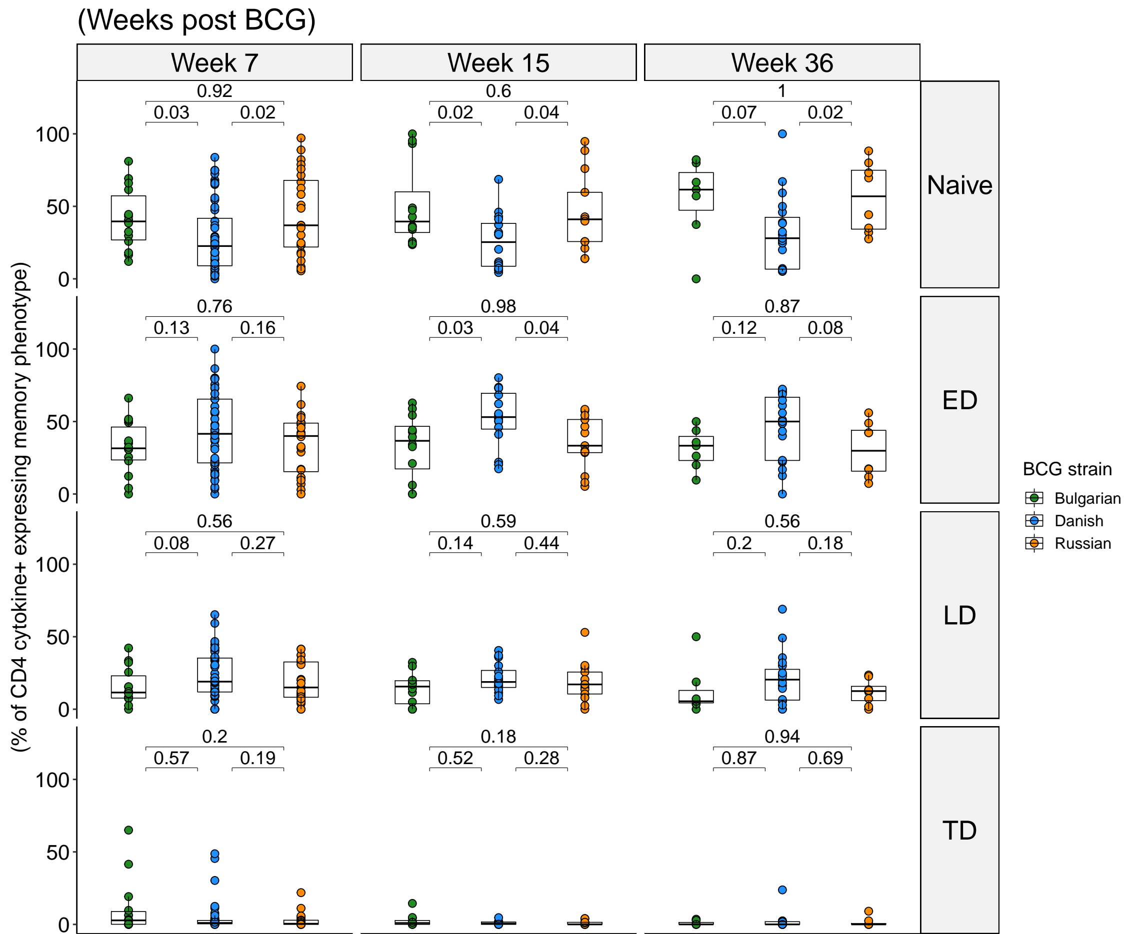

Figure 5.5: BCG-Denmark induces a more differentiated T cell memory phenotype compared to BCG-Russia or BCG-Bulgaria strains. Frequency of mycobacterial-specific memory subsets stratified by BCG immunising strain. Data point colors: Green (BCG-Bulgaria), blue (BCG-Denmark), orange (BCG-Russia), show the proportion of cytokine+ cells co-expressing combinations of memory markers CD45RA and CD27 that define memory subsets (as described in Figure 7.6). Wilcoxon Rank Sum Test was used to test for differences by BCG strain. $P<0.001$ were considered significant.

5.3.5 Strain of BCG impacts CD4+ responses to heterologous antigens

Given that BCG has been shown to have heterologous effects to non-BCG antigens [285, 145], we examined the effect of BCG strain on CD4+ T cell responses to TT, BP and PHA (Table 5.3 and Figures 5.6 & 5.7). We focused only on CT infants and compared those who received BCG-Denmark versus BCG-Russia strains, and thus removed the potential confounding effects of geography, genetic background of participants and different vaccination strategies. When stratified by HIV exposure

status, an interesting pattern was observed: differences in the magnitudes of CD4 cytokine responses to heterologous antigens (TT, BP and PHA) were most apparent the HEU group (Table 5.3 and Figure 5.6). Specifically at week 15 post BCG, HEU infants receiving BCG-Denmark strain had significantly higher responses than those receiving BCG-Russia (TT median= 0.26% vs. 0.05%, p<0.001; BP median = 0.14% vs. 0.06%, p=.002; PHA median = 0.86% vs. 0.18%, p=0.003, respectively). Among HU infants the most significant difference was observed at week 7 for responses to TT, where BCG-Denmark induced higher responses compared to BCG-Russia (TT median= 0.1% vs. 0.02%, p=0.001, respectively) and to a lesser extent week 15 responses to PHA (PHA median = 0.73% vs. 0.15%, p=0.007), though this did not hold after adjustment for multiple comparisons. These results suggest that the heterologous effects of BCG strain impact HEU infants more so than HU infants.

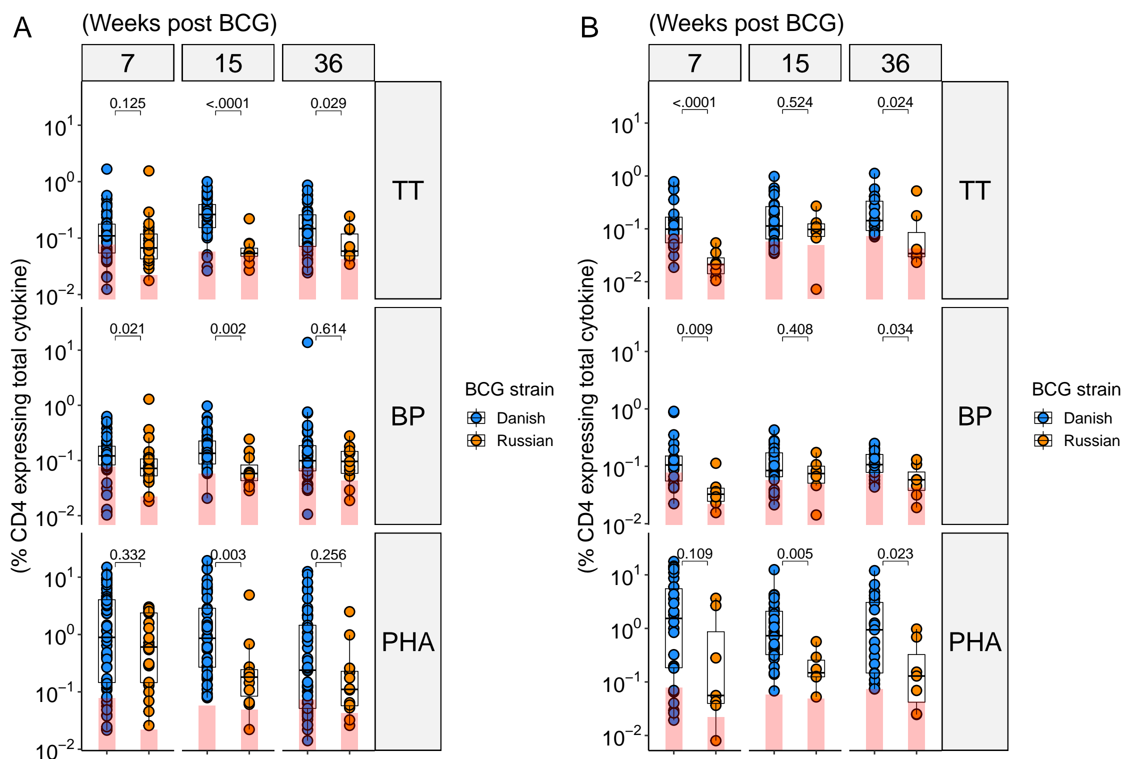

Figure 5.6: BCG immunising strain impacts the magnitude of heterologous antigen responses. Cross-sectional analysis of heterologous antigen-specific CD4 cytokine responses between BCG-Denmark vs. BCG-Russia immunised infants. A: Responses among HEU groups. B: Responses among HU groups. Weeks indicate the time point post BCG vaccination. Y axes show the frequencies (%) of CD4+ cells producing total cytokine (any combination of IFN-γ, IL-2 or TNF-α). Shaded bars showing median value of unstimulated samples. Wilcoxon Rank Sum Test was used to compare cross-sectional data, with p values <0.01 were significant after adjustment for multiple comparisons.

