EARLY LIFE IMMUNE ONTOGENY IN A VULNERABLE POPULATION

by

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ABSTRACT

The first year of life represents a time of marked susceptibility to infections, particularly in resource-poor regions and regions where HIV is endemic, such as sub-Saharan Africa. Infants that are exposed to HIV are among the World's most vulnerable to infectious morbidity and mortality. Immune defense mechanisms may differ between global regions with high rates of childhood death and disease. This dissertation examines innate immune development throughout the first year of life in HIV unexposed (UE) South African infants, and compares their immune development with HIV-exposed uninfected (HEU) South African infants. Clinical outcomes and adaptive immune responses to vaccination were also compared between HEU and UE infants. Specifically, infection incidence and severity were correlated with multidimensional assessments of immune profiles, including innate immune responses to pathogen-associated stimuli, as well as antibody responses to vaccination, all contrasting HEU and UE infants.

I found that monocyte, classical dendritic cell and plasmacytoid dendritic cell responses in our cohort were mostly similar to responses measured in other global regions. However, responses to endotoxin matured more rapidly in South African infants. HEU mononuclear cells demonstrated enhanced pro-inflammatory responses when compared to UE, particularly during the first 6 weeks of life, and altered responses were specific to pathogen type. HEU vaccine responses were similar, but in some instances developed protective titers more rapidly than UE.

Incidence of infection was equivalent between HEU and UE. However, HEU infants experienced more severe infections, and peak relative risk occurred within the first 12 months of life.

This cohort study represents the first prospective, longitudinal description of immune ontogeny throughout the first year of life. My PhD dissertation provides a thorough examination of innate immune development during this time period. HEU are a population that suffers disproportionately from infectious disease, and our findings suggest HEU infants have altered immune defense mechanisms. This collection of work provides a foundation to mechanistically dissect etiological factors that predispose high-risk groups, such as HEU infants, to suffer increased infectious morbidity and mortality.

PREFACE

Apart from Chapters 1 and 6, which comprise the General Introduction and Discussion, respectively, this dissertation's chapters (2-5) and Appendix A are comprised of published manuscripts that have been modified to facilitate their inclusion into a unified document. The titles, authorship and the authors' respective contributions for these published works are as follows:

Chapter 2: A version of Chapter 2 has been published [Amy L. Slogrove, **Brian A. Reikie**, Shalena Naidoo, Corena de Beer, Kevin Ho, Mark F. Cotton, Julie Bettinger, David P. Speert, Monika Esser & Tobias R. Kollmann. **HIV exposed uninfected infants are at increased risk for severe infections in the first year of life**. ***Journal of Tropical Pediatrics*** **2012** Dec;58(6):505-8. This manuscript is used with permission from Slogrove et al. (2012) of which I am an author. With the supervisory authors, T.R.K. and M.E., I was a lead investigator for the overall cohort study, responsible for conceptualization of the cohort on which the manuscript was based, acquiring ethics approval, and to assume a leading role in forming the physical cohort. Alongside M.E., I was a team leader who managed the cohort study, patient recruitment, transportation and clinic visits. With C.d.B., I organized and participated in the acquisition of biological samples and clinical data. A.S. and J.B. provided the primary conceptual input for analyzing the clinical data comprising this manuscript. I assisted A.S. and T.R.K in analyzing the data, and in writing and editing the manuscript. S.N and K.H. significantly contributed to data collection, data

management and data analysis. M.F.C. and D.P.S. were instrumental in the realization of the cohort study, and provided significant intellectual contributions for optimal interpretation and presentation of data.

Chapter 3: A version of Chapter 3 has been published [**Brian A. Reikie**, Adams RC, Ruck CE, Ho K, Leligdowicz A, Pillay S, Naidoo S, Fortuno ES 3rd, de Beer C, Preiser W, Cotton MF, Speert DP, Esser M, Kollmann TR. **Ontogeny of Toll-like receptor mediated cytokine responses of South African infants throughout the first year of life**. ***PLoS One*** **2012**;7(9):e44763. In collaboration with the supervisory authors, T.R.K and M.E., I was a lead investigator, responsible for all major areas of concept formation, cohort development, study coordination, data collection and analysis, as well as manuscript composition. R.A., C.E.R., K.H., A.L., S.P., S.N., E.S.F. and C.d.B. significantly contributed to various aspects of data collection, data management and data analysis. W.P., M.F.C. and D.P.S., were instrumental in the realization of the cohort study, and provided significant intellectual contributions for optimal interpretation and presentation of data.

Chapter 4: A version of Chapter 4 has been published [**Brian A. Reikie**, Adams RC, Leligdowicz A, Ho K, Naidoo S, Ruck CE, Pillay S, de Beer C, Preiser W, Cotton MF, Speert DP, Esser M & Kollmann TR. **Altered innate immune development in HIV-exposed uninfected infants**. J Acquir Immune Defic Syndr. 2014 Jul 1;66(3):245-55. In collaboration with the supervisory authors, T.R.K and M.E., I was a lead investigator, responsible for all major areas of concept formation, cohort

development, study coordination, data collection and analysis, as well as manuscript composition. R.C.A., A.L., K.H., S.N., C.E.R., S.P. and C.d.B. significantly contributed to data collection, data management and data analysis. W.P., M.F.C. and D.P.S., were instrumental in the realization of the cohort study, and provided significant intellectual contributions for optimal interpretation and presentation of data.

Chapter 5: A version of chapter 5 has been published [**Brian A. Reikie**, Naidoo S, Ruck CE, Slogrove AL, de Beer C, la Grange H, Adams RC, Ho K, Smolen K, Speert DP, Cotton MF, Preiser W, Esser M, Kollmann TR. **Antibody responses to vaccination among South African HIV-exposed and unexposed uninfected infants over the first 2 years of life**. ***Clin Vaccine Immunol*** **2013** Jan:20(1):33-8. In collaboration with the supervisory authors, T.R.K and M.E., I was a lead investigator, responsible for all major areas of concept formation, cohort development, study coordination, data collection and analysis, as well as manuscript composition. S.N. significantly contributed to coordinating clinical visits, biological sample collection and data analysis. A.S. contributed to conceptualization of the study and interpretation of results. C.d.B., H.l.G., R.A., K.H. and S.K. performed data collection, facilitated data management and assisted with data analysis. D.P., M.F.C. and W.P. were instrumental in the realization of the cohort study, and provided significant intellectual contributions for optimal interpretation and presentation of data.

Appendix A: A version of Appendix A has been published [Kirstin Jansen, Darren Blimkie, Jeff Furlong, Adeline Hajjar, Annie Rein-Weston, Juliet Crabtree, **Brian**

Reikie, Christopher Wilson, and Tobias Kollmann. **Polychromatic flow cytometric high-throughput assay to analyze the innate immune response to Toll-like receptor stimulation**. ***Journal of Immunological Methods*** **2008** Jul 31;336(2): 183-92]. This manuscript is used with permission from Jansen et al. (2008) of which I am an author. This manuscript communicated new methods in immune analysis, which I assisted in developing. With D.B., I carried out the assays and gathered the data included in this manuscript. Data analyzed by K.J. was transferred to me to generate each figure. I assisted in editing the completed manuscript. This manuscript communicates the development of assays that provided the foundation for the immune analysis component of this dissertation.

Peer reviewed, published manuscripts authored by Brian Reikie, which are not directly incorporated in this dissertation, but are referenced within, and were foundational for our understanding of early life immune development and thus for the development of this dissertation, are as follows:

Brian A. Reikie, Kinga K. Smolen, Edgardo S. Fortuno III, Daniela I.M. Loeffler, Bing Cai, Darren Blimkie, and Tobias R. Kollmann. **A Single Immunization Near Birth Protects Immediately and For Life**. ***Vaccine*** **2010** Dec 10;29(1):83-90.

Kinga K. Smolen, Daniela I.M. Loeffler, **Brian A. Reikie**, Laura Aplin, Bing Cai, Edgardo S. Fortuno III, and Tobias R. Kollmann. Neonatal immunization with Listeria monocytogenes induces T cells with an adult-like avidity, sensitivity, and

TCR-V<beta> repertoire, and does not adversely impact the response to boosting. *Vaccine* 2009 Dec 10;28(1):235-42.

Tobias R. Kollmann, **Brian Reikie**, Darren Blimkie, Sing Sing Way, Adeline M. Hajjar, Kiea Arispe, Angela Shaulov, and Christopher B. Wilson. **Induction of Protective Immunity to *Listeria monocytogenes* in Neonates. *Journal of Immunology* 2007** Mar 15;178(6):3695-701.

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LIST OF ABBREVIATIONS

APC	antigen presenting cell
AIDS	acquired immunodeficiency syndrome
ART	antiretroviral therapy
ARV	antiretroviral
BCG	*Mycobacterium bovis* bacillus Calmette Guerin
BD	Becton Dickinson
BFA	brefeldin A
Bp	*Bordetella pertussis*
CAP	community acquired pneumonia
cART	combination antiretroviral therapy
CD	cluster of differentiation
cDC	conventional (or classical) dendritic cell
CI	confidence interval
CpG	cytosine-phosphodiester-guanine oligodeoxynucleotides
DAIDS	Division of AIDS
DNA	deoxyribonucleic acid
DOHaD	developmental origins of health and disease
dsRNA	double stranded ribonucleic acid
DTaP-IPV	diphtheria/tetanus/acellular pertussis/inactivated polio vaccine (combined)
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPI	expanded program for immunization
FACS	fluorescence-activated cell sorting
HBsAg	hepatitis B surface antigen
HepB	hepatitis B vaccine
HEU	HIV exposed but uninfected
Hib	*Haemophilus influenzae* type B vaccine
HIV	human immunodeficiency virus
ICC	intracellular cytokine cytometry
ICS	intracellular cytokine stimulation
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP-10	interferon-g inducible protein 10

IRAK	interleukin-1 receptor associated kinase
LAZ	length-for-age Z-score
LPS	lipopolysaccharide (also known as endotoxin)
LRTI	lower respiratory infection
MBL	mannose binding lectin
MCP	monocyte chemoattractant protein
mDC	myeloid dendritic cell
MDG	millennium development goal
Men-C	meningococcal C vaccine
MFI	mean fluorescence intensity
MHC	major histocompatability complex
MIFlowCyt	minimum information about a flow cytometry experiment
MIP	macrophage inflammatory protein
MyD88	myeloid differentiation primary response gene 88
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	cells natural killer cells
NLR	NOD-like receptor
NOD	nucleotide-binding oligomerization domain
OPV	oral polio vaccine
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PBSAN	phosphate buffered saline with BSA and sodium azide
PCR	polymerase chain reaction
PCV	pneumococcal conjugated vaccine
pDC	plasmacytoid dendritic cell
PFD	polyfunctional degree
PGN	peptidoglycan
PGNSA	peptidoglycan from *Staphylococcus aureus*
pI:C	polyinosinic:polycytidylic acid
PJP	*Pneumocystis jirovecii* pneumonia
PMN	polymorphonuclear
PMTCT	prevention of mother to child transmission
PRR	pattern recognition receptor
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute media

RR	relative risk
RV	rotavirus vaccine
SEM	standard error of the mean
ssRNA	single stranded ribonucleic acid
TAH	Tygerberg academic hospital
TB	tuberculosis
TCR	T-cell receptor
Td	tetanus and reduced strength diphtheria vaccine
TDaP	tetanus, diphtheria and acellular pertussis vaccine
Th	T-helper cell
TIR	TLR/IL-1 Receptor
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha
UE	HIV unexposed
UN	United Nations
Var	varicella vaccine
WAZ	weight-for-age Z-score
WB	whole blood
WBC	white blood cell
WHO	World Health Organization
WLZ	weight-for-length Z-score

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Forward motion has never been a challenge, but to say I have an intrinsic sense of linear direction would be an overstatement. Thankfully, I have known a number of inspiring navigators who offered me guidance. I am filled with immense gratitude towards my doctoral supervisor, Professor Tobias Kollmann, who inspired and empowered the majority of my professional growth, while encouraging perseverance and humility through many of life's extracurricular challenges and successes. Thank you for your friendship and mentorship, and for leading by example with an undying and infectious passion for the betterment of health of vulnerable populations. The "Kollmann Laboratory" was a vibrant environment for growth, with numerous exceptional characters. I am especially thankful for Darren Blimkie's help and camaraderie. He provided me with a friend and confidant throughout much of my training.

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DEDICATION

In loving memory of my paternal grandparents, Bob & Lois Reikie, maternal grandparents Jackson & Jean Ball, and my adopted grandmother Mona Sparling.

1. GENERAL INTRODUCTION

1.1 Overview of Thesis Aims

The work comprising this thesis aimed to assess immune development and clinical outcomes over the first year of life in infants born in a developing nation's setting. We aimed to describe normal infant innate immune development and to subsequently compare innate immune development and adaptive immune development between HIV-exposed but uninfected (HEU) and HIV unexposed (UE) infants.

The **specific aims** of this thesis were to:

1) develop a cohort composed of HEU and UE newborns in South Africa and contrast their clinical outcomes over the first year of life.

2) provide a comprehensive analysis of innate immune development in a cohort of South African infants.

3) compare differences in innate immune development, and differences in adaptive immune development between HEU and UE infants.