Table 5.3: Magnitudes of heterologous antigen responses by BCG immunising strain

Age (weeks)	Exposure	Antigen	Median CD4 cyk (IQR) Danish	Russian	*P*-values
7	HEU	TT	0.11 (0.05-0.18)	0.07 (0.04-0.12)	0.125
		BP	0.12 (0.08-0.18)	0.07 (0.05-0.11)	0.021
		PHA	0.89 (0.15-4.04)	0.61 (0.14-2.38)	0.332
15		TT	**0.26** (0.15-0.39)	**0.05** (0.05-0.07)	**<.0001**
		BP	**0.14** (0.09-0.23)	**0.06** (0.04-0.09)	**0.002**
		PHA	**0.86** (0.27-2.87)	**0.18** (0.09-0.25)	**0.003**
36		TT	0.15 (0.07-0.26)	0.06 (0.05-0.12)	0.029
		BP	0.1 (0.06-0.19)	0.1 (0.06-0.15)	0.614
		PHA	0.24 (0.05-1.45)	0.11 (0.06-0.23)	0.252
7	HU	TT	**0.1** (0.05-0.17)	**0.02** (0.01-0.03)	**0.001**
		BP	0.11 (0.06-0.15)	0.03 (0.02-0.04)	0.012
		PHA	1.56 (0.18-5.56)	0.06 (0.04-1.48)	0.108
15		TT	0.11 (0.06-0.26)	0.1 (0.07-0.12)	0.515
		BP	0.08 (0.07-0.17)	0.08 (0.05-0.1)	0.398
		PHA	0.73 (0.32-2.09)	0.15 (0.12-0.26)	0.007
36		TT	0.14 (0.09-0.34)	0.03 (0.03-0.11)	0.027
		BP	0.11 (0.08-0.16)	0.06 (0.04-0.08)	0.036
		PHA	0.94 (0.15-3.06)	0.13 (0.05-0.42)	0.026

BCG-Denmark and BCG-Russia were infants from the Cape Town cohort. Numbers represent median frequencies of CD4+ cells producing total cytokine (any combination of IFN-γ, IL-2 or TNF-α) with interquartile ranges in brackets. Time is expressed as weeks post BCG vaccination with week 0 the pre-vaccination time point (birth for BCG-Denmark and BCG-Russia, and Week 1 after birth for BCG Bulgaria). Wilcoxon Rank Sum Test was used to compare strains (B-D; Bulgarian vs. Danish, D-R; Danish vs. Russian, B-R; Bulgarian vs. Russian) with p<0.005 significant after multiple comparison using Holm's step down method.

We examined the cytokine profiles of these responses at week 7, when the BCG response itself peaked. At this time point the most significant differences in cytokine profiles to heterologous antigens between BCG immunising strains was observed and are shown in Figure 5.7. BCG-Denmark strain induced significantly higher proportions of polyfunctional (IFN-γ+ and TNF-α+ and IL-2+) CD4 cells to TT (median = 1.7% vs. 0%, p=0.001), BP (1.6% vs. 0%, p<.0001) and dual-functional (TNF-α+ and IL-2+) to PHA (16.2% vs. 2.6%, p<.0001) (Figure 5.7B). In addition, lower proportions of single TNF-α+ responding CD4+ T cells among BCG-Denmark immunised infants compared to BCG-Russia for TT (median = 14.1% vs. 39.6%, p=0.02), BP (14.7% vs. 39%, p<.001) and PHA (56.1% vs. 81.9%, p=0.03).

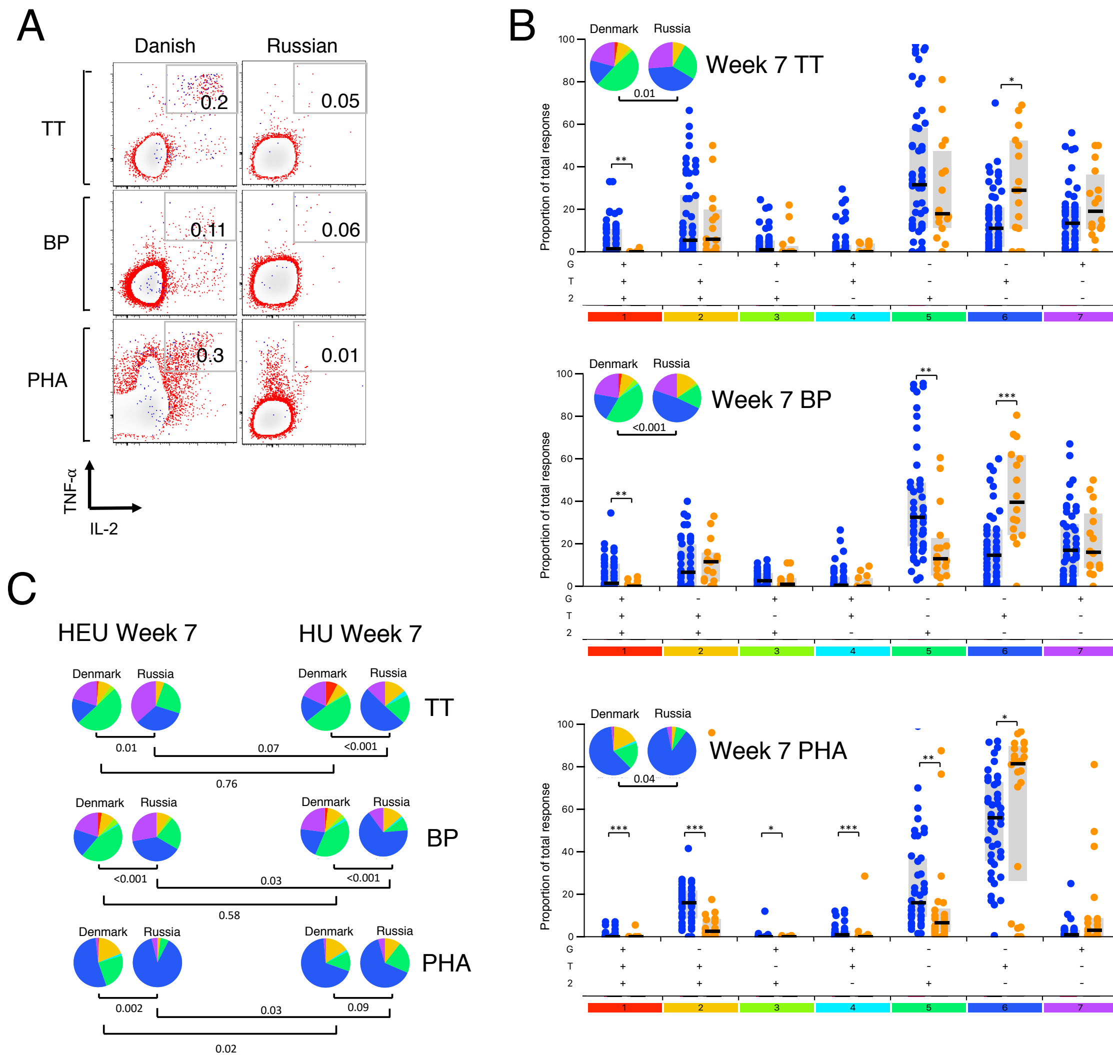

Figure 5.7: Cytokine profiles to heterologous antigens at week 7.(A) Representative flow cytometry plots showing heterolgous antigen responses (TT: Tetanus Toxoid, BP: Bordetella Pertussis, PHA: Phytohaemagluttinin) stratified by BCG immunising strain. Axes show IL-2+ vs. TNF-α and IFN-γ overlaid in blue dots. (B) Jitter points show the median proportion of each cytokine combination (G = IFN-γ, 2 = IL-2, T = TNF-α) per infant as a fraction of total cytokine+ cells per infant responding to TT, BP and PHA and are colour-coded by BCG immunising strain (blue = Denmark, orange = Russia) irrespective of HIV exposure status. Shaded bars show interquartile ranges with line showing median. Wilcoxon Rank Sum test was used to compare BCG strains, $P<0.05$ were considered significant ***$P<0.001$, **$P<0.01$, *$P<0.05$. Pie charts are a summary of the median proportions of cytokine combinations (G = IFN-γ, 2 = IL-2, T = TNF-α) as a fraction of the total response in each BCG strain group irrespective of HIV exposure status by SPICE analysis. Pie charts are compared using the SPICE permutations test with P<.01 considered significant.(C) Pie charts summarising week 7 cytokine profiles by HIV exposure status and BCG immunising strain.

When stratified by both BCG strain and HIV exposure, the impact of strain was still evident for these heterologous antigens (Figure 5.8), where differences in the cytokine profiles occurred equally for both HEU and HU infants. Interestingly, cytokine profiles did not differ beyond week 7 post BCG (data not shown), suggesting that the heterologous effects of BCG strain on the types of cytokines induced occurs

short-lived. Collectively, our data show that the strain used for BCG vaccination has a profound impact on the magnitude and polyfunctionality of CD4+ T cell responses to unrelated heterologous vaccine antigens although the effect is short-lived.

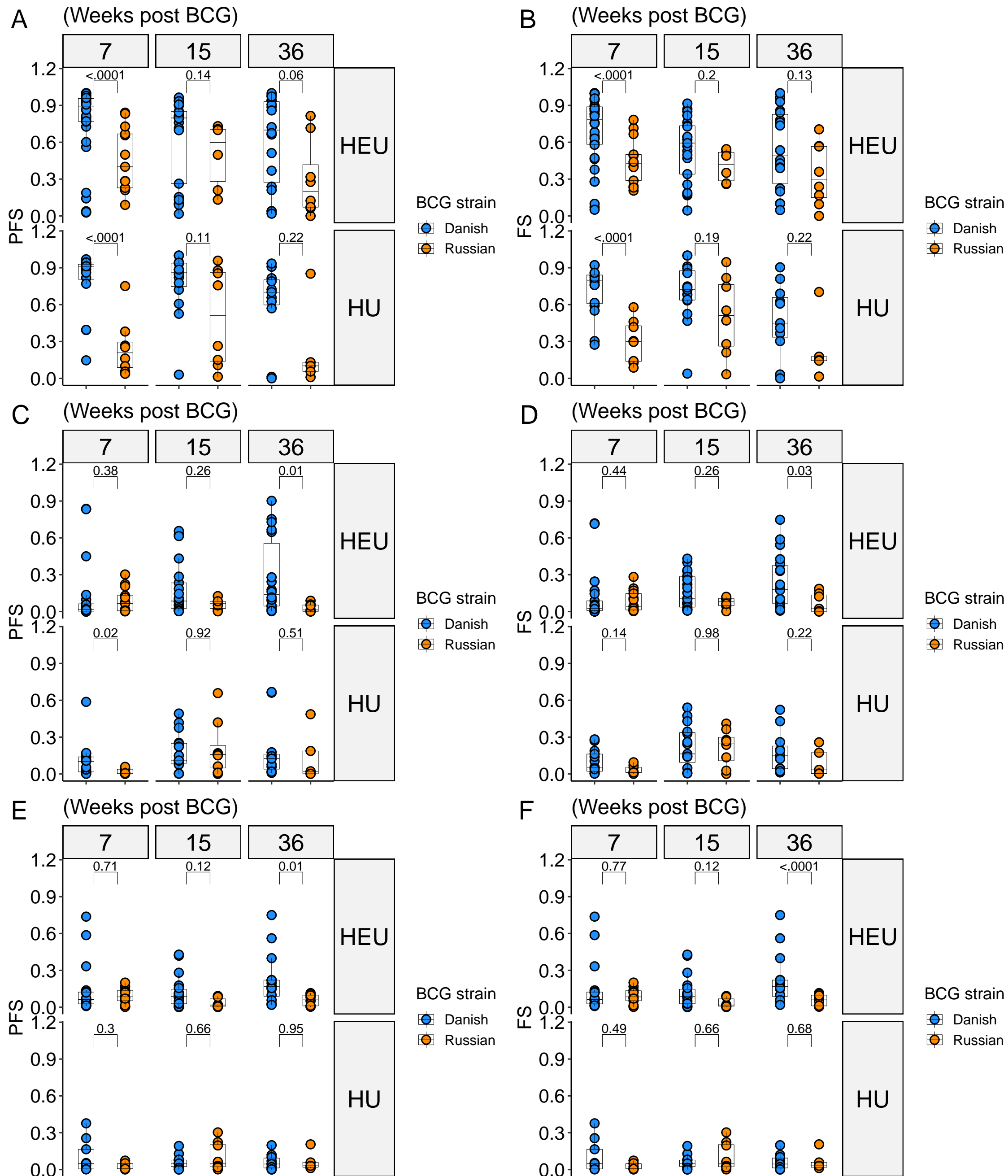

Figure 5.8: BCG immunising strain impacts on heterologous antigen responses: polyfunctional and functional scores by COMPASS. Cross-sectional analysis of heterologous antigen polyfunctional (A, C, E) and functional scores (B, D, F) between BCG-Denmark vs. BCG-Russia immunised infants. Scores are stratifed by HIV exposure status (HEU: HIV-exposed and uninfected and HU: HIV-unexposed). A & B (BCG scores), C & D (Tetanus Toxoid scores), E & F (*Bordetella pertussis* scores). Weeks indicate the time point post BCG vaccination. Y axes show the score values. Wilcoxon Rank Sum Test was used to compare strains, with p-values<0.01 were significant after adjustment for multiple comparisons.

5.4 Discussion

This chapter shows that the magnitude and polyfunctional nature of CD4+ T cell responses to BCG and other heterologous antigens in the first few months of life depends on the immunising strain of BCG itself irregardless of HIV exposure status. BCG-Denmark is the most immunogenic compared to BCG-Russia and BCG-Bulgaria strains and causes an early differentiated phenotype in memory CD4+ T cells. The latter strains appear to allow the accumulation of naïve-like responsive cells, which appear to induce mono-functional responses. The important aspect of our study is that responding CD4+ T cells to all vaccine antigens, including Tetanus and Pertussis, were significantly affected by the strain of immunising BCG, with the Denmark strain inducing the highest magnitude of responses and a Th1 polyfunctional subset. Furthermore, the relatively advanced stage of CD4+ T cell memory maturation in response to BCG-Denmark may explain the phenomena of infant cells responding with greater magnitude and Th1 polyfunction. We acknowledge that this was purely an observational study, and a confirmatory study in the form of a randomised control trial is needed. Both specific and non-specific immune responses to the BCG vaccine by strain have been shown in a randomised control trial [286], however long-term culture assays were used to measure cytokine in culture supernatant—which does not inform the cellular source of the response. Here we identify T cells as being part of the effector response that defines differences in antigenicity across BCG strains.