1.2 Early-Life Susceptibility to Infectious Disease

Infants and children carry the greatest burden of infectious disease. The World Health Association (WHO) estimates that 10.6 million children under the age of 5 die every year[1]. The highest mortality rate occurs in the first month of life and the next highest is between the 1st and 12th month of life [2], making the first year a period of exceptional susceptibility. The majority of child death is preventable and is caused by infectious disease [1,3]. In an effort to combat child mortality, the United Nations (UN) hosted the Millennium Summit in 2000 and set the stage for a worldwide effort to reduce child mortality. Eight health and development goals were agreed upon by 189 member states (Millennium Development Goals (MDGs)), the fourth of which was to reduce under-five mortality rates by two-thirds between 1990 and 2015. Tremendous efforts have been undertaken towards identifying 'where?' and 'who?' the populations are, and specifically 'when?' and from 'what?' they are most at risk. The essential next step is to illuminate 'why?' the most vulnerable populations are susceptible. This knowledge would be utilized to decrease morbidity and mortality from preventable disease in children.

The majority of child deaths are from neonatal, infant and childhood pneumonia (19%), diarrhea (18%), malaria (8%) and neonatal sepsis (10%) [1]. The highest mortality rate from these diseases is during the first year of life [2], and by far the greatest burden of infant morbidity and mortality (99%) occurs in developing

nations [2]. Furthermore, endemic disease such as HIV and Tuberculosis (TB) is worst in the world's poorest nations, and these diseases contribute to making sub-Saharan Africa the region of the world that is hardest hit by under 5 mortality [3].

1.2.1 Global Infectious Disease Burden in Infants

Morbidity and mortality in infants under 5 years is not shared equally across the globe. The World Health Organization Child Health Epidemiology Reference Group has identified that 41% of deaths under 5 occur in sub-Saharan Africa, 34% in South Asia, and 13% in the Eastern Mediterranean nations [3]. The worldwide infant death rate (per 1000 births) is estimated at 42. In more developed regions the rate is estimated at 6 (Canada is 4), and in the least developed regions it is 72. The infant mortality rate in sub-Saharan Africa is 79 (Figure 1) [2].

1.2.2 Infectious Causes of Morbidity and Mortality Early in Life in South African Infants

An initial step towards reducing infectious morbidity and mortality in infants is to identify the diseases infants are suffering and dying from. The leading cause of infant morbidity and mortality in every major region globally is lower respiratory tract infections (LRTIs) [1]. However, the collection of mortality data in neonates (<1 month) and infants (<1 year) is challenging, and data pertaining to the specific causes (e.g. infectious etiology) of neonatal and infant deaths is limited. Data collection can be particularly challenging in developing regions. Severe infections

may carry out their course at home without the help of medical personnel. Vital statistics are often more readily available, but these data only report causes of mortality, and not morbidity [3].

Contrasting mortality data, information pertaining to clinical illnesses causing neonatal and infant morbidity is more readily available, particularly when the illness leads to hospitalization. Global data for severe illnesses suggests that, similar to children under 5, neonatal pneumonia is responsible for a large proportion of deaths (~10%)[1]. The WHO Young Infant Study Group was formed to study serious infections in young infants and the most common pathogenic bacteria in neonatal disease were identified. *Streptococcus pneumonia*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Echerichia coli*, *Neisseria meningitidis*, and *Haemophilus influenza* were the bacterial pathogens that caused the most severe illness in this age group [4]. However, when interpreting these data we must consider the lack of microbial diagnosis when community acquired pneumonia (CAP) is treated in an outpatient setting, which is the most common scenario [5]. Furthermore, even when microbiologic diagnosis is pursued (in or out of hospital), the traditional diagnostic tests have poor diagnostic yield [6,7]. Another common cause of morbidity and mortality in children in developing nations is diarrhea, but similar to CAP, supportive therapy without microbial diagnosis is recommended in most cases for diarrheal disease [8]. Therefore a similar paucity of infectious disease etiological data is encountered.

Risk factors leading to neonatal and infant hospitalization for infectious diseases have also been documented [9]. Factors contributing to morbidity and mortality in this age group include, but are not limited to, maternal HIV infection [10], malnutrition [11], lack of breastfeeding [12] and lack of clean, running water [9]. Lower socioeconomic status is a general risk factor that has long been considered a risk factor for poor health outcomes in infants [9]. Given that an estimated 30% of babies born in southern Africa are born from mothers with HIV, and this population is disproportionately exposed to the other above mentioned risk factors [13], a particular focus on infectious morbidity and mortality in HIV-exposed infants is warranted.

1.2.3 HIV-Exposed Uninfected Infants: A Population at Increased Risk for Infectious Morbidity and Mortality

Areas that carry the greatest burden of infectious disease are often the same regions with the highest prevalence of HIV. South Africa's antenatal HIV seroprevalence is 30% [13,14]. However, with prevention of mother to child transmission (PMTCT) protocols (antiretroviral therapy for mother and child), the vast majority of the newborns are HIV negative (>95%) [15]. These infants do not seroconvert and remain polymerase chain reaction (PCR) negative for HIV. However, these infants mount HIV-specific adaptive immune responses, thus indicating they were HIV-exposed but remained uninfected (HEU) [16-18].

Over the past decade more than 2 million HEU infants were born each year, and in sub-Saharan Africa HEU infants suffer up to 4 times higher risk of dying in the first 2 years of life. HEU are also at increased risk for infectious morbidity, and are hospitalized for infections more often than UE infants [19,20]. The etiology of increased susceptibility of HEU infants to infectious disease remains undetermined.

1.2.4 Infectious Causes of Morbidity and Mortality Early in Life in South African HEU Infants

The leading cause of infectious mortality is acute respiratory infections followed by diarrheal disease in all infants regardless of HIV exposure [1,21]. The risk of severe lower respiratory tract infection (LRTI) is greatest during life's earliest months [19,22,23], and risk for severe diarrheal disease is highest after 6 months of age and coincides with cessation of breastfeeding [24].

There is limited data on the infectious etiology of LRTIs and diarrheal disease in HEU infants. Some studies have attempted to characterize and compare the microbial cause of respiratory disease between HIV-infected and HIV-uninfected infants [25], but only a single study has directly compared bacterial etiology of disease with HIV unexposed infants [26]. The results of this study are summarized in Table 1. Community acquired lower respiratory infection in HIV unexposed newborn and infant populations are usually caused by *Streptococcus pneumonia* [26]. An alarming observation in HEU infants with LRTIs is the prevalence of *Pneumocystis jirovecii*

pneumonia (PJP) infections [20,26,27]. PJP is an opportunistic infection that afflicts immunocompromised patients and is one of the more common LRTI pathogens in patients with advance stage HIV infections/acquired immunodeficiency syndrome (AIDS). In immunocompetent patients, however, PJP infection is exceedingly rare. HEU infant susceptibility to PJP, and to LRTIs in general, suggests that they are a population that is at least transiently immunocompromised.

A recent cross-sectional study in Kenya examined infectious etiology of diarrheal disease in 6 to 22 month old HEU and UE infants (Table 2). For both groups, the five most common organisms cultured in infants presenting to hospital with diarrheal disease were enteroaggregative *E. coli*, *Giardia* species, *Campylobacter* species, enteropathogenic *E. coli* and *Cryptosporidium*. The frequency of these infections ranged from 3.7% to 13.3%. HIV-exposure was associated with increased *Cryptosporidium* infections in children less than 24 months of age and intriguingly HIV infection was not associated with increased *Cryptosporidium* infections. One limitation of this study was the lack of data on when infants were weaned from breastfeeding. A study based in Uganda demonstrated a relative risk of 6.4 for HEU infants developing severe diarrheal illness if the child was not breastfed between 6 and 11 months of age [28].

1.2.5 Potential Etiologies for Increased Susceptibility to Infections in HIV-Exposed Uninfected Infants

The etiology of increased susceptibility to infection in HEU infants is poorly understood and is most likely multifactorial. Risk factors that have been identified include, but are not limited to malnutrition, dietary deficiency, poor breastfeeding practices, poor hygiene, low socioeconomic status, water and air pollution, poor maternal health (or low CD4+ T cell count) or neutropenia at the time of birth [13,29-34].

Consideration of environmental exposures is paramount to understanding infectious disease susceptibility in HEU infants. In areas where HIV is endemic, poorer communities, with higher HIV rates, have unique environmental exposures. There are social, economic and infectious burdens to HIV infection within the household and community [35]. When a child is exposed to disease, regardless of the setting, a healthy immune system is required to control infection. Importantly, when immune development is examined on a global scale, and compared between resource rich [36-38] versus resource poor settings [39-42], variability in immune development is observed [43]. Concordantly, factors indicating relative wealth or poverty within a community (e.g. lack of access to running water, or indoor plumbing) have also been shown to correlate with variability in innate immune responses [44].

Pre-natal and early-life medical therapy differs between HEU and UE infants. Antiretroviral therapy (ART) is offered in the pre- and- post-natal period for the

prevention of mother to child transmission (PMTCT) of HIV [45], and ART exposure *in utero* and in the perinatal period is associated with anemia, neutropenia, relative lymphopenia and down regulation of select genes encoding innate immune cell receptors[46-49]. The type and quantity of ART administered to the mother and the newborn varies within the PMTCT protocol depending on maternal knowledge of HIV infection, viral titer and compliance with PMTCT [45]. The effect of ART on newborn immunity is likely dependent on the extent of drug exposure, and there are currently no data measuring the effect of ART exposure on functional innate immune ontogeny early in life.

Different ethnic groups, with varied genetic backgrounds by extrapolation, can exhibit varied innate immune responses to, and protection from, infectious challenge[50,51]. There are likely several genetic factors that result in variability of immune development, and even within a single heterogeneous population in South Africa, polymorphisms in genes that encode innate immune receptors were associated with heterogeneity of innate immune responses[52].

Differences in breastfeeding practices may also impact immune development. Breast milk contains antimicrobial proteins and peptides, nucleotides, oligosaccharides and immunoglobulins [53,54]. These compounds confer protection from microbes and clinical evidence indicate that the time period of increased morbidity from diarrheal infections in HEU infants coincides with the average time of weaning[29]. The components of breast milk also have immunomodulatory effects.

For example, breastfeeding practices were shown to impact responses to early life vaccination [55], and components of breast milk have also been shown to impact innate immune responses to stimulation with pathogen associated molecular patterns (PAMPs) [53,54]. The WHO has recently changed recommended breastfeeding practices to include HIV positive women in developing nations because evidence suggests that the benefits of breast milk outweigh the risk of contracting HIV from breastfeeding [12,20]. Despite these recommended guidelines there remains heterogeneity in breastfeeding practices, especially in the HEU population.

Elucidating specific risk factors in highly susceptible populations, and identifying how these factors impact immune development may move us closer to identifying targeted strategies to decrease infectious morbidity and mortality. It is essential to describe immune development in regions with the greatest burden of infant morbidity and mortality, and then to compare with immune development in the most vulnerable subgroups, exposed to highly prevalent risk factors, such as the HIV-exposed South African infant.

1.3 Early-Life Innate Immune Ontogeny

Early life is a time of increased susceptibility to infections and both innate and adaptive immune responses may have a suboptimal capacity to control some infections, or to prime effective, protective long-term immune responses [56-58].

Newborns and infants have an impaired capacity to control infection and deficiencies in host defenses are detected at various stages in the first year of life. Neonatal and infant innate immune defenses are unique due to factors such as skin and gastric pH changes, decreased complement, poor recruitment of neutrophils, macrophages and monocytes to sites of infection, decreased antigen presenting cell responses to some stimuli, altered cytokine production, and impaired T and B cell function.

Understanding early life immune development is essential in our quest to develop better strategies for monitoring, prevention from and/or treatment of pediatric infectious disease. Furthermore, a better understanding of immune development in populations with average risk for infectious morbidity and mortality (relative to their surroundings) is a necessary first step for examination of populations at increased risk, such as HIV-exposed infants.

1.3.1 Overview of Innate Immune Defense Mechanisms in Neonates and Infants

The first year of life represents a dynamic period for development of the innate immune system [56-59]. These dramatic and rapid changes represent a window of vulnerability (possibly explaining at least in part the increased risk to suffer and die from infection early in life) as well as opportunity (the chance to direct the

trajectory of the immune system in a direction supporting homeostasis, health and well being for life) [60].

Three main components comprise the body's protection from infectious disease: i) barrier function, including skin as well as intestinal and respiratory mucosa; ii) innate immunity and iii) adaptive immunity. Innate immunity directs the adaptive immune response following pathogen exposure (or vaccination); and innate immune cells are located along our physical barriers and intimately interact with mucosal and integumentary protective components. The clinically observed increased risk for infectious morbidity and mortality, as well as suboptimal response to vaccination in neonates and infants, may be due to an altered function of any of these components. Given the centrality of innate immune components for all three compartments, it is reasonable to hypothesize that altered innate immunity is at least partially responsible for the observed clinical differences in infectious disease susceptibility. [59-65]

1.3.1.1 Integumentary and Mucosal Barriers / Antimicrobial Barriers

Neonates and infants exhibit differences in first line defenses from pathogenic microbes. Epithelia form a physical barrier against infection by preventing direct entry of microbes while biochemical protection is provided from secreted chemical agents that protect from infection. Mechanisms of protection include, but are not limited to, mucociliary clearance, enzymatic protection, collectins and defensins.

Epithelia also provide a surface for the colonization of commensal flora that forms a biological barrier from pathogenic microbes.