Prior studies have suggested that BCG-Denmark may be more immunogenic than other widely available BCG strains. A randomised control trial in Uganda administering the same BCG strains used in this study showed that BCG-Denmark induced the highest levels of IFN-γ, IL-13 and IL-5 in culture supernatants to BCG, TT and PHA stimulation [286]. The cellular source of cytokine production, however, was not measured due to the nature of the assay used. In support of our data, CD4 polyfunctionality among infants randomised to receive BCG-Denmark, -Japan and -Russia at birth showed higher polyfunctional (IFN-γ+TNF-α+IL-2+) responses

after BCG-Denmark strain immunisation [281]. Our findings are similar, except that our cohorts are in some of the highest TB endemic areas in Africa [194, 186, 126, 106] and that the early differentiated memory state may also be important for the heterologous non-BCG antigen effects we observe with BCG-Denmark. Although CD4+ Th1 cytokine responses are not cognate correlates of protection against Tetanus and Pertussis, they are likely important measures of overall immune responsiveness to heterologous antigens. BCG itself has well documented "training" effects where enhanced monocyte mediated responses against other pathogens are central [291, 285]. The molecular mechanisms are thought to include recognition by the NOD2 receptor in monocytes, the induction of pro-inflammatory cytokines—3 months after vaccination—and the epigenetic reprogramming of monocyte populations [117, 285]. Heterologous effects of BCG vaccination on Th1 and Th17 immunity have been shown to persist for up to a year after vaccination [287]. The immunological basis for different heterologous effects by BCG strain is not known. The attenuation of BCG is the result of the loss of virulent elements in the genome, with some strains having more regions of deletions than others [292, 280, 293]. BCG-Denmark, a member of the DU2-III group, has more deletions than both BCG-Russia and BCG-Bulgaria from group DU2-I [280]. The better quality and quantity of responses induced by BCG-Denmark are unlikely-related to antigenic coverage as this strain has less T cell epitopes than BCG-Russia [293]. Certain BCG strains are known to replicate and persist longer in tissues after immunisation in animal models [294, 277] and this may result in different antigen priming between strains. However, there are most likely numerous factors other than antigen load, as BCG-Russia has been found to replicate and persist in tissues of immunised mice over and above BCG-Denmark related strains (BCG-Prague and BCG-Glaxo strains) [294]. There are likely numerous possible reasons for differences in vaccine immunogenicity between CT and Jos. We do not regard the six-fold lower CFU dose of BCG-Bulgaria, compared with BCG-Denmark/Russia, as a contributing factor (Table 7.3). Suboptimal vaccination

doses affect the magnitudes of Th1 responses in adults [295], but not infants [296]. Differences in immune responses between cohorts may also depend on population differences; i.e. environmental antigen exposure and/or genetics [276, 17, 32]. For example helminth and malaria co-infections, which are common in Nigeria, are associated with IL-10 responses and a skewing to Th2 response to Mtb [40], but we did not evaluate helminth infections in our infant cohort. Shipping of cells from Nigeria to South Africa may have impacted on their integrity. However, our finding that BCG-Russia—which is closely related to BCG-Bulgaria [297]—also resulted in a similar set of low magnitude and polyfunctional responses in CT infants led us to regard BCG strain differences as a main determinant of vaccine immunogenicity. Finally, although the proportion of HIV-exposed infants was unbalanced between the groups, this would have favoured better responses in the BCG-Bulgarian infants. In 2016, many countries around the world experienced a shortage of BCG-Denmark and had to transition to other strains [298]. Our results suggest that BCG vaccination, given to over 100 million infants annually around the globe, has variable immunogenicity according to strain, and that heterologous effects may be specific to BCG-Denmark. We do not discount the possibility that differences between strains may be related to the number of live replicating bacilli—which may be different across manufacturers. These findings have implications for vaccine policy makers, manufacturers and programs worldwide. These findings also suggest that BCG-Denmark, the first vaccine received in many African infants, has both specific and non-specific heterologous effects to HEUs in the first few months of life, and may provide an immune priming benefit to other EPI vaccines.

Chapter 6

Thesis summary and conclusions

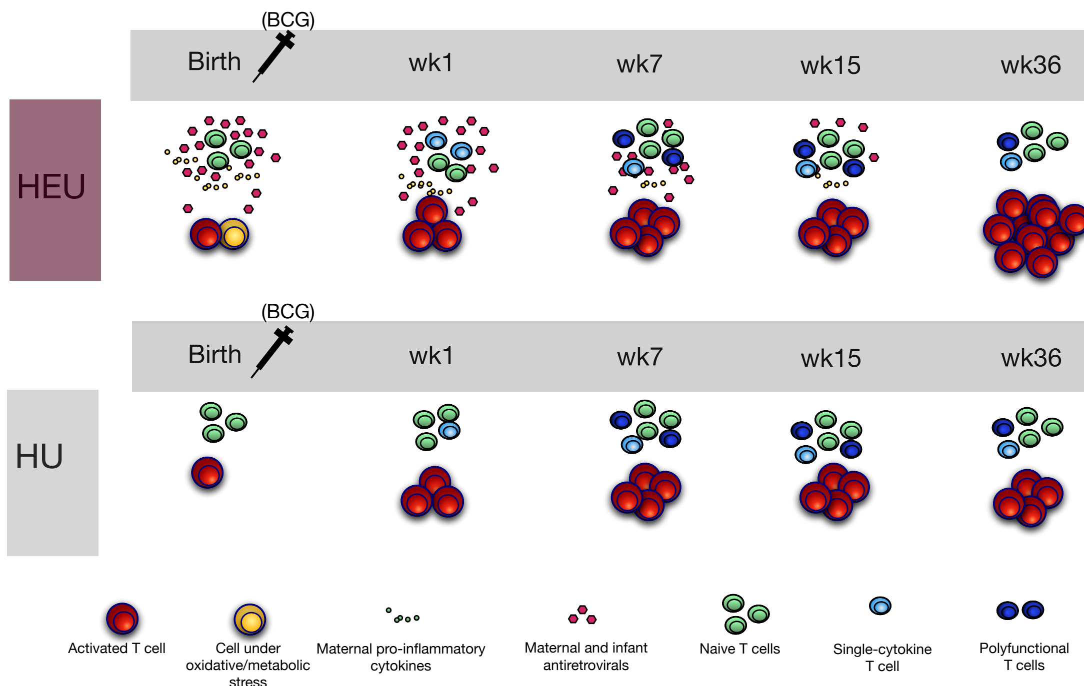

Figure 6.1: Synopsis of thesis. We hypothesised that as a result of exposure to maternal pro-inflammatory signals and ART drug regimens *in utero* and postpartum, the immune milieu of children born to HIV infected mothers (cHEU) is (*i*) activated, (*ii*) has an altered transcriptional profile and (*ii*) is functionally perturbed in response to vaccination. At birth, two gene homologues synonymous with cellular stress were increased in peripheral blood among HEUs, with no significant enrichment or suppression of biological pathways. For BCG vaccine moderate differences in single cytokine producing cells were observed one week after vaccination in those with detectable immune responses. Later however (weeks 7-36), BCG immunity was indistinguishable from un-exposed infants in both magnitude and polyfunctional phenotypes concomitant with hypothesized lower ART exposure (neonatal and via breast milk). Most activated T cell phenotypes followed similar kinetics, however increased activated CD8 T cells (expressing CD25 or HLA-DR) were elevated in HEUs and in particular at week 36 of age.