Antimicrobial peptides found on the epithelia of the skin, gut and respiratory tract differ between newborns and adults. For example, α- and β-defensins, which are broadly protective against bacteria, fungi, viruses and protozoa, are decreased in concentration in the neonatal gut, while the mucosal pH is less acidic compared to later in life. Contrasting these pro-microbial conditions other defensins, such as cathelicidin (LL-37), are increased in this age group, likely providing compensatory anti-microbial protection [66]. While the unique biochemical and anti-microbial environment may facilitate early-life establishment of gut flora, compensatory mechanisms may be imperfect, thus potentially leaving the newborn and young infant at an increased susceptibility to infectious disease.

1.3.1.2 Humoral Mediators of Innate Immunity

When microbes gain entry past the epithelial barriers, they are next confronted with components of humoral immunity. Humoral mediators of innate immunity include, but are not limited to, acute phase proteins such as mannose-binding lectin (MBL), and complement serum proteins which function via any of 3 mechanistic pathways (classical, alternative and lectin). Neonates have decreased activity in the alternative and classic complement pathways, thus complement-mediated opsonization of microbes and complement-derived chemotaxis is impaired. MBL levels are reduced in some newborns, and low-levels of MBL are associated with increased risk of

sepsis and pneumonia. In addition to altered levels peptides that directly mediate humoral immunity, other factors differ such as an elevated level of adenosine in neonatal plasma. High adenosine in the plasma has been shown to increase intracellular cyclic adenosine monophosphate levels, which in turn inhibits Toll-like receptor (TLR)-mediated tumor necrosis factor (TNF)-α production (thereby decreasing antimicrobial responses) [67].

1.3.1.3 Polymorphonuclear Granulocytes

Neutrophils, eosinophils and basophils are polymorphonuclear (PMN) granulocytes and are central to innate immune defenses from bacterial (neutrophils) or parasitic infections (eosinophils and basophils). Neutrophils reside in the bloodstream as the most abundant leukocyte (60 – 70% of leukocytes are neutrophils [68]), until they are recruited into infected or injured tissue. Neutrophils bind, phagocytose and kill bacteria once they reach the site of infection. Newborns have a similar number of neutrophils available to respond to infection, however neonatal activated neutrophils exhibit impaired trancytosis and a limited capacity to induce neutrophil proliferation [69]. Neonatal eosinophils and basophils are less numerous in the blood than in adults, and represent 1-6% and <1% of white blood cells respectively (WBCs). Both cell types are integral to anti-helminth responses, but eosinophils are also involved in anti-viral immunity. In newborns there is a preferential differentiation of eosinophil/basophil precursors towards the eosinophil lineage [70]. The altered differentiation may be due to increased production of IL-5, a key Th1

cytokine, which is associated with higher levels of eosinophils and total immunoglobuolin (Ig) E [71,72].

Preterm neonates exhibit a pattern of bacterial infections that closely parallels that of severely neutropenic patients, which suggest that immature neutrophil function is a clinically significant phenomenon of early life development [73]. While neutrophil function may be somewhat impaired early in life, thus potentially increasing vulnerability to bacterial infections, basophil and eosinophil function may also be increased, providing polymorphonuclear granulocyte mediated protection from parasites and viruses early in life. Further research is required to delineate the relative importance of altered neutrophil, eosinophil and basophil function with respect to early life susceptibility to infectious disease.

1.3.1.4 Natural Killer Cells

Natural Killer (NK) cells survey the body for virally infected cells or tumor cells. The concentration of NK cells is similar in term babies to what is found in adults, however function of NK cells may differ in an age dependent manner. NK cells modulate immune responses by producing TNF-α and interferon (IFN)-γ early in infection, and while IFN-γ production, which is essential to antiviral responses, remains intact in neonates, NK cell mediated TNF-α production is decreased early in life and may decrease cytotoxicity or resistance to infection from some viruses [74]. Decreased TNF-α from NK cells may also alter dendritic cell mediated cytokine

production and differentiation early in life [75]. Furthermore, neonatal NK cells demonstrate impaired cytotoxic responses towards HSV and CMV-infected cells. NK cell mediated antiviral immunity is therefore impaired in newborns; however, the NK cell functional deficiencies are likely viral strain specific.

1.3.1.5 Antigen Presenting Cells

Antigen presenting cells (APCs) are central to linking the innate and adaptive immune systems [76,77]. The major human APCs are monocytes, classical dendritic cells and plasmacytoid dendritic cells, as well as B cells (B cells are discussed in section 1.3.1.6). APCs continually survey their environment and when PAMPs are detected the APC can upregulate the expression of costimulatory molecules and cytokine production. APCs respond in a pathogen-specific manner to coordinate appropriate adaptive immune responses [78].

1.3.1.5.1 Monocytes and Macrophages

Monocytes are mononuclear phagocytes produced in the bone marrow. When monocytes leave the bone marrow they circulate in the blood for 1 to 3 days prior to migration into tissues. In the tissues monocytes differentiate into macrophages or cDC. For modest infectious insults macrophages are the first and only necessary line of defense. Macrophages are tissue resident cells that respond rapidly to infection, even before neutrophils. Macrophages also act as antigen presenting cells, although to a lesser degree than dendritic cells, and mediate immune responses by producing

cytokines in response to microbial exposure. In addition, macrophages play a major role in clearing dead host cells, phagocytosis of microbes and secretion of inflammatory mediators.

Term babies have similar concentrations of macrophages and monocytes as adults do, with the exception of alveolar macrophages, which are decreased in numbers in life's earliest weeks. Neonatal monocytes and macrophages are slower to migrate into infected tissues. Expression of pattern recognition receptors (PRRs) is similar between newborns and adults, however responses to PRR stimulation differ. For example, production of interleukin (IL)-12 and type I IFNs (IFN-α and IFN-β) is decreased. TNF-α production is also decreased in response to some stimuli in neonates. Contrasting these diminished responses, neonatal monocytes and macrophages produce increased amounts of IL-23, which is central to anti-bacterial and anti-fungal immune responses. IL-12, Type I IFN, TNF-α and IL-23 are all important cytokines for guiding both innate and subsequent adaptive immune responses, and altered expression of these cytokines early in life may significantly impact susceptibility or resistance to infectious disease.

1.3.1.5.2 Dendritic cells

Dendritic cells are APCs whose primary function is to orchestrate adaptive immune responses. They are found in blood and tissues throughout the body, and they are the cell type with the most major histocompatibility complex (MHC)-II receptors.

There are two main types of dendritic cells, conventional (or classical) dendritic cells (cDC), which were previously known as myeloid dendritic cells, and plasmacytoid dendritic cells (pDC). There are also tissue specific dendritic cells, such as Langerhan cells, which are DCs that reside in the epidermis.

cDCs play a central role in the initiation and modulation of adaptive immune responses. They are essential in maintaining tolerance to self-antigens in secondary lymphoid tissues, and on the other hand they are the most impactful cell type for guiding antimicrobial T cell responses. They activate T cells and determine what type of response should be mounted, i.e. T-helper 1 (T_H1) vs. T-helper 2 (T_H2) vs. T-helper 17 (T_H17), amongst others. T_H1 generally help to protect from viruses and other intracellular pathogens. T_H2 are essential for antiparasitic responses and promote T-cell dependent B-cell responses and subsequent antibody production. T_H17 are central to anti-fungal and extracellular bacteria immune responses. The specific and varied roles of T-helper subsets underscore the importance of pathogen-specific responses from dendritic cells. cDCs are able to steer adaptive immune responses down the appropriate pathway by expressing the appropriate cytokines and co-receptors, while presenting antigen within the appropriate MHC-type.

In newborns the concentration of cDCs in peripheral blood is similar to concentrations observed in adult blood. Expression of MHCII and costimulatory molecules increases in response to stimulation of TLRs. However, this upregulation

is not as pronounced in newborns relative to adults. Similar to observations in monocytes, neonatal cDC produce less type I IFN and IL-12, and more IL-23 in response to PRR stimulation. Generally, this may indicate impaired defense from intracellular pathogens, but enhanced reactivity to extracellular bacteria and fungi.

pDCs perform a similar role to cDCs with respect to generating pathogen specific immune modulatory responses. However, the specific function of pDCs is quite distinct from cDCs. The pDC is specifically essential for modulating antiviral responses. Large amounts of type I interferon (IFN-α and IFN-β) are secreted in response to viruses. An additional important role for pDC may be to promote cDC antibacterial responses. IL-15 dependent cross-talk between pDC and cDC was recently demonstrated as an important component to antibacterial immunity in animal models [79]. pDCs were shown to assume an almost T-helper cell-type role in promoting cDC mediated clearance of *Listeria monocytogenes* by stimulating cDC with CD40 ligand and paracrine secretion of IL-12 [79].

Concentration of pDCs is higher in the neonatal period relative to pDC in adult peripheral blood. Neonatal pDC are deficient in type I IFN production, much like in neonatal cDC and monocytes. pDC are primary producers of type I IFNs, which in turn induce IFN-γ production and activation of Th1 T-cells. Early-life deficiencies in type I IFN production may provide an explanation for suboptimal Th1 responses to some pathogenic challenges.

1.3.1.6 B cells: an Adaptive Cell with Antigen Presentation Function

B cells are lymphocytes capable of antigen presentation. B cells mature in the bone marrow and spleen. The white pulp of the spleen provides an important microenvironment for dendritic cells bearing antigen, and B cells and T helper cells to interact. When activated, B cells proliferate and form germinal centers where they differentiate into memory B cells or plasma cells [80]. B cells also reside in peripheral tissues where they await antigen encounter and activation. B cells require either one or two signals to differentiate into memory B cells or plasma cells. Signal one is provided by antigen binding the B cell receptor, which is then endocytosed, broken down and loaded into MHC II molecules. In thymus-dependent activation signal two involves B cells performing APC function by presenting the antigen/MHC II complex to T-helper cells; T helper cells are activated and provide the second activation signal (in the form of cytokine) to the B cell. B cells can also be activated in a thymus-independent fashion simply by binding antigens such as LPS and flagellin. The primary function of plasma cells is the secretion of antibodies.

B cells are functionally deficient at birth and function matures rapidly over the first months of life. Deficiencies in the anatomic microenvironment in the white pulp of the spleen may be partly responsible for poor antibody responses near the time of birth. Isotype expression by B cells is limited in response to thymus-dependent antigens. Limitations of B-cell maturation likely impact isotype conversion. Interestingly, the neonate is not deficient in all antibody responses. While early-life production of antibody in response to polysaccharides is impaired, IgM production, and to a lesser degree IgG production in response to protein antigens is relatively

intact. Interestingly, postnatal age is more closely correlated with maturation of Thymus dependent B cell responses than gestational age. This indicates that environmental exposures early in life are likely instrumental in the maturation of B cell responses.

Maternal factors can also impact newborn B cell responses. Maternal antibody crosses the placenta in the prenatal period, and circulating maternal IgG in the neonatal plasma confers passive immunity from pathogens to which the mother is immune. However, maternal antibody in the newborn can interfere with antigens (including vaccines) encountered by the neonatal and young infant immune system, thereby decreasing newborn B cell responses [55]

1.3.2 Evaluating Innate Immune Development

Innate immune responses can be examined using several methods. Techniques employed to evaluate innate immune development include, but are not limited to, stimulation assays that measure protein expression, e.g. detection of intracellular cytokine production, cytokine secretion or cell receptor expression. Advances in flow cytometric technologies allow comprehensive high throughput platforms for innate immune analysis. Methods to analyze production of multiple innate cytokines in key functional categories utilizing both multiplex bead array and intracellular cytokine cytometric strategies have been highly standardized [81-83].

Several groups have employed multiparameter flow cytometric strategies to examine early-life immune responses [36,37,40,63,82,84,85]. Although robust techniques have been developed which allow comprehensive evaluation and analysis [86], important differences exist in the parameters measured, and in methods utilized between groups. For example, some studies have been conducted on peripheral blood mononuclear cells (PBMC) while others are conducted with whole blood, which can lead to significantly different responses [83]. Isolating PBMCs can allow more focused examination of mononuclear cell function and can simplify assays by eliminating variables from other cell types or proteins in the blood. However, retaining other factors found in whole blood provides a milieu that is less artificial and may more closely resemble innate immune function in vivo. Some groups have focused on fewer PAMPs, examining only responses to viral patterns, while others have examined responses to a wider array of PAMPs.

Various timelines of immune development have been examined. Studies have been performed by examining blood from study subjects at ages ranging from birth (cord blood) through infancy [38]. The first year of life marks the time that the immune system is most rapidly changing/developing, and even small changes in age can represent large changes in immune development. This is an important consideration because time-restricted differences in innate immune responses to PAMPs may be pertinent to corresponding periods of increased susceptibility of infants to infectious diseases, or to suboptimal vaccine induced protection from infectious disease [84,87]. Some studies examined multiple time points but were cross-sectional

[36,40], while others were prospective cohort studies [38]. Cohort studies have the advantage of providing longitudinal data for each study subject, thereby limiting the confounding variable of inter-individual differences in immune development. Previous to the work comprising this thesis no longitudinal cohort had examined immune development throughout the first year of life.

Environmental and genetic variability between study groups is another essential consideration. Genetic polymorphisms, even within similar cultural groups, have been shown to impact innate immune responses [52]. Therefore, variability must be expected between infants with racially different backgrounds. Furthermore, factors such as access to running water or exposure to disease or different vaccines [51], e.g. TB or *Mycobacterium bovis* bacillus Calmette Guerin (BCG) respectively, also impact immune development. There was a recent global collaborative group formed to compare immune development between infants from various genetic backgrounds, also with different widely varied environmental exposures [43].

1.3.2.1 Pattern Recognition Receptors

Threat recognition at the cellular level and initiation of appropriate immune responses is central to the maintenance of homeostasis. PRRs have emerged as an essential component of the innate immune system for the recognition of molecular patterns that are associated with threats, such as PAMPs. PAMPs binding to PRRs trigger local and systemic responses that compose the initial response to an invading pathogen; this ligand–receptor interaction and the downstream signal

mechanisms are fundamental to promoting protective adaptive immune responses [76,77,88]. Central to the APCs function is phagocytosis, digestion and processing of pathogens for presentation on the cell surface within the context of MHC molecules. Antigen-presentation can be referred to as 'signal 1', and can be accompanied by expression of co-stimulatory molecules (signal 2) and production of cytokines (signal 3). The APC is able to achieve pathogen appropriate specificity by varying the strength and character of signal 1, 2 and 3 to guide innate immune cell function and to program adaptive immune cells. The recognition of pathogen (versus non-pathogen) and pathogen-type is largely mediated by PRRs. PRRs therefore act as gatekeepers, which are essential in the strategic unlocking of APCs versatile capabilities (Figure 2).

When PRRs encounter PAMPs either on the APC cell surface, within an endosome or in the cytoplasm, there is induction of antigen presentation, expression of costimulatory molecules and secretion of cytokines. Of the PRRs, TLRs are the best-studied [78]. A summary of the best-studied TLRs, their location (cell surface/endosomal), their cognate antigen and the immune responses they elicit is provided in Table 3.

Dysregulation of TLR signaling leads to aberrant innate immune cell function and can cause pathologic inflammation, compromised immune defenses or induction of autoimmunity. Regulation of TLR signaling is mediated through TLR/IL-1 receptor (TIR)-domain containing adaptor molecules such as Myd88, TRAM, MAL/TIRAP and

TRIF [89]. TLR signaling specificity is achieved through differential utilization of these various adaptors at the cytoplasmic domains of TLRs. The two major pathways of TLR signaling are known as Myd88-dependent and Myd88-independent (TRIF-dependent) signaling pathways. Downstream from Myd88 or TRIF are the IRF7 and IRF3 nuclear transcription factors, respectively. These nuclear factors are phosphorylated and then translocate into the nucleus to promote Type I IFN gene and IFN-inducible gene expression. TLR-mediated expression of other genes that encode cytokines is largely regulated by the downstream degradation of the NFκB inhibitor molecule IκB. Degradation of IκB liberates NFκB to translocate into the nucleus. NFκB family transcription factors control the expression of a large number of target genes that mediate inflammatory responses to environmental stimuli.

In addition to TLRs, there are other PRRs that allow APCs to respond to their environment, such as nucleotide-binding domain–containing and leucine-rich repeat–containing receptors (NLRs) (e.g. nucleotide-binding oligomerization domain (NOD)-1, NOD-2 and NALP3); C-type lectin receptors include DC-SIGN, Dectin-1, and Dectin-2; Retinoic acid gene like receptors include RIG-1, MDA-5 and LGP2. NOD1 and NOD2 activate mitogen-activated protein kinase and NFκB pathways leading to proinflammatory cytokine production. This activity is shown to act in synergy with TLRs, in particular NOD1 and NOD2 respond to similar stimuli as TLR2/1, such as peptidoglycan from Gram negative or positive bacteria. Recent evidence suggests NLRs also participate as “signaling partners”, either enhancing or suppressing NFκB expression in response to viral and parasitic infections as well [90].

NALP3 recognizes components of bacterial peptidoglycan, bacterial RNA and DNA, and some endogenous danger signals such as uric acid, and alum (used as a vaccine adjuvant) [91,92]. NALP3 signals differently than NOD-1 and NOD-2. NALP 3 activates a macromolecular complex known as the inflammasome, leading to the activation of caspase 1, and subsequent secretion of the proinflammatory cytokines IL-1β and IL-18 [93]. DC-SIGN is involved in interactions between DCs and HIV. Dectin-1 and Dectin-2 act with TLR2 in response to fungi [94]. RIG-I, MDA-5, and LGP2 are intracytoplasmic in most cell types and provide rapid antiviral surveillance; these 3 receptors mediate responses to distinct varieties of viruses.

Altered signaling through the above-mentioned receptors, or aberrant activation of their downstream nuclear factors underlies various disease states [78]. Understanding how environmental exposures impact the precise regulation of innate immune receptor signaling, including the activation and termination of these signals, would provide insight into how environmental and infectious stimuli can shift normal innate immune cell function to pathological.

1.3.2.2 Pattern Recognition Receptor Responses in Neonates and Infants

Several research groups have begun to examine neonatal and infant immune development over the past decade. With improving technologies, increasingly comprehensive studies of neonatal and infant immunity have been undertaken; these studies have been primarily focused on variability of PRR responses to stimulation.

Newborn basal expression of monocyte TLRs is similar to adult monocytes, however their response to TLR stimulation is distinct in an age-specific manner [39]. Differential TLR function suggests that TLR signaling may be distinct in early life. TLR signaling is dependent on interleukin-1 receptor associated kinase (IRAK)-4 and myeloid differentiation primary response gene 88 (MyD88) signaling molecules, both of which have been demonstrated to be deficient in early life [39,95,96].

Cytokine expression differs between newborns and adults. Neonates exhibit impaired Th1 cytokine responses (e.g. TNF-α and IFN-α) in a stimulus dependent manner [36,37,39,67,97-100]. Interestingly, newborns produce adult-like TNF-α levels in response to TLR8 agonists. Interestingly, neonates produce higher levels of acute phase cytokines such as IL-6 and IL-1β and also IL-10 [39]. A decreased TNF-α:IL6 ratio and more IL-23 likely leads to stronger Th2 and Th17 differentiation [67,99]. Elevated IL-10 suggests enhanced anti-inflammatory function in newborns, which is potentially a mechanism that keeps immune responses in check during the exposure to the plethora of new antigens that occurs in early life [101]. In addition to changing with age, PRR mediated responses likely vary between resource rich versus resource poor settings. Studies conducted in resource poor compared to those performed in resource rich settings demonstrated a relatively rapid increase of TLR4 mediated responses within the first year of life [36,102-104], possibly indicating a polarization to Th1 phenotype earlier in life in resource poor settings. The hygiene hypothesis supports these observations [39,102,105,106]. A study conducted in Ecuador

demonstrated decreasing responsiveness of TLRs between 1 and 2 years of life [41], and contrastingly a study undertaken in Papa New Guinea showed no decline in TLR responses over the same time frame [40]. Although these studies were aimed at investigating innate immune development, they were all cross-sectional in nature and were therefore susceptible to inter-individual variability. Rhandawa et al. demonstrated that polymorphisms in TLRs within a relatively homogeneous population lead to statistically different immune responses, thus suggesting that a longitudinal cohort study would have greatly enhanced utility in investigating differences in innate immune function between populations with differing environmental exposures. One such study had been performed in Canada, which followed children from birth to 2 years [38]. Similar to the cross-sectional studies in The Gambia, Ecuador and Belgium the Canadian longitudinal study demonstrated an increase from birth up to 2 years of age in Th1 and pro-inflammatory cytokines [38].

1.3.2.3 Key Differences in Immune Development Study Type and Assay Techniques

The majority of studies investigating innate immune development early in life examined cytokine levels in culture supernatants [36,39,40,99]. Detecting cytokines in culture supernatants is less specific than ICC analysis, however, our prior studies with PBMCs demonstrated that results are largely congruent between the two techniques [38]. The cellular composition of blood changes over the first year of life, therefore it is difficult to extrapolate results of supernatant analysis to changes occurring at the cellular level. Furthermore, while an overview of production of inflammatory cytokines may appear similar between supernatant and ICC analysis,

patterns of expression can be more readily detected with ICC analysis [38,82,107]. Our previous study in North America represented the first cohort study aimed at identifying the cellular source of cytokines detected in supernatants extending and complementing previous studies of innate immune ontogeny which only performed secreted cytokine analysis [36,37,39].

ICC stimulation assays performed in PBMC culture may introduce additional artifact due to removal of the non-white blood cell components of blood when compared with assays performed in whole blood. With the exception of studies of infant immune development in Belgium and in Papa New Guinea, who analyzed infant whole blood, previous studies analyzed responses from isolated PBMCs. Although stimulation of PBMCs in culture can enhance sensitivity, the other components of blood are subtracted from this *in vitro* assay and may not represent the *in vivo* environment as closely as a stimulation assay performed in whole blood. Our previous results with North American infants were in line with studies of European or Papa New Guinean infants that used whole blood and followed infants throughout the first year of life, e.g. increased Th1 cytokine production in response to TLR4 stimulation was observed over the first year of life [36,37,40].

1.3.2.4 Global Variability in Innate Immune Development

Striking differences have been observed in South African infant responses at 2 years of age when contrasted with infants from South America, North America and Europe [43]. Infants from each continent have different environmental exposures and possess

varied genetic backgrounds. South African infants differed from all other groups more than infants from the other 3 continents differed from each other [43]. Furthermore, there is evidence that the immune system develops differently early in life depending on rural or urban living, and differences between these two groups are observed both in geographically distinct developing and developed nations [108-110]. This suggests environmental exposures can play a dominant role in shaping the immune system, possibly overshadowing genetic differences as a guiding force for variability in immune ontogeny. On the other hand, differences in ethnicity (genetic differences by extrapolation) are also associated with different innate immune responses to malaria infection in children in West Africa [50]. Differing host genetics between South African and e.g. North American or European infants are still likely an important source for differences in immune function [111].

Socioeconomic status is a non-specific factor that correlates with environmental exposures, and more broadly, environmental exposures can drastically vary for infants in resource-rich versus resource-poor settings [44,110]. There are several potentially contributory mechanisms for differences in infant innate immune responses detected in cohorts around the globe. Environmental exposures that may impact immune development include iatrogenic exposures as well, such as live vaccines given at or near birth. For example, infants from Europe or North America do not receive BCG or oral polio vaccine (OPV) at birth, while children in both South Africa and The Gambia receive both BCG and OPV at birth. Infants in a study from Papa New Guinea received BCG at birth, but were mixed with respect to a neonatal

dose of oral polio vaccine; and infants in a study from Ecuador received only BCG at birth. An Australian study examined specific exposures within a single ethnic group, and demonstrated differences in innate cytokine production based on different living conditions [44]. Similarly, a Brazilian study demonstrated that access to running water and basic sanitation is significantly correlated with altered immune responses in a cytokine specific manner [44].

Infectious disease exposures vary widely across the globe and may have a profound effect on early life innate immune development. For example, the prevalence of HIV infection in women of childbearing age in South Africa is approximately 30%, and sub-Saharan Africa as a whole bears the majority of the global HIV burden. HIV exposure results in virus-specific adaptive immune responses [112]. Therefore, it is plausible that early life exposure to HIV affects development of the immune system. Few groups have compared innate immune development in HEU infants throughout the first year of life, and there has been no cohort study to compare HIV-exposed and unexposed innate immune responses throughout the first year of life.

The data strongly suggest that differences in innate immune ontogeny early in life between children born and raised in different regions of the world exist. The relative contribution of environmental exposures, vaccine exposures, and host genetics, on differences in immune development is yet to be determined.

1.4 HIV-Exposed but Uninfected Infant Immune Development

HEU infants are at an increased risk of morbidity and mortality compared to UE infants [19,20,34,113-116]. The etiology of the increased infection risk in HEU infants is unknown.

Innate immunity orchestrates the initial response to pathogens while shaping future adaptive responses to prevent or clear infections [60]. Differences in early life innate immunity between HEU and UE infants may be associated with increased risk of infectious morbidity [117]. Early life vaccination is an integral component to avoidance of infectious morbidity and mortality, especially in settings with the highest risk [57], therefore it is also essential to understand immunologic outcomes in terms of vaccine responses in both HEU and UE infants in sub-Saharan Africa.

1.4.1 HIV-Exposed but Uninfected Infant Innate Immune Development

Few studies have examined HEU innate immune development, and no previous group has followed a cohort of infants throughout the first year of life. As illustrated by studies of HIV unexposed infant innate immune development in previous cross-sectional studies, the first year marks a period of rapid change in infant innate immune responses, therefore this is likely a critical period of life to study in HEU infants. Morbidity and mortality data supports the first year of life as a critical

period to study immune development, e.g. the first year is when HEU infants suffer the highest risk of morbidity and mortality from several types of infections, including LRTIs, which are the leading cause of death in this age group [1,19,21,22]. A careful examination of innate immune ontogeny in HEU infants would therefore be a useful tool to help us understand HEU infants' increased susceptibility to infection.

Previous comparisons of early life innate immunity in HEU and UE infants demonstrate altered secretion of immune mediating cytokines. For example, increased IL-12 production has been observed in HEU infants [118]. More specific to differences in innate immune cells, upregulation of cell surface receptors, such as increased MHCII expression has been identified on unstimulated HEU APCs relative to UE APCs [119]. Functional comparison of NK cell activity at one month of age also demonstrates an increase of an intermediate NK phenotype for activation and perforin expression in HEU vs. UE, which 'normalizes' by one year [120].

Due to social and financial burdens associated with HIV, HEU infants may be exposed to different risk factors in the household. For example, access to running water may differ depending on income, and lacking access to running water correlates positively with expression of IL-10 in childhood [44]. HEU infants may also be exposed to different pathogens in the household, as immunosuppressed HIV positive individual(s) in the home carry a heavier burden of disease [115]. Different microbial exposures in early life can impact immune development [121].