6.1 Thesis summary

This thesis began in Chapter 2 with the exploration of possible transcriptomic signatures in blood that distinguish cHEU from cHU controls at birth. A small set of differentially expressed genes were identified but did not translate to any differences in gene pathways in the HEU. We then explored the hyothesis that cHEUs would have different frequencies of activated, proliferating or gut homing T cells compared to HU in Chapter 3. On the whole, the kinetics in the frequencies of T cell phenotypes followed similar patterns of peaking and waning between HIV exposure groups between birth and 36 weeks. Two markers were significantly increased in CD8 T cells of HEU across all time-points: HLA-DR and CD25, the latter significantly at 36 weeks in cross-sectional analyses. In Chapter 4, we explored whether T cell function was perturbed in the HEU by analysing the magnitudes and cytokine profiles of T cells responding to vaccine antigen using a short-term *in vitro* assay. There was a trend towards differences in quality of vaccine-specific T cell responses by HIV exposure status, depending on the stimulating antigen, however these did not hold when applying the more robust COMPASS algorithm. Two key observations prompted a further interrogation of BCG responses and was the focus of Chapter 5: (*i*) BCG response magnitudes were differed by cohort site and (*ii*) CT infants had been vaccinated with one of two strains of BCG (Denmark or Russian), whereas Jos infants had received the Bulgarian strain—genetically identical to the Russian strain. We hypothesized that the immunising strain of BCG resulted in different BCG response profiles and therefore stratified BCG response magnitudes and cytokine profiles to the strains used to immunise infants. BCG-Denmark immunisation in CT infants resulted in higher mycobacterial-specific CD4 responses with greater polyfunctional profiles and more memory differentiated BCG-specific phenotype compared to either CT infants receiving BCG-Russia or Jos infants receiving BCG-Bulgaria strains. Furthermore, there were heterologous effects of BCG Denmark, meaning that this immunogenic strain extended to boosting the non-mycobacterial antigens of TT

and BP (as well as the PHA positive control stimulus). These observations were independent of HIV exposure status.

6.1.1 Limitations of the study

A major limitation of this study was the use of whole blood to address the thesis aims regarding immune ontogeny in the HEU infant. Peripheral blood is relatively non-invasive can be cryopreserved for batch measurements and has a wide range of commercially available reagent toolkits. There is however, discordance between peripheral blood and sites of infection/disease in terms of immune subsets, functional profiles, differentiation states and thresholds for antigenic stimulation [299, 300, 301, 302, 303]. Another limitation was that flow cytometry design constraints only allowed us to measure Th1 cytokines in our whole blood assay. Given Th2 skweing in early life [1] and that all antigens in this study can induce Th2 responses [276, 304], we missed a more comprehensive evaluation of T cell function in the HEU. In addition, some samples from the Jos cohort were compromised, possibly during shipping or due to technical error during processing. This limited our sample sizes from this cohort in some assays.

6.1.2 Concluding remarks

The sites in which these study cohorts were derived have extensive HIV care, counselling and management services for women during pregnancy and post-partum [305, 306]. Both have implemented WHO's Option B+ which is lifelong care for all HIV pregnant women regardless of CD4 count. In addition, maternal breast feeding rates among HIV infected women were high in both sites [98], meaning that HIV exposed babies benefited from nutrition and passive immunity provided by breast-milk. Therefore, our study populations may represent some of the 'healthier' HIV exposed infant cohorts compared to previous cohorts [307]. Equal rates of infectious morbidities between HEU and HU groups were previously demonstrated in

the same cohorts used here (hazard ratio 1.01; 95% CI 0.78 - 1.32) [98]. Thus, in the context of our specific cohort, this thesis can surmise that: (*i*) *in utero* ART/HIV exposure does not perturb immune pathways HEU newborn, (*ii*) ART/HIV exposure does not significantly alter vaccine-specific T cell responses over the first 9 months of life, (*iii*) ART/HIV exposure induces an activated T cell phenotype compared to un-exposed controls during the first 9 months of life.

The negative results for gene transcription and functional immunity presented here demonstrate that effective ART has no deleterious consequences for the general immune biology in the HEU and results in a parity of T cell vaccine immunity with unexposed infants. We rather showed that the immunising strain of BCG had significant mycobacterial and off-target effects in the HEU. The effect of BCG "trained immunity" to off-target has been well-documented in animal and human models [285, 308, 287, 309]. Whether the strain of BCG administered will ultimately mean disproportionate levels of protection to other infections in HEU can only be determined by large, randomised control trials with well-defined clinical endpoints. Our observation that bulk CD8 cells from HEUs have an activated phenotype relative to HUs is in line with previous reports on activated immune subsets in HEUs (Table 3.5). This warrants further investigation as to the underlying mechanisms and *in vitro* experiments where PBMCs would be exposed to escalating doses of ART, for example, could partially address this. Importantly, whether *in vivo* CD8 activation levels represent a correlate of infection risk in HEUs beyond the first year of life should be investigated in observational studies. Overall, this thesis provides evidence for immune competence in our cohorts and demonstrates how beneficial Option B+ is to the health of infants during the first year of life.

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Chapter 7

Appendix

Table 7.1:
Combined study participant characteristics

	HEU, N=137	**HUU, N=57**	***P*-values**
Infant			
Gestation, weeks	38 (37 - 39)	38 (38 - 39)	0.55
Birth weigh, g	3025 (2778 - 3330)	2930 (2840 - 3230)	0.95
Length, cm	48 (46 - 50)	48 (47 - 50)	0.40
% Female	58	32	0.20
Maternal			
Weight, kg	70 (63 - 79)	70 (63 - 75)	0.55
Age, years	29 (25 - 33)	26 (23 - 30)	0.05
CD4, cells/mL	447 (316 - 561)	n/a	
% EBF to 6 months	81	88	0.18

Numbers indicate median and (IQR) unless otherwise stated. *P*-values calculated using Wilcoxon Rank Sum test. Chi-squared test of homogeneity was used to compared distribution of gender and exclusive breastfeeding rates (% EBF) between exposure groups.

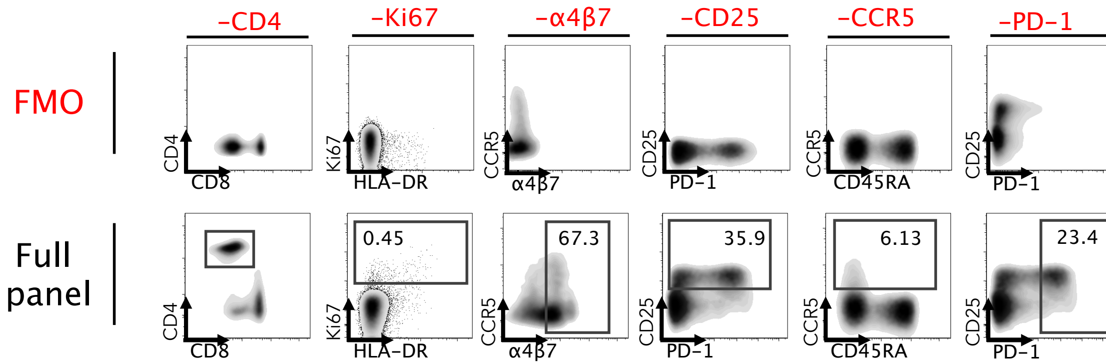

Figure 7.1: Fluorescence minus one controls for activation/proliferation marker gating