In ideal circumstances HEU infants are exposed to antiretroviral therapy (ART) as part of PMTCT. In adults, ART is associated with anemia, neutropenia, relative lymphopenia, and down regulation of select pattern recognition receptor genes [46-49]. Similar effects have been seen in newborns exposed to PMTCT [122]. Furthermore, altered secretion of soluble markers of inflammation is observed in infants with perinatal exposure to ARVs [123]. ART exposure may therefore impact HEU immune development; the effect of ART exposure on functional innate immune ontogeny early in life has not yet been determined.

Different ethnic groups (with varied genetic backgrounds by extrapolation) can exhibit varied innate immune responses to, and protection from, infectious challenge [50,51]. More specifically, TLR polymorphisms are associated with heterogeneity of innate responses [52]. Therefore, in comparing HEU and UE immune development it is essential to consider any differences in the racial distribution of populations where HIV is more versus less prevalent.

The WHO now recommends that HIV positive women continue to breastfeed while receiving ART [124]. However, differences likely persist between breast feeding practices of HIV positive and HIV negative mothers [125]. Breast milk contains compounds that modulate PRR-mediated immune responses, including immunoglobulins, cytokines, antimicrobial proteins/peptides, nucleotides and oligosaccharides [53,54,126]. Breast milk immune composition is impacted by maternal nutritional status, exclusivity and duration of breastfeeding and maternal or infant

infections [127,128]. However, maternal HIV-seropositivity does not appear to be an independent determinant of altered breast milk immune composition or quality [129]. Immune composition of breast milk, or of breastfeeding infants' serum, has been compared between HEU and UE infants and their mothers, but our understanding of the clinical significance of these data will remain limited until specific correlates of immune protection have been identified [130-132]. Nonetheless, clinical evidence indicate that the time period of increased morbidity from diarrheal infections in HEU infants coincides with the average time of weaning [29].

Future studies are needed to examine relative contributions of potential etiological factors (ranging from infectious disease exposure to variables associated with host genetics) to changes in innate immune ontogeny, and how those changes in immune development contribute to early life susceptibility to infectious disease [117].

1.4.2 HIV-Exposed Uninfected Infant Adaptive Immune Outcomes: Vaccine Responses

HEU infants are at increased risk for infectious morbidity and mortality and immunization is crucial to combat infectious susceptibility in children under 5 years of age [133]. Increased vulnerability of infants to infections has prompted the WHO to recommend an earlier vaccination schedule in an attempt to curb infant mortality in areas where the risk of infection is highest [134] (Table 4). A challenge to optimal early life vaccination is the unique immune response to routinely administered vaccines

in early infancy. The infant immune response to immunization is often suboptimal and frequently requires boosting to reach protective levels that are readily achieved in older children or adults [135]. Development of safe and effective vaccines for early life is hampered by a limited understanding of ways to optimally induce early and long-lasting protective immune responses in the context of the neonatal immune system [136]. This lends further support for better understanding early life immune development in, specifically, infants at the highest risk of infection, like most HEU infants are.

Suboptimal response to vaccination has been suggested as a potential contributor to the increased infectious burden of HEU [55,137-139]. Prior to initial immunization antibody titers can be lower due to decreased passive transfer of antibody while in utero because of lower maternal titers, placental dysfunction resulting in decreased antibody transfer, or lower half lives of the maternal antibodies [55,137,139-141]. This may be a mechanism behind higher initial response to immunization with the pertussis vaccine at 4 months of age [55]. The relative protective quality of these antibodies produced by HEU is unknown and may also be serotype specific for multivalent vaccines. For example, HEU were shown to have superior antibody levels for one of the serotypes for the 7-valent pneumococcal vaccine (6B), but HEU antibody were less effective than UE antibody in a functional opsonophagocytic killing assays [142], i.e. quantity may not indicate quality.

To date, however, studies have only investigated vaccine responses in either short-term cohorts or cross-sectional studies [55,137-139], thereby neither addressing the efficacy of booster doses or long-term vaccine induced immunity [143]. A longitudinal analysis of HEU responses to vaccination is required to better understand how the rapidly expanding population of HEU infants responds to childhood vaccination.

1.5 Summary

The **overall aim** of this study is to comprehensively examine immune development over the first year of life, to compare immune development in HEU and UE infants, and to observe their clinical and immunologic outcomes later in childhood.

The **overall hypothesis** for this study is that South African infant innate immune responses differ from infants raised in other environments. In HEU infants, there is likely altered innate immune development, when compared to UE infants early in the first year of life, which is a time of increased infectious disease susceptibility in HEU infants.

This dissertation addressed three separate objectives.

Objective 1: develop a cohort composed of HEU and UE newborns in South Africa and contrast their clinical outcomes over the first year of life.

Objective 2: provide a comprehensive analysis of innate immune development in a cohort of South African infants.

Objective 3: compare differences in innate immune development, and differences in adaptive immune development between HEU and UE infants.

1.6 Chapter One Tables and Figures

Table 1: Microbial Diagnosis for Severe Pneumonia in HEU relative to HIV-Positive and HIV-Unexposed Infants

[Adapted from: McNally et al. 2007 [26]]

Response to initial antibiotic therapy	Culture type	Most common pathogen 1st>2nd>3rd ...	Age	Frequency of patients (%)		
				HIV +	HEU	UE
RESPIRATORY INFECTIONS						
Responded	Blood	*Streptococcus* pneumonia > *Staph aureus* > Viridans group streptococci	<1year	↑ – –	– ↑ ↑	– – –
Failed initial therapy	Blood/ Respiratory*	*Pneumocystis jirovecii* > *Mycobacterium tuberculosis* > *Cytomegalovirus*	<1year	↑↑↑ ↑↑ ↑↑↑	↑↑↑ ↑↑ ↑↑↑	–

*Respiratory cultures include, nasopharyngeal aspirate, bronchoalveolar lavage, gastric washings or pleural aspirate. **-** <5% of cases; ↑ 5-10% of cases; ↑↑ 10-20% of cases; ↑↑↑ >20% of cases

Table 2: Microbial Diagnosis for Severe Diarrhea in HEU relative to HIV-Positive and HIV-Unexposed Infants

[Adapted from: Pavlinac 2014 [144] and Kotloff 2013 [145]]

Study	Culture type	Most common pathogen 1st>2nd>3rd ...	Age	Frequency of patients (%)		
				HIV +	HEU	UE + HEU
Palvinac *et al.*, 2014	Fecal	enteroaggregative *E. coli* >	<24 months	↑↑↑	↑↑↑	↑↑
		Giardia species >		↑↑↑	↑↑↑	↑↑↑
		Campylobacter species >		↑↑	↑↑	↑↑
		enteropathogenic *E. coli* >		↑↑	↑↑#	↑↑
		Cryptosporidium		↑	↑↑*	↑
				All infants		
Kotloff *et al.*, 2013	Fecal	Rotavirus >	<24 months	↑↑↑		
		Cryptosporidium >		↑↑		
		Enteroaggregative *E. coli* >		↑↑		
		enteropathogenic *E. coli* >		↑↑		
		Shigella		↑		

<3% of cases; ↑ 3-5% of cases; ↑↑ 5-10
% of cases; ↑↑↑ 10-15%; ↑↑↑↑ 15-20% of cases
* prevalence ratio compared to unexposed = 2.81; # prevalence ratio compared to uninfected = 3.7

Table 3: Pattern Recognition Receptors and Respective Ligands, Adaptor Proteins, Transcription Factors and Resulting Immune Response

PRR	Ligand	Synthetic analogue	Site of interaction	Relative Expression (TLR#)	Resulting Immune Response
TLR2/1 **(TLR2/6)**	Bacterial PGN, lipoteichoic acid, lipopeptides, mycobacterial lipoarabinomannan. -Recognition is mediated by heterodimers of TLR2/TLR1 or TLR2/TLR6	PAM3Csk4	Cell surface	Mo: ++ (1) ++ (2) ++ (6) cDC: ++ (1) ++ (2) ++ (6) pDC: + (1) – (2) ++ (6) (TLR1) Mo and B cells > cDCs (TLR2) Mo and B cells > cDCs (TLR6) Mo and cDCs, B cells > pDCs	Innate: ↑ TNF-α, IL-6, IL-12, IL-8 and NO_2 and promotes mast cell degranulation Adaptive: ↑ IFN-γ, IL-2, IL-6, IL-10, IL-17 from T cells Promotes Th1 and Th17 differentiation
TLR3	Double-stranded RNA	Poly I:C	Endosome	Mo: – cDC: ++ pDC: – cDCs	Innate: ↑ type I IFNs, TNF-α, IL-6 and IL-12 Adaptive: ↑ IFN-γ, TNF-α and IL-2 from CD8 T cells
TLR4	LPS, RSV	N/A*	Cell surface	Mo: ++ cDC: – pDC: – Mo > cDCs	Innate: ↑ IL-6, IL-12 and TNF-α ↑ costimulatory receptor and type I IFN production by dendritic cells Adaptive: ↑ IFN-γ-producing CD8+ T cells; ↑ TNF-α, IL-12, IFN-γ, IL-2 and IL-6
TLR5	Flagellin	N/A*	Cell surface	Mo: ++ cDC: + pDC: – Mo > cDCs	Innate: Induces strong IgM and IgG responses Adaptive: Not determined
Continued on following page					

PRR	Ligand	Synthetic analogue	Site of interaction	Relative Expression (TLR#)	Resulting Immune Response
TLR7/8	Single-stranded RNA	R848	Endosome	Mo: + (7) ++ (8) cDC: + (7) ++ (8) pDC: ++ (7) + (8) (7) pDCs and B cells ≫ cDCs > Mo (8) Mo and cDCs	Innate: ↑ IFN-α, IL-12 and TNF-α; -recruits DCs and CTLs Adaptive: ↑ mature dendritic cells in draining lymph nodes ↑ antigen-specific CD4+ and CD8+ T-cell responses ↑ TH1 bias
TLR9	Unmethylated CpG DNA	CpG oligonucleotides	Endosome	Mo: – cDC: – pDC: ++ pDCs and B cells	Innate: Activates immune cells and cytokine production for strong TH1-type responses Adaptive: Increased the number of IFN-γ-producing antigen-specific CD8+ T cells and the level of cytolysis; improved protection against vaccinia virus infection compared with separately administered antigen and adjuvant

+, ++ and – indicate moderate, high and low/no mRNA expression for each TLR, respectively
References: Hubbell, 2009 [146]; Medzhitov, 2001 [88]; West, 2006 [147]; Iwasaki, 2004 [148]; Takeuchi, 1999 [149]; Morgan, 2014 [150]; Takeda, 2005 [89]
#mRNA expression from freshly isolated APCs

Table 4: Developing and Developed Nation Immunization Schedule: South Africa and Canada

Adapted from: [151,152]

South Africa		Canada	
Age	**Scheduled Vaccination (Dose)**	**Age**	**Scheduled Vaccination (Dose)**
Birth	OPV (1) / BCG (1)	**Birth**	N/A
6 weeks	OPV (2) / RV (1) / DTaP-IPV (1) / Hib (1) / HepB (1) / PCV (1)	**6 weeks**	N/A
10 weeks	DTaP-IPV (2) / Hib(2) / HepB (2)	**8 weeks**	DTaP-IPV (1) / Hib (1) / HepB (1) / PCV (1) / Men-C (1)
14 weeks	DTaP-IPV (3) / Hib (3) / RV (2) / HepB (3) / PCV (2)	**16 weeks**	DTaP-IPV (2) / Hib (2) / HepB (2) / PCV (2) / Men-C (2)
6 months	N/A	**6 months**	DTaP-IPV (3) / Hib (3) / HepB (3) / PCV (3)
9 months	Measles (1) / PCV (3)		N/A
12 months	N/A		MMR (1) / PCV (4) / Var (1)
18 months	DTaP-IPV(4) / Hib(4) / Measles(2)	**18 months**	DTaP-IPV (4) / Hib (4) / MMR (2)
6 years	Td (1)	**4-6 years**	DTaP-IPV (5) / MMR (3)
12 years	Td (2)	**12 years**	N/A
14-16 years	N/A	**14-16 years**	TDaP (1)

Oral polio vaccine (OPV); Baccile Calmette-Guerin vaccine (BCG); Respiratory syncytial virus vaccine (RV); Diphtheria, tetanus, acellular pertussis (combined with) inactivated polio virus vaccine (DTaP-IPV); *Haemophilus influenzae* b vaccine (Hib); Hepatitis B vaccine (HepB); Pneumococcal conjugate vaccine (PCV); Meningococcal C vaccine (Men-C); Measles vaccine (Measles); Measles mumps rubella vaccine (MMR); Varicella vaccine (Var); Tetanus, diphtheria vaccine (Td); Tetanus, diphtheria, acellular pertussis vaccine (TDaP)