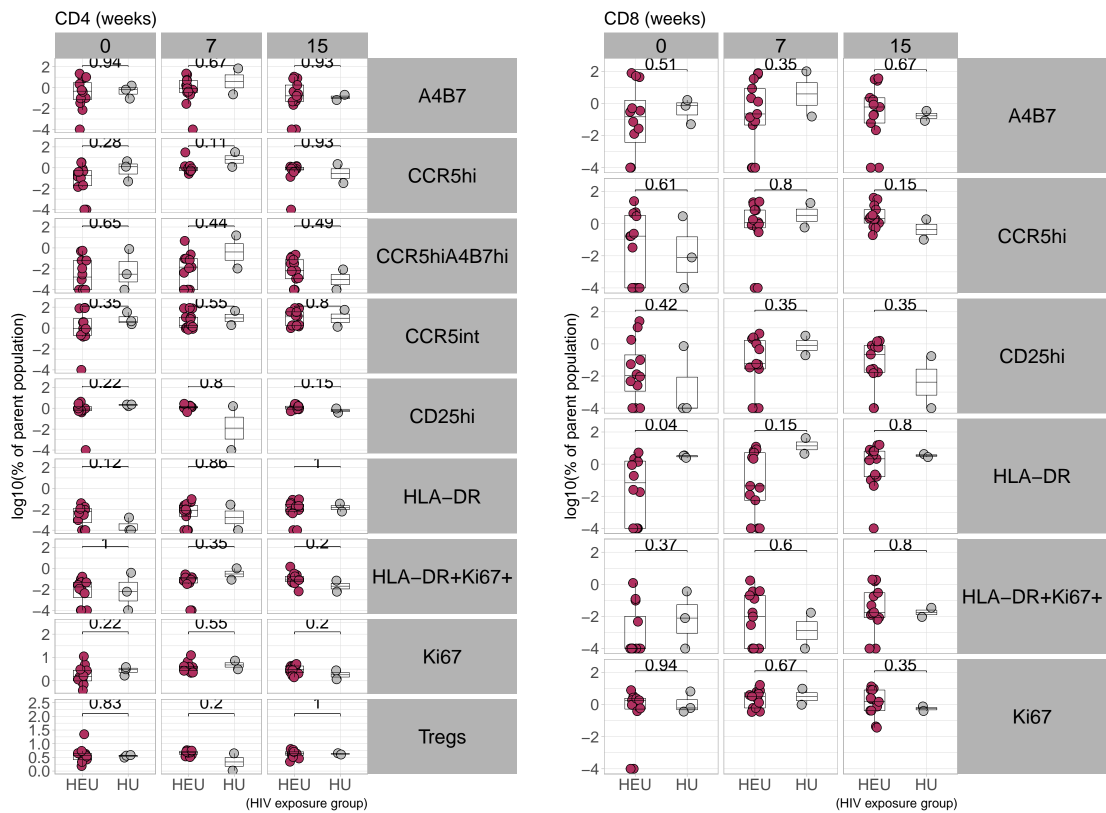

Figure 7.2: T cell markers of activation, proliferation and gut homing in Jos infants. Ex vivo markers in matched samples were measured from birth to 36 weeks for cHEU (maroon circles, N=11) and cHU (grey circles, N=16) and are expressed as a percentage of the parent T cell population (CD4 or CD8). A4B7 = α4β7. CCR5hiA4B7hi= CCR5hi+α4β7hi (potential HIV target cells). CD25 were CD127hi and Treg-like cells were CD25+CD127lo. A paired Wilcoxon Ranked sum test was used to compare expression between CD4 and CD8 populations. P values<0.001 were significant after adjusting for multiple comparisons using the Holm's step down method.

Table 7.2: Results of post-hoc Friedman test comparing expression of T cell markers between time intervals

CD4+	Age interval	group 1	*p*-adj 1	group 2	*p*-adj 2	CD8+	Age interval	group 3	*p*-adj 3	group 4	*p*-adj 4
CCR5hi	0-7	HEU	0.02	HU	<0.001		0-7	HEU	0.06	HU	0.06
	0-15	HEU	0.02	HU	0.08		0-15	HEU	0.03	HU	0.001
	0-36	HEU	0.003	HU	0.19		0-36	HEU	0.006	HU	0.26
	15-36	HEU	0.94	HU	0.98		15-36	HEU	0.94	HU	0.22
	7-15	HEU	1	HU	0.35		7-15	HEU	1	HU	0.57
	7-36	HEU	0.94	HU	0.19		7-36	HEU	0.87	HU	0.92
CD25	0-7	HEU	NA	HU	NA		0-7	HEU	0.14	HU	NA
	0-15	HEU	NA	HU	NA		0-15	HEU	0.65	HU	NA
	0-36	HEU	NA	HU	NA		0-36	HEU	0.02	HU	NA
	15-36	HEU	NA	HU	NA		15-36	HEU	0.77	HU	NA
	7-15	HEU	NA	HU	NA		7-15	HEU	0.87	HU	NA
HLA-DR	0-7	HEU	NA	HU	0.01		0-7	HEU	0.06	HU	0.30
	0-15	HEU	NA	HU	0.19		0-15	HEU	0.09	HU	0.02
	0-36	HEU	NA	HU	<0.001		0-36	HEU	0.001	HU	0.04
	7-15	HEU	NA	HU	0.69		15-36	HEU	0.52	HU	1
	7-36	HEU	NA	HU	0.80		7-15	HEU	1	HU	0.63
	15-36	HEU	NA	HU	0.19		7-36	HEU	0.65	HU	0.80
	7-36	HEU	NA	HU	. 0.80		7-36	HEU	0.30	HU	0.80
Ki67	0-7	HEU	0.02	HU	0.003		0-7	HEU	NA	HU	NA
	0-15	HEU	0.01	HU	0.26		0-15	HEU	NA	HU	NA
	0-36	HEU	0.004	HU	0.80		0-36	HEU	NA	HU	NA
	15-36	HEU	0.98	HU	0.80		15-36	HEU	NA	HU	NA
	7-15	HEU	1	HU	0.35		7-15	HEU	NA	HU	NA
	7-36	HEU	0.96	HU	0.05		7-36	HEU	NA	HU	NA
HLA-DR+Ki67+	0-7	HEU	0.03	HU	0.001		0-7	HEU	0.14	HU	0.03
	0-15	HEU	0.003	HU	0.05		0-15	HEU	0.001	HU	<0.001
	0-36	HEU	0.007	HU	0.02		0-36	HEU	0.006	HU	0.02
	15-36	HEU	1	HU	0.98		15-36	HEU	0.94	HU	0.58
	7-15	HEU	0.90	HU	0.69		7-15	HEU	0.30	HU	0.46
	7-36	HEU	0.96	HU	0.89		7-36	HEU	0.65	HU	1
α4β7hi	0-7	HEU	0.042	HU	0.13		0-7	HEU	0.02	HU	NA
	0-15	HEU	0.13	HU	0.26		0-15	HEU	0.41	HU	NA
	0-36	HEU	0.01	HU	0.002		0-36	HEU	0.06	HU	NA
	15-36	HEU	0.82	HU	0.26		15-36	HEU	0.53	HU	NA
	7-15	HEU	0.96	HU	0.98		7-15	HEU	0.98	HU	NA
	7-36	HEU	0.98	HU	0.46		7-36	HEU	0.77	HU	NA
CCR5hi+α4β7hi+	0-7	HEU	0.06	HU	0.01		0-7	HEU	0.04	HU	0.01
	0-15	HEU	0.03	HU	0.08		0-15	HEU	0.06	HU	0.08
	0-36	HEU	0.005	HU	<0.001		0-36	HEU	0.001	HU	0.02
	15-36	HEU	0.94	HU	0.19		15-36	HEU	0.59	HU	0.95
	7-15	HEU	1	HU	0.89		7-15	HEU	1	HU	0.89
	7-36	HEU	0.87	HU	0.58		7-36	HEU	0.65	HU	1
PD-1	0-7	HEU	0.53	HU	0.58		0-7	HEU	0.80	HU	NA
	0-15	HEU	0.006	HU	0.003		0-15	HEU	0.03	HU	NA
	0-36	HEU	0.001	HU	0.002		0-36	HEU	0.26	HU	NA
	15-36	HEU	0.94	HU	1		15-36	HEU	0.26	HU	NA
	7-15	HEU	0.21	HU	0.13		7-15	HEU	0.80	HU	NA
	7-36	HEU	0.06	HU	0.08		7-36	HEU	0.80	HU	NA
Treg-like	0-7	HEU	0.06	HU	0.001		0-7	HEU	ND	HU	ND
	0-15	HEU	0.09	HU	0.08		0-15	HEU	ND	HU	ND
	0-36	HEU	0.002	HU	0.01		0-36	HEU	ND	HU	ND
	15-36	HEU	0.05	HU	0.89		15-36	HEU	ND	HU	ND
	7-15	HEU	1	HU	0.58		7-15	HEU	ND	HU	ND
	7-36	HEU	0.65	HU	0.95		7-36	HEU	ND	HU	ND

P-adjusted values are post-hoc *p*-values following a Friedman test. Age intervals listed are in weeks. If overall *p*-values for a particular marker were not significant, then values are listed as NA (not applicable). ND = Not determined.