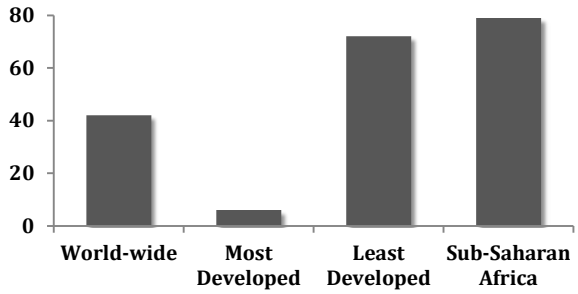
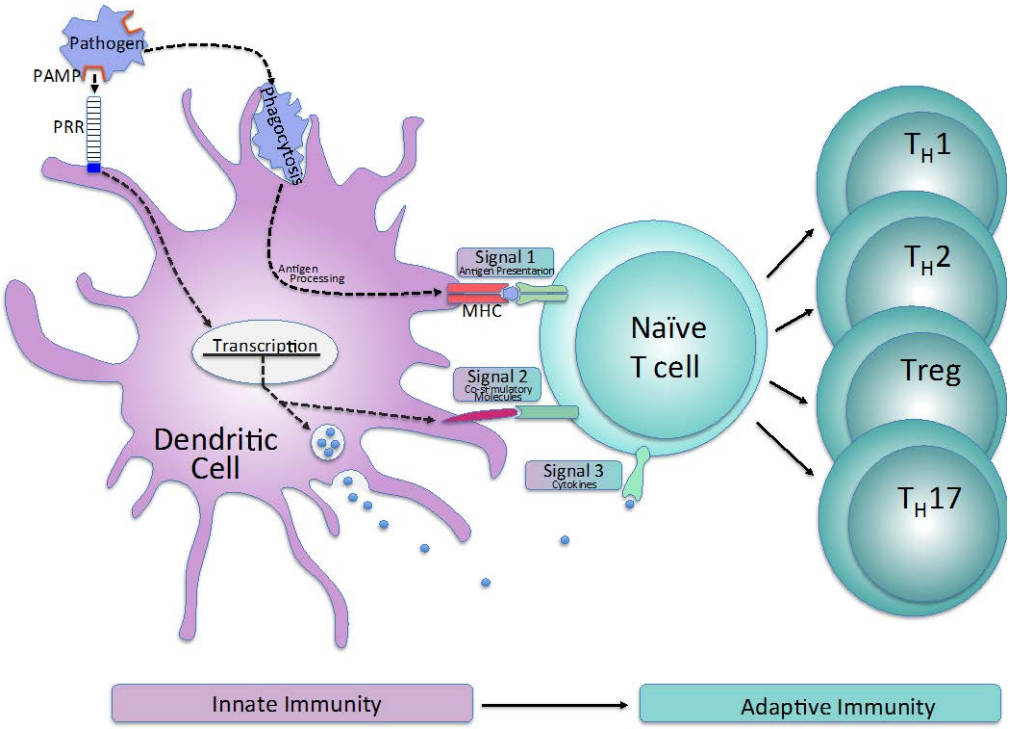


Figure 1: Infant Death Per 1000 Births [2]



Adapted from Kapsenberg, 2003 [153] and Takeda et al., 2005 [89]

Figure 2: Pathogen Detection by Pattern Recognition Receptors Drives Dendritic Cell Responses and Subsequent Polarization of Antigen-Specific Adaptive Immunity

2. HIV-EXPOSED UNINFECTED INFANTS ARE AT INCREASED RISK FOR SEVERE INFECTIONS IN THE FIRST YEAR OF LIFE

2.1 Introduction

Globally, 1.5 million children are born to HIV-infected mothers each year. One fifth of these births occur in South Africa (SA), with the highest antenatal HIV seroprevalence worldwide, unchanged at close to 30% since 2004 [154]. PMTCT interventions have lowered in-utero and intra-partum HIV infection to < 5% [15]. Therefore more than a quarter of SA's newborns are HEU. However, increased mortality and morbidity in formula and breast fed HEU infants has been described [19,155]. The underlying reasons for these poor outcomes are probably multifactorial [10,156,157]. Avoidance of breastfeeding has proven catastrophic in areas with a high burden of infectious diseases and malnutrition [157,158]. But it is not only formula fed HEU infants that experience more frequent infections and death. The ZVITAMBO study, the largest prospective cohort directly comparing HEU and UE infants showed an increased mortality and morbidity in breastfed Zimbabwean HEU infants prior to the availability of a PMTCT program [19,115]. After documenting a case series of severe opportunistic infections in HEU infants [159], we initiated a pilot cohort study to explore reasons for their compromised health. This report aims to compare infection-related clinical outcomes of HEU and UE infants in the first year of life.

During this study PMTCT prophylaxis in SA consisted of maternal and infant short-course zidovudine with single dose nevirapine. Maternal CD4 count < 200 cells/µl

qualified for lifelong combination antiretroviral therapy (cART). The program provided six months of infant formula for mothers choosing not to breastfeed.

2.2 Methods

2.2.1 Ethics Statement

This study was specifically approved by the Research Ethics Committee of Stellenbosch University and the IRB of the University of British Columbia (Protocol H09-02064 and H11-01947). Informed written consent from the next of kin, care givers or guardians on the behalf of the minors/children participants involved in our study was obtained for all study participants.

2.2.2 Enrollment

Infants were recruited in 2009 from the labor ward of Tygerberg Academic Hospital, a tertiary center in the Western Cape. Infants were assessed at 2, 6, 12, 24 and 52 weeks for clinical outcomes. HIV-uninfected infants born to HIV-infected mothers were classified as HEU. Infants born to HIV-uninfected mothers were classified as UE. All infants were confirmed as HIV-uninfected by HIV-DNA-PCR (Amplicore version 1.5, Roche Diagnostics, USA) at 2, 6 and 12 weeks.

2.2.3 Clinical Data Collection

Maternal demographic, obstetric and basic social characteristics were collected at 2 weeks through a structured interview. HIV-uninfected mother's HIV-infection status was confirmed on presentation in labor using standard HIV testing algorithms [45]. Infant health history including caregiver report of any infant infectious event, anthropometric measurements, confirmation of immunizations from infant immunization cards, and physical examinations were conducted by unblinded medical professionals and recorded onto a standardized report form. A trained research nurse measured weight and length. The primary clinical outcome was the type and severity of infection-associated hospitalizations, which were extracted from the hospital chart after caregiver self-report. Only hospitalizations for presumed infections based on clinical features, and/or positive microbiological cultures performed as local standard of care, were included. Hospitalizations were graded for severity by an unblinded pediatrician from 1 (mild) to 5 (fatal) according to the Division of AIDS (DAIDS) Table for Grading the Severity of Adult and Pediatric Adverse Events (12). Severe anemia was defined as DAIDS grade $\geq$ 3.

2.2.4 Data Analysis

Anthropometry was compared as WHO Z-scores. Continuous variables were analyzed using the Student's t-test and categorical variables the Chi-square or Fisher's exact test. The incidence rate of any caregiver-reported infectious event included the total number of reported infectious events over the total number of

months of infant follow-up. The relative risk (RR) for infection-associated hospitalization was calculated comparing the number of infants hospitalized at least once in each group. Both a cumulative incidence ratio, including only infants with complete follow-up to 12 months, as well as an incidence rate ratio, including all infants with any duration of follow-up with the denominator as number of months of follow-up, were calculated. Two-sided alpha was set at 0.05 and Bonferroni adjustment for multiple comparisons was applied. Statistical analysis was performed using R version 2.13.1 (The R Foundation for Statistical Computing; Vienna, Austria).

2.3 Results

Fifty-five infants were enrolled at birth and retained at 2 weeks of age, 27 were HEU and 28 UE. All HEU infants remained HIV-uninfected at 6 and 12 weeks in the absence of breastfeeding. Maternal and infant characteristics are shown in Table 5. The median duration of exclusive breastfeeding in UE infants was 12 weeks. Three infants, 2 UE infants at 6 months and 1 HEU at 12 months had moderate acute malnutrition with a weight-for-length Z-score (WLZ) < – 2.

There was no difference in the number of caregiver-reported infectious events; 14.7 (95% CI11.4-18.9) compared to 13.9 (95% CI 10.7-18.1) events per 100 months of infant follow-up in HEU and UE respectively. Fourteen infants were hospitalized at least once: 14 hospitalizations in 10 HEU infants and four hospitalizations in four UE

infants. Nine of 14 (64%) HEU and three of four (75%) UE hospitalizations occurred during the first six months. Among infants who completed follow-up to 12 months (23 HEU and 21 UE) the RR to be hospitalized was 2.74 (0.85-8.78) times greater for HEU than UE. Considering all infants observed for any duration, HEU infants experienced an incidence rate of 3.4 (1.86-6.26) hospitalized infants per 100 infant-months, compared to 1.4 (0.57-3.60) in UE infants for an RR of 2.42 (0.70-10.59).

Lower respiratory infections accounted for 50% (9/18) of hospitalization events: 4 bronchiolitis (4 HEU, 0 UE), 4 pneumonia (2 HEU, 2 UE) and 1 probable pulmonary tuberculosis (1 HEU, 0 UE). The remaining events were severe gastroenteritis (3 HEU, 0 UE), culture confirmed urinary tract infections (2 HEU and 1 UE), neonatal sepsis (0 HEU, 1 UE), varicella zoster (1 HEU, 0 UE) and measles (1 HEU, 0 UE). The median DAIDS grade for hospitalizations was 3 in each group. Two HEU and one UE infants were hospitalized for events graded < 3. One grade 4 event occurred in an HEU infant and one grade 5 (fatal) event in an UE infant, the latter due to adenovirus pneumonia at 8 months.

All 14 hospitalized infants were appropriately immunized, were not severely anemic and had 6-month WLZ above the mean for the cohort (Table 5). Three fully immunized HEU infants with normal growth each experienced $\geq$ 2 hospitalizations. One of these three mothers was on cART, one on PMTCT prophylaxis and one received no antenatal care. The two mothers receiving antenatal care had CD4 counts > 350 cells/μl.

2.4 Discussion

We observed no difference in the number of caregiver-reported infectious events in HEU compared to UE infants. Twice as many HEU had at least one infection-associated hospitalization in the first year of life. This difference in hospitalizations did not reach statistical significance, but the confidence intervals were wide due to the small sample. Thus, our preliminary observation suggests that although HEU infants are not necessarily experiencing a greater number of infectious events, they may be at higher risk for more severe infectious events requiring hospitalization. This is consistent with findings of marginally increased morbidity [19] but impressively increased mortality [115,130] in HEU from other African settings where access to healthcare, and hospitalization, may be less readily available than in South Africa. The observed infection risk in the present cohort occurred in the absence of a number of factors postulated to place HEU infants at higher risk. Low birth weight or prematurity, missed immunizations, malnutrition, infant anemia and severe maternal immunosuppression did not account for the infectious morbidity in this cohort. Comparison by feeding mode was not possible with only one breastfed HEU infant. Severe gastroenteritis occurred only in HEU infants, which may be related to formula feeding. However HEU infants were not compromised nutritionally as measured by anthropometry, their growth was not inferior to UE infants and the majority of severe infections were respiratory (7 of 14), not gastrointestinal (3 of 14). In early infancy breastfeeding protection against diarrhea is substantially

greater than protection against respiratory infections [12] suggesting that this cohort of infants were not particularly disadvantaged due to absence of breastfeeding alone. Measles was observed in a 7-month old HEU infant prior to receiving measles immunization. Poor measles IgG transplacental transfer has been described in HIV-infected mothers [160]. However, vaccine preventable infections were not implicated in any of the other hospitalizations in our study.

Over-hospitalization of HEU infants could have introduced bias as admitting clinicians were not blinded to HIV exposure status. However, the DAIDS grade of hospitalization events $\geq$ 3 (75% UE and 80% HEU), suggests at a minimum, limited if not absent indication bias. Significantly more HEU infants were of African descent. We are not aware of ethnically determined genetic variations that would account for this infection risk. Detailed socioeconomic data were not available to determine the role of social and household circumstances. However, the entire cohort accessed free government health services and maternal education was similar, diminishing the likelihood of gross differences in socioeconomic position and quality of health care.

Although small, the strength of this cohort is the direct comparison of HEU to UE infants, which has not previously been conducted in South Africa. It is recognized that a gradient of HEU risk determined largely, but not exclusively, by feeding practice and maternal HIV disease severity exists [10], however this risk has not been well quantified against UE infants. With up to 25% of South African infants being

HEU, it is crucial for public health planning to determine the increased burden of disease this group experiences above that of UE infants.

This cohort of HEU infants showed a trend toward increased infection-related hospitalizations in the first year of life in the absence of an increased number of infectious events, advanced maternal HIV disease or infant malnutrition. HEU infants experienced 2.74 (0.85-8.78) times greater risk of hospitalization in the first year. The current WHO infant feeding guidelines [124] recommending breast milk with postnatal prophylaxis for HIV-exposed infants should improve HIV-exposed infant outcomes, however as even breastfed HEU infants still experience greater infectious morbidity and mortality than UE infants [19,115] consideration of other environmental and infant host risk factors is required. Prospective comparisons between HEU and UE are urgently required to determine relative contributions of HEU infant immune response and environmental exposure.

2.5 Chapter Two Tables and Figures

Table 5: Maternal and Infant Characteristics

	Total (55)	**HEU (27)**	**UE (28)**	**P-value**
Maternal Characteristics				
Age (in years) – mean (SD[1])	26.6 (6.3)	25.9 (6.8)	27.3 (5.7)	0.4
Multigravida (%)	40 (73)	21 (78)	19 (68)	0.6
Received antenatal care (%)	48 (87)	24 (89)	24 (86)	1
Completed secondary education (%)	15 (27)	6 (22)	9 (32)	0.6
Smoked during pregnancy (%)	14 (25)	3 (11)	11 (39)	0.17*
Consumed alcohol during pregnancy (%)	4 (7)	0 (0)	4 (14)	0.11
HIV diagnosed during pregnancy (%)		14 (52)		
Antenatal CD4 count in cells/µl – median (range)		337 (131-673)		
Antiretroviral exposure				
None (%)		3 (11)		
cART[2] (%)		4 (15)		
PMTCT[3] prophylaxis (%)		19 (70)		
Unknown		1 (4)		
Infant Characteristics				
Male (%)	22 (40)	7 (26)	15 (54)	0.35*
Ethnicity				0.001*
African[4] (%)	30 (55)	22 (81)	8 (29)	
Mixed or Caucasian[5] (%)	25 (45)	5 (19)	20 (71)	
Birth weight in g – mean (95% CI[6])	2966 (2857-3075)	2945 (2866-3024)	2986 (2830-3142)	0.7
Gestational age in weeks - mean (95% CI[6])	37.8 (37.1-38.4)	37.7 (36.7-38.7)	37.9 (37.0-38.7)	0.8
Received any breastfeeding (%)	29 (53)	1 (4)	28 (100)	<0.001*
Infant Anthropometry				
6 months - N	47	25	22	
WAZ[7] (SD[1])	-0.25 (1.07)	+0.17 (0.95)	-0.73 (1.16)	0.03*

Continued on following page

	Total (55)	HEU (27)	UE (28)	P-value
Infant Anthropometry				
LAZ[8] (SD[1])	-0.64 (1.26)	-0.44 (1.05)	-0.87 (1.61)	0.42
WLZ[9] (SD[1])	+0.32 (1.16)	+0.68 (1.05)	-0.09 (1.17)	0.13*
12 months - N	44	23	21	
WAZ[7] (SD[1])	-0.09 (1.16)	+0.26 (1.13)	-0.47 (1.09)	0.18*
LAZ[8] (SD[1])	-0.20 (1.29)	+0.10 (1.09)	-0.53 (1.43)	0.22
WLZ[9] (SD[1])	+0.04 (1.15)	+0.32 (1.05)	-0.26 (1.19)	0.10

Key: (1) SD – standard deviation; (2) cART – combination antiretroviral therapy; (3) PMTCT – vertical transmission prevention; (4) African includes infants of Xhosa speaking South African [n=27], Malawian [n=2] and Zimbabwean [n=1] descent (5) All mixed ethnicity except 1 Caucasian UE (6) CI – confidence interval; (7) WAZ – weight-for-age Z-score; (8) LAZ – length-for-age Z-score; (9) WLZ – weight-for-length Z-score

* Adjusted for multiple comparisons

3. ONTOGENY OF TOLL-LIKE RECEPTOR MEDIATED CYTOKINE RESPONSES OF SOUTH AFRICAN INFANTS THROUGHOUT THE FIRST YEAR OF LIFE

3.1 Introduction

The first year of life represents one of the most important periods of change for the immune system [56-59]. Increased risk for infectious related morbidity and mortality, as well as suboptimal response to vaccination, is likely related to less effective innate immune function in neonates and infants as compared to adults [59,61-64].

Antigen presenting cells represent the key link between the innate and adaptive immune system [76,77]. We previously developed a stringently controlled high-throughput platform that enables robust investigation of changes in APC function over time (described in Appendix A) [81-83]. Using this platform, we had examined the TLR response of the four major human APC in a cohort of children born and raised in North America and found that instead of lower innate immune responses to TLR stimulation in early life, strikingly different response patterns exist that dynamically change over the first 2 years of life [38,161]. These findings were in agreement with studies of infants born and raised in other resource-rich settings [36,37].

In our current study we prospectively followed a cohort of infants born and raised in South Africa over the first year of life. Our previously established comprehensive platform was used to analyze production of innate cytokines in key functional

categories utilizing both multiplex bead array and intracellular cytokine cytometric strategies [81-83]. Dynamic and unique development of innate immunity was identified in our cohort, which changed with time and with the stimulus employed. Given these as well as similar previous accounts [39-41], it is likely that dramatic differences in innate immune ontogeny exist between different populations.

3.2 Methods

3.2.1 Prospective Birth Cohort Study Design

A prospective, longitudinal cohort study commenced in 2009 in Cape Town, South Africa, to evaluate immune function in infants early in life. Infant recruitment, and infant and mother serological HIV testing was performed as described in Chapter 2 Methods. Equal numbers of HEU and UE were recruited into the study concurrently. Exclusion criteria included: (1) diagnosis of a significant chronic medical condition including: immunosuppression by disease or medication; cancer; bone marrow or organ transplantation; blood product administration within the last 3 months; bleeding disorder; known congenital malformation or genetic disorder. (2) if parent or legal guardian were unable to read and/or comprehend the consent process. Additionally, any febrile illness within the last 24 h, or brief (<1 months) immunosuppressive medication use within one month, would result in a deferral of the blood draw to a later date. Fifty-four percent of enrolled subjects were male; the mean gestational age of enrolled infants in weeks was 37.9 (37.0-38.7 CI), and the

mean birth weight in grams was 2986 (2830-3142 CI). Maternal and infant characteristics are summarized in Table 5. Infants were followed at 0.5, 1.5, 3, 6, and 12 months (3 month innate responses were not analyzed). We initially enrolled 29 UE infants (concurrently enrolled with HEU infants, who were not analyzed for this study) and were able to analyze 28 of them at 0.5 months (one infant did not provide a blood sample), 26 at 1.5 months, 23 at 6 months and 20 at 12 months. We were unable to locate and contact the 8 infants that were lost to follow up over the first year of life. Infants received their vaccinations according to South Africa's Expanded Program for Immunization (EPI). At each visit, infant health history was obtained, receipt of immunizations on the infant immunization card verified, and physical examinations conducted by medical professionals. The median duration of exclusive breastfeeding was 12 weeks. Healthy adults, unrelated to the infants, aged 24 to 47, of equal male-female ratio and ethnic background as the infant study subjects were recruited at Stellenbosch University.

3.2.2 Blood Sample Processing

3–5ml of peripheral blood was drawn into sodium-heparin tubes and then immediately processed as described previously [82,83]. Samples were diluted 1:1 with RPMI-1640 media and added to prefabricated stimulation plates containing PAMPs at concentrations that elicit optimal cytokine expression [82]. Six TLR and NOD agonists were used, which represent canonical bacterial vs. viral stimuli, e.g. bacteria (CpG, PAM), Gram negative bacteria (LPS), Gram positive bacteria (PGN),

single stranded RNA viruses (R848), or double stranded RNA viruses (pI:C). CpGA (CpG_{Coley}) stimulates signaling through TLR9, PAM3CSK4 ($PAM_{EMC\ microcollections}$) signals via TLR2/1, 0111:B4 LPS ($LPS_{InvivoGen}$) through TLR4, Peptidoglycan ($PGN_{InvivoGen}$) via TLR2 and NOD1/2, TLR7/8 is stimulated by $R848_{InvivoGen}$, and $pI:C_{Amersham}$ through TLR3 [162]. Stimulation plates contained the vesicle transport inhibitor BFA (added at T=0h for PGN, PAM, LPS and R848, or at T=3h for pI:C and CpG as described [82]) and were incubated for 6h at 37°C in 5% CO2. Cell pellets were stored frozen in FACS Lysing Solution$_{BD\ Biosciences}$ as described [82]. An identical set of plates was incubated in parallel for 18 h without BFA; at 18 h, these plates were spun and 100 µl of supernatant was removed and frozen at -80°C for later Luminex analysis.

3.2.3 Assessment of Cytokines in Culture Supernatant

Supernatants were thawed at room temperature, and filtered through a 1.2-µm filter plate (Millipore) into a clean 96-well plate to remove any remaining cellular debris using a multi-screen HTS vacuum manifold (Millipore). The Luminex assay was performed using the Upstate/Millipore "Flex Kit" system using the high-biotin protocol and overnight incubation at 4°C. Cytokines measured were IFN-α2, IFN-γ, Interferon-γ inducible protein (IP)-10, IL-12p70, IL-12p40, IL-6, TNF-α, IL-1β, IL-8, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, IL-10. Samples were diluted 1-to-1 (10- or 20-fold if needed to fall within the standard curve) with RPMI 1640 supplemented with 10% human AB

serum. Assays were read using Luminex 200 Total System (Luminex) running either the Bio-plex (Bio-Rad) or the MasterPlex (MiraiBio) softwares, and the downstream analysis was performed using Excel (Microsoft) and an in-house database. To determine the IL-23 concentration, filtered supernatants were diluted 1:4 in diluent contained in the human IL-23 (p19/p40) enzyme-linked immunosorbent assay (ELISA) kit (eBioscience), and assays were performed according to the manufacturer's specifications. Plates were read at 450 nm with 570-nm subtraction. A sigmoid logistic curve was used to generate the standard curve. The responses observed in the unstimulated (negative) controls were negligible for all subjects, and subtracted from the stimulated samples for each individual.

3.2.4 Staining, Acquisition, and Flow Cytometric Analysis

A detailed description of antibodies (source, clone, and dilution), machine set up, and data acquisition compliant with MiFlowCyt reporting standards [163,164] is provided in supplementary methods. Samples were prepared for flow cytometric analysis as previously described [82]. Stained and fixed cells were analyzed on a FACSAria flow cytometer$_{\text{BD Biosciences}}$ set up using a biological standard, according to published guidelines[164]. B-cells, monocytes, cDCs and pDCs were differentiated, and intracellular TNF-α, IL-6, IL-12 and IFN-α were detected. 300,000 events were acquired per sample for analysis with FlowJo software$_{\text{Tree Star}}$ and gates were set based on the fluorescence-minus-one principle [165]. Negative controls from

unstimulated samples that produced cytokine above cutoffs were negligible and were subtracted from stimulated samples [165].

3.2.5 Statistical Analysis

Graphs were prepared using Excel (Microsoft). Statistical differences between infant pairs were analyzed using the Wilcoxon matched-pair signed rank test. To correct for multiple comparisons of the secreted cytokine analysis, we computed the Bonferroni corrected acceptable Type I error rate (alpha) as 0.05/6 stimulation conditions = 0.01 (0.0083), as these would yield false discoveries at a rate of < 0.05 (for a total of 6 comparisons for each data set) [166]. Accordingly, p-values of <0.01 were considered significant. To assess polyfunctionality (i.e., the ability of an individual cell to produce one vs. more than one cytokine in response to a specific stimulus), we computed the percentage of cells in a given cytokine-combination category (e.g., there are 7 possible cytokine-combination categories for 3 cytokines in which at least one cytokine is positive (23 – 1 = 7)). The percentages of reactive cells that were positive for only one of the 3 cytokines were added up to give the polyfunctional degree (PFD) 1 (PFD1); and separately, the percentages of reactive cells expressing any 2 of the 3 cytokines or all 3 cytokines were computed to give PFD2 and PFD3 values, respectively. Given that values of polyfunctional subsets in flow cytometry represent qualitative composites of several categories of quantitative data, and statistical analysis would thus be of little value, the flow

cytometric analysis of polyfunctional cytokine production was not subjected to statistical examination.

3.3 Results

3.3.1 Striking Stimulus-Dependent and Age-Dependent Differences in Secreted Cytokine Production in Response to PRR Stimulation of South African Infants' Whole Blood Within the First Year of Life

Stimulation of PRRs with PAMPs allows cells expressing the relevant receptors to respond with changes in cytokine production [167]. In Figure 3 and Figure 4 we grouped the 14 cytokines we analyzed based on their major known functions, i.e. those promoting either Th1 (IFN-α, IFN-γ and IL-12p70, as well as interferon-γ inducible protein 10 (IP-10)), or Th17 development of CD4 T cells (IL-12p40, IL-23 and IL-6), those considered to be general pro-inflammatory or chemoattractant (TNF-α, IL1-β, IL-8, MCP-1, MIP-1α, MIP-1β) or anti-inflammatory (IL-10).

Only TLR3, TLR7/8 or TLR9 agonists induced production of the Th1 promoting cytokines IFN-α, IFN-γ or IL-12p70 (Figure 3; Supplemental Table 6). Specifically, TLR7/8 stimulation with R848 led to IFN-α secretion in younger infant samples that was significantly higher compared to the 12 month-old infant and adult samples. TLR9-induced IFN-α production followed a similar trend, from a high at 2 weeks to an adult-low by 12 months of age. TLR3-induced production of IFN-γ and IL-12p70

in infant samples at 2 weeks, 6 weeks and 6 months of age was found at levels above those of adults, with the 12-month samples approaching the adult-low level. TLR7/8-induced IFN-γ production was also highest in the 2 and 6 week infant samples, but samples from infants 6 and 12 months of age already secreted IFN-γ at or below adult samples by 6 months of age. As expected, the production of IP-10 largely followed the trends of IFN-γ, with the notable exception of a peak around 6 months of age in response to TLR7/8 stimulation.

Production of Th17 promoting cytokines (IL-23 (consisting of IL-12p40 and p19) and IL-6) occurred in response to a broader range of stimuli as compared to Th1-promoting cytokines (Figure 3; Supplemental Table 6). NOD1/2 & TLR2 stimulation induced high levels of both IL-23 and IL-6 in infant samples that decreased by 12 months to an adult-low. TLR2/1 activation induced a strong IL-6 and IL-23 response that followed a similar trend, but did not induce IL-12p40 above background. TLR3 stimulation led to IL-23 production in the early infant samples that decreased to an adult low by 12 months of age yet induced no IL-6 secretion in any of the age groups tested. The TLR7/8 agonist R848 induced IL-6 secretion that dropped from an early infant high to an adult low by 12 months, but sustained higher levels of IL-23 in infant samples as compared to adult samples. TLR4 stimulation induced sustained high IL-6 production, but, contrary to all other trends, an increase in IL-23 production from a 2 and 6 week low to above adult levels at 6 and 12 months of age.