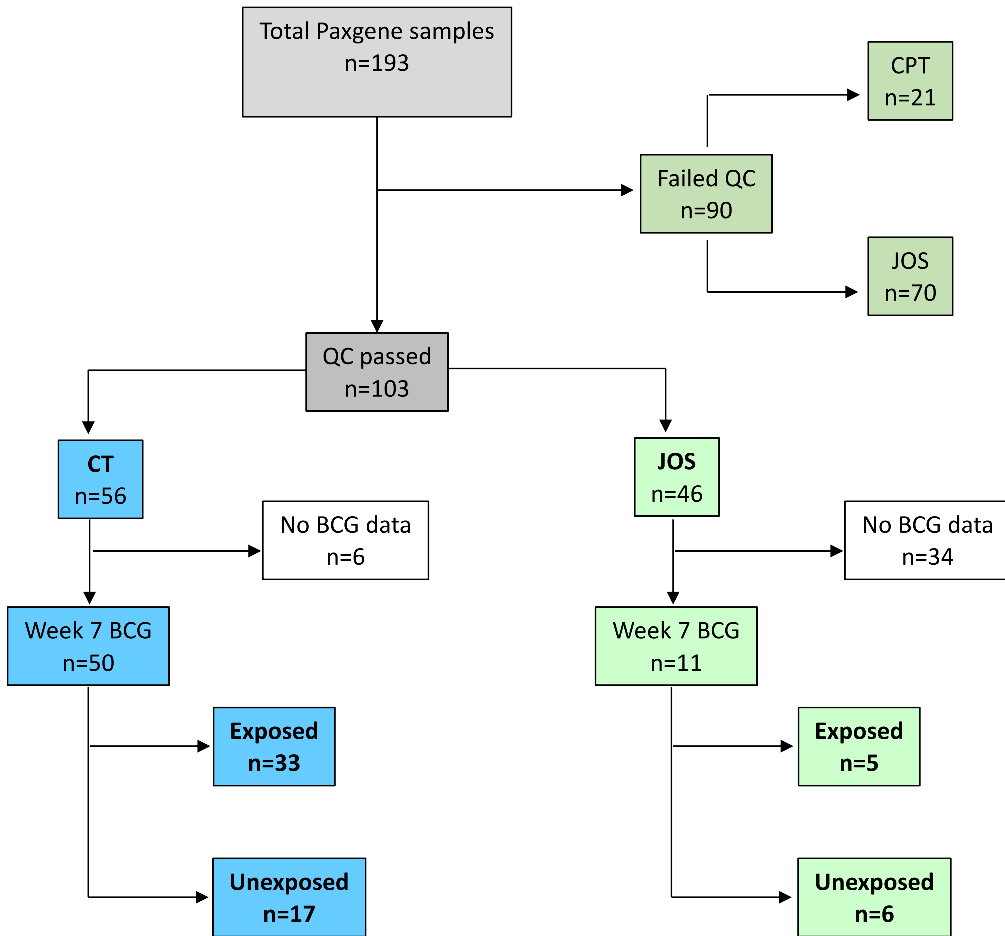

Figure 7.3: Selection of birth whole blood samples for RNA Seq analyses.

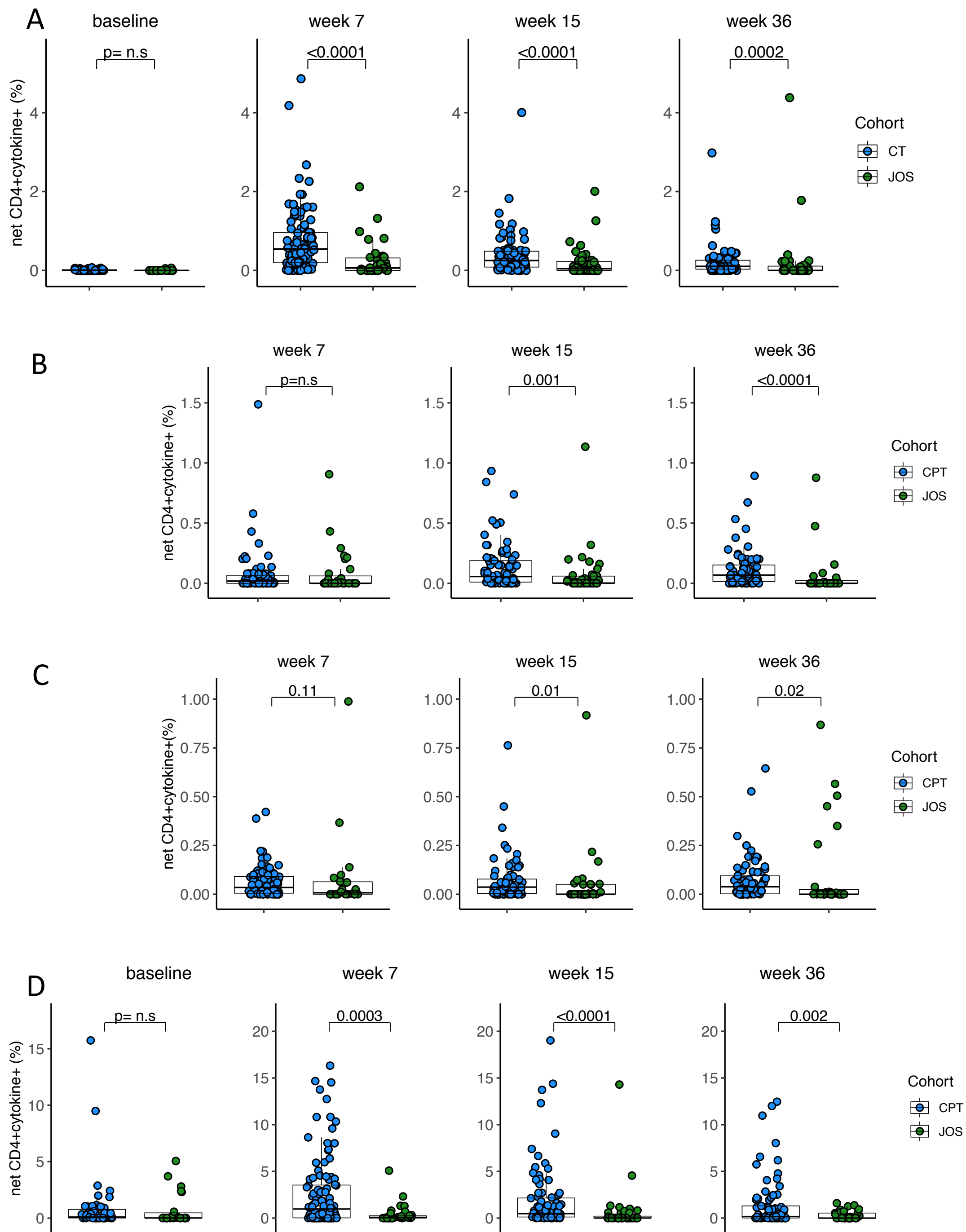

Figure 7.4: Comparison of vaccine responses by cohort. Infants for each cohort were grouped irrespective of HIV exposure. (A) BCG, (B) Tetanus Toxoid, (C) *Bordetella Pertussis* and (D) PHA responses are shown for CD4 cells. A Wilcoxon Ranked Sum test was applied to test differences by cohort and $P<0.01$ were considered significant after multiple comparisons correction.

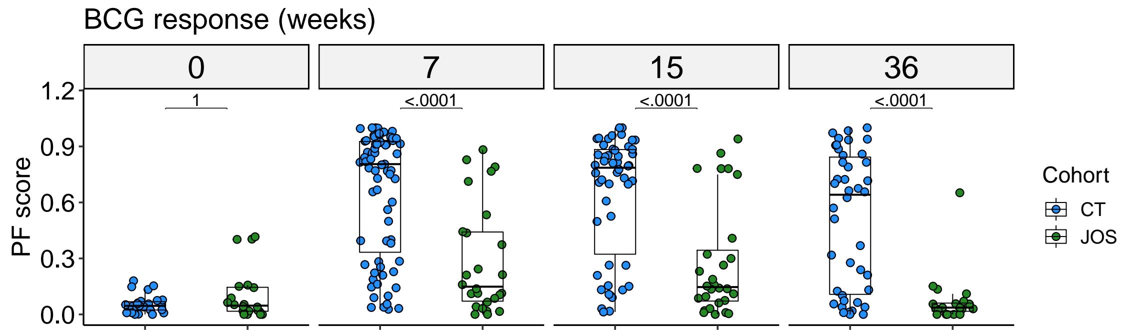

Figure 7.5: Comparison of COMPASS polyfunctional scores for BCG responses. Infants for each cohort were grouped irrespective of HIV exposure. A Wilcoxon Ranked Sum test was applied to test differences by cohort. $P<0.01$ were considered significant after multiple adjustment correction using Holm's step down method.

Table 7.3: Vaccine and whole blood assay antigen dose

Cohor	Vaccine dose	*In vitro* antigen concentration
CT	Denmark 1331 (~2 x 10^5 CFU)	Denmark 1331(~12 x 10^5 CFU/mL)
	Tetanus Toxoid (>40 IU)	Tetanus Toxoid antigen (5 IU/mL)
	Acellular Pertussis (25μg)	Bordetella antigens (0.1%v/v)
Jos	Bulgaria SL 222 Sofia (~0.3 x 10^5 CFU)	Bulgaria SL 222 Sofia (144 CFU/mL)
	Tetanus Toxoid (>40 IU)	Tetanus Toxoid antigen (5 IU/mL)
	Whole cell Pertussis (>4.0IU)	Bordetella antigens (0.1%v/v)

Numbers represent approximate *in vivo* vaccine dose per infant and *in vitro* antigen concentration per mL of whole blood in whole blood assay.

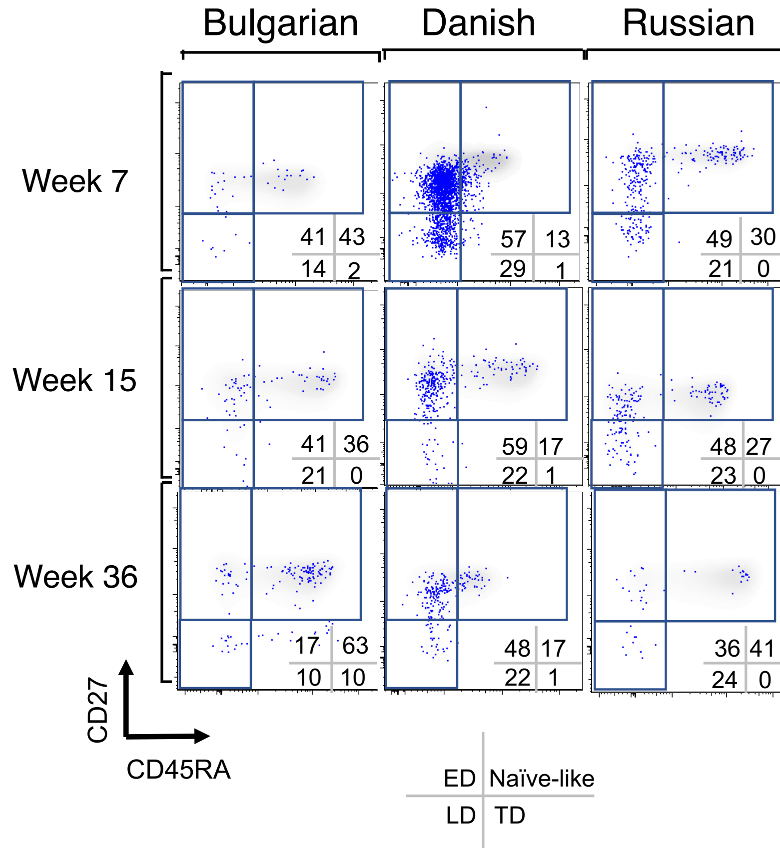

Figure 7.6: BCG-Denmark induces a more differentiated T cell memory phenotype compared to BCG-Russia or BCG-Bulgaria strains. Representative flow cytometry plots showing the memory profile of mycobacterial-specific (cytokine positive) CD4 cytokine responses stratified by BCG immunizing strain. Axes show CD27 vs. CD45RA expression and blue dots are cytokine+ cells responding to BCG overlaid against a background of total CD4+ cells. CD45RA+CD27+ represent naïve-like, CD45RA-CD27+ early differentiated (ED), CD45RA-CD27- late differentiated (LD) and CD45RA+CD27- terminally differentiated (TD) phenotypes.

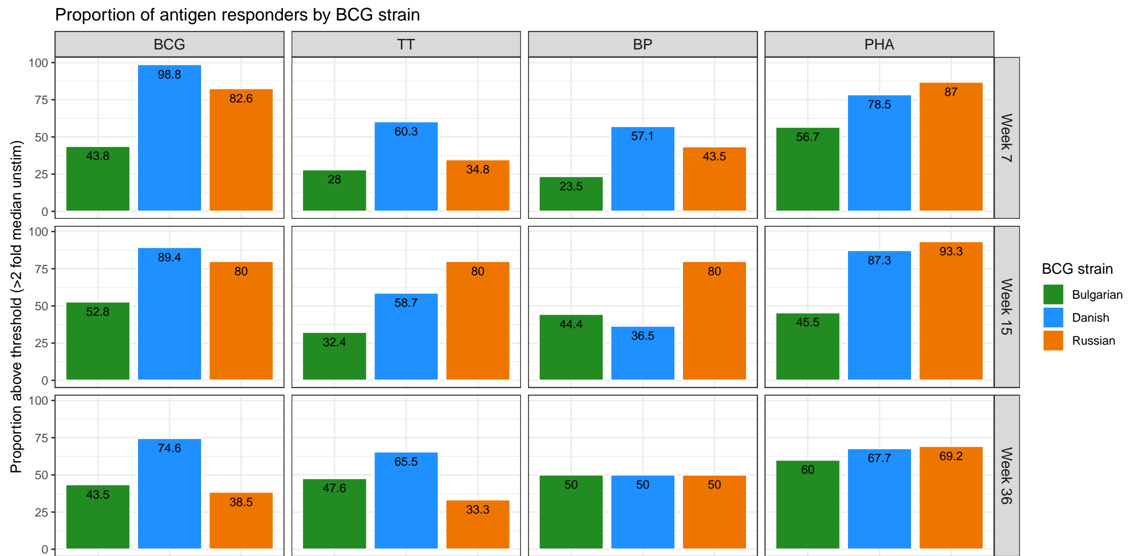

Figure 7.7: Proportion of antigen responders by BCG immunising strain. Bar plots show the frequency of responders defined as >2-fold above background (unstimulated CD4+cytokine frequency) for each of the antigens measured in the whole blood assay.