Secretion of pro-inflammatory/chemoattractant cytokines occurred in response to the widest range of stimuli (Figure 4; Supplemental Table 6). For example, NOD1/2 & TLR2, TLR2/1 as well as TLR7/8 induced production of TNF-α, IL-1β, IL-8, MCP-1, MIP-1α, and MIP-1β that was high in the younger age groups, decreasing over the first year of life to reach adult-low levels around 12 months of age. TLR3 and TLR9 stimulation on the other hand only led to production of MCP-1, but followed a similar trend over time. TLR4 was again the only pathway that displayed opposite trends in comparison to other PRR. Specifically, TLR4-induced production of TNF-α, IL-1β, MIP-1α, and MIP-1β all increased from a low early in life to a high at 12 months of age; IL-8 production in response to TLR4 stimulation was overall low but higher in the infant as compared to adult samples. MCP-1 was the only cytokine produced in response to TLR4 stimulation that followed the more common trend of an early high response decreasing to an adult low by 12 months of age. Secretion of the anti-inflammatory cytokine IL-10 occurred only in response to NOD1/2 & TLR2, TLR2/1 and TLR7/8 stimulation, and generally decreased from a 2 week high over the first year of life to reach adult-low levels by 12 months of age.

When comparing the level of PRR-induced cytokine secretion between each successive age group, an increase in the number of statistically significant differences was detected with increasing age for all PRR responses except for TLR4 (Supplementary Table 6). Specifically, there was no difference in the production of any cytokine between 2 and 6 week-old infant samples after NOD1/2 & TLR2 stimulation. Only 2 cytokines (IL-10 and MCP-1) differed significantly between 6

weeks and 6 months, but 7 cytokines (IL-10, IL-23, IL-6, MCP-1, MIP-1α, MIP-1β, and TNF-α) displayed statistically significant differences between the infants' response to NOD1/2 & TLR2 stimulation at 6 month and 12 month of age. A similar increase in the number of significant differences with advancing age was also observed following TLR2/1, TLR3, TLR7/8 and TLR9 stimulation. Again, only the response to TLR4 stimulation did not follow this trend, as cytokine secretion following TLR4 stimulation did not differ significantly between 2 and 6 week old infants, nor between 6 month and 12 month old infants, and only 2 cytokines (IL-23 and MIP-1α) differed significantly between the infants at 6 weeks and 6 months of age.

3.3.2 Single Cell Analysis of Intracellular Cytokine Production Within the First Year of Life

To determine the cellular source of key cytokines detected in the culture supernatant, intracellular cytokine cytometry (ICC) was employed as previously described [38,82,161,164]. Our panel of surface anchor markers allowed identification of the major APC target populations in whole blood (monocytes, B cells, cDC and pDC, Supplemental Figure 9 and Figure 10), in parallel to the expression of cytokines in each of these major APC subsets (Supplemental Figure 11). A rapid rise in the fraction of B cells was detected after 2 weeks of age with a slow decline in the fraction of monocytes over the same time period; cDC and pDC populations however, remained relatively stable over the first year of life (Supplemental Figure 10). Such

changes in cellular composition together with changes in cellular compartments not analyzed by our flow cytometry-based approach, might affect the cytokine secretion measured in the culture supernatant described above, and thus must be considered as a variable during interpretation of results.

The polychromatic single cell flow cytometric approach enabled assessment of TNF-α, IL-6, IL-12/23p40 and IFN-α production in response to PRR stimulation of monocytes, cDC, pDC and B cells. In this study, B cells did not produce any of these cytokines under any of the conditions tested (data not shown). Monocytes and cDC did not respond to TLR3 (polyinosinic:polycytidylic acid, pI:C) or TLR9 (CpG) stimulation above background, while pDC responded to only TLR7/8 (R848) (data not shown). Therefore, analysis was focused on monocyte and cDC responses following stimulation of TLR2 & NOD1/2 (PGN), TLR1/2 stimulation (PAM), TLR4 (LPS), and TLR7/8 (R848) (Figure 5, Figure 6 and Figure 7), and on pDC responses, after TLR7/8 (R848) stimulation (Figure 8). TLR/NOD stimulation of monocytes and cDC elicited TNF-α, IL-6, and IL-12/23p40 responses, but not IFN-α, whereas TLR7/8 stimulation of pDC elicited TNF-α, IL-6, and IFN-α responses, but not IL-12/23p40. Therefore, data only for the 3 cytokines produced by each respective cell type is displayed. Visualization of the degree of polyfunctionality is further augmented for each of the innate cell subsets with line graphs illustrating the proportion of cells producing either 1, 2, or 3 cytokines in response to PRR stimulation. The IL-12 family members p35, p19, p28 and EBI3 could not be reliably detected above background by ICC with currently available reagents (data not

shown); only IL-12p40 could be detected as we have previously described [38,82,83,161]. This precludes differentiation of IL-12p70 vs. IL-23 by ICC nor could we exclude detection of IL-12p40 homodimers by ICC.

3.3.3 High Level of Monocyte and cDC Cytokine Response to PRR Stimulation Decreased Over the First Year of Life for All Pathways Except TLR4

Monocytes and cDC displayed an overall very similar response pattern (Figure 5 and Figure 6). For example, NOD1/2 & TLR2 and TLR2/1 both induced production of TNF-α, IL-6, or IL-12/23p40 that was statistically significantly higher in 2 and 6-week samples as compared the 6 and 12-month infant or adult samples. While the overall response level to NOD1/2 & TLR2 and TLR2/1 stimulation in monocytes was maintained for the 6-month samples, cDCs in the same 6-month samples already displayed a statistically significant decrease to near adult levels. The 12-month infant samples displayed an overall response to NOD1/2 & TLR2 and TLR2/1 stimulation that was at or near adult responses. For cDC however, the observed response at 12 months was below the adult and the other infant age-group responses. The response to TLR7/8 stimulation resulted in maximal stimulation and this high level of response was maintained throughout infancy; only cDC in 6 and 12-month samples responded to TLR7/8 stimulation at a level statistically significantly below that of the 6 week old samples. The response pattern of monocytes and cDC to TLR4 stimulation again displayed a different pattern

compared to all other stimuli: while all infant samples revealed a higher overall response as compared to adult samples, the maximal response to TLR4 stimulation was detected in the 6 month samples for both monocytes and cDC; this peak at 6 months however was not significantly different as compared to either the 6 week or the 12 month samples.

Despite similarities in response to NOD1/2 & TLR2 and TLR2/1 stimulation, we detected substantial differences in combinations of the particular cytokines produced (Figure 5 and Figure 6). While TNF-α-only producing monocytes and cDC represented the major population of responders to both NOD1/2 & TLR2 and TLR2/1 stimulation, TLR2/1 stimulation induced larger fractions of IL-6- or IL-12-only producing cells as compared to NOD1/2 & TLR2 stimulation. Both NOD1/2 & TLR2 and TLR2/1 stimulation induced substantial fractions of cells that produced TNF-α and IL-6, and a large proportion of monocytes and cDC produced all three cytokines. These trends were clearly evident in PFD line graphs for NOD1/2 & TLR2 and TLR2/1 stimulation for both monocytes and cDC. TLR7/8 stimulation, however, induced the largest fraction of monocytes and cDCs producing all three cytokines.

The level of expression per cell (i.e. the mean fluorescence intensity, or MFI) of IL-12 in monocytes and cDC decreased in response to every stimulus from a 2 week old high to and adult-low by 12 months of age (Figure 7). While a similar trend was observed for the MFI of IL-6 in monocytes and cDC in response to NOD1/2 & TLR2, TLR2/1 and TLR7/8 stimulation, the IL-6 MFI increased again in monocytes from a

6-week low. For the TNF-α MFI in monocytes and cDC our findings suggest an overall similar level of expression per cell across all age groups in response to NOD1/2 & TLR2, TLR2/1 and TLR7/8 stimulation, and even an increase from 2 weeks to 12 months in response to TLR4 pathway activation.

3.3.4 pDC Cytokine Production in Response to PRR Stimulation Remained High Throughout the First Year of Life

The fraction of pDC responding to TLR7/8 stimulation was similar in all age groups we tested, both in overall response as well as cytokine-combination specific subsets (Figure 8A). For example, the largest fraction of pDCs were positive for both TNF-α and INF-α, followed by cells positive for INF-α only. This was also reflected in the line graph displaying PFD (Figure 8B). However, while the level of expression of INF-α per cell decreased from a 2 week high to a 12 month low, the MFI for TNF-α decreased from a 2 week high to a 6 month low, only to increase again by 12 months of age (Figure 8C).

3.4 Discussion

This is the first longitudinal cohort study analyzing human innate immune ontogeny over the first year of life in a developing nation, and complements similar themed cross-sectional studies from resource poor settings in The Gambia [39], Ecuador [41], and Papa New Guinea [40]. Several well-characterized PRR ligands were used to

stimulate whole blood samples collected in the same cohort of infants at 2 and 6 weeks, 6 and 12 months of age and compared to adult samples according to a robust innate immune profiling platform. A striking decrease from an early high to an adult low by 12 months of age was detected for nearly all cytokines analyzed in response to nearly all stimuli. Specifically, cytokines promoting Th1 or Th17 development such as IFN-α, IFN-γ, IL-12p70 or IL-23 and IL-6 decreased from a high in the first half-year of life to an adult low by 12 months of age. We observed the same age-dependent trend in response to PGN (NOD1/2, TLR2), PAM (TLR2/1), pI:C (TLR3) or R848 (TLR7/8) stimulation for the proinflammatory cytokines or chemoattractants (TNF-α, IL-1β, IL-8, MCP-1, MIP-1α and MIP-1β) as well as the anti-inflammatory IL-10. Only the response to TLR4 stimulation with LPS did not follow this trend, in that production of IL-23, IL-6, TNF-α, IL-1β, MIP-1α, MIP-1β either increased from an early low to a high at 12 months of age or maintained a higher than adult production throughout the first year of life (e.g. IL-8). LPS (TLR4) induced MCP-1 production was the only response that followed the more common age-dependent decline from an early high to an adult-low by 12 months of age. Singe-cell intracellular cytokine cytometry overall confirmed the secreted cytokine pattern, in that PGN (NOD1/2, TLR2), PAM (TLR2/1) and R848 (TLR7/8) stimulation of monocytes and cDC led to production of TNF-α, IL-6 and IL-12/23p40 that decreased from an early high to a low by 12 months of age. Again, only the response of monocytes and cDC to LPS (TLR4) followed the opposite direction of the more common age-dependent trend. The percentage of pDC responding to TLR7/8 (R848) stimulation with INF-α and TNF-α expression remained relatively constant

throughout infancy. The level of expression of each cytokine per cell, i.e. the mean fluorescent intensity, reinforced these findings as only expression of TNF-α per cell remained relatively constant in all cell types across the infant age groups.

The unique pattern of increasing sensitivity to TLR4 stimulation over the first year of life is supported by previous observations that cord blood was poorly responsive to LPS as compared to adults [67]. However, the underlying mechanisms that led to the divergent trends for LPS vs. other PAMPs are currently unclear. Further investigation correlating PAMP responses and susceptibility to infectious morbidity from e.g. Gram negative (via TLR4) versus viral (via e.g. TLR3) may yield valuable information.

Given the changes in cellular composition of WB over the first year of life (Supplemental Figure 9), it is difficult to interpret age-associated changes of cytokine levels detected in culture supernatants. Our previous study in North America and this current study in South Africa represent the first cohort studies to attempt identifying the cellular source of cytokines detected in supernatants extending and complementing previous studies of innate immune ontogeny which only performed secreted cytokine analysis [36,37,39]. We had demonstrated in prior experiments, that multiplex bead array-based analysis is largely congruent with ICC, but does not always produce the exact same pattern of cytokine expression as ICC analysis [38,107]. The comparison of intracellular and secreted cytokine production allows contextualization of cellular responses and provides a comprehensive

approach to describing innate immune ontogeny. While the trends we observed in our South African cohort in innate immune responses evaluated by ICC and multiplex bead array were the same, the magnitude of the change in response with age was much less apparent by ICC than by multiplex bead array. For example, the level of TNF-α detected in culture supernatant in response to TLR2/1 stimulation decreased dramatically between 6 months and 12 months of age (Figure 4), while the number of cells that produced of TNF-α in response to PAM actually remained steady (Figure 5 and Figure 6) and in fact increased on a per cell basis (MFI, Figure 7). This suggests that either cellular sources were involved in cytokine production that we did not assess by flow cytometry (e.g. NK, NKT, or γσ-T cells), or that cell-cell interactions and responses to secreted cytokines were inhibited by the secretion inhibitors used in the experimental set up for flow cytometry. Both of these hypotheses can be interrogated by expanding the range of target cells for ICC and a time course of addition of cytokine secretion inhibitors. We were unable to detect our target cytokines in B cells in response to PAMP stimulation, which confirms previous findings [38], but stands in contrast to data from animal studies [168] which demonstrated cytokine production from marginal zone and follicular B cells in response to TLR stimulation. This may indicate differing responses in white blood cells in peripheral blood vs. tissue resident cells or differences between humans vs. mice.

Our detailed, broadly functional as well as single-cell based analysis of early life innate immune ontogeny adds insight into human innate immune development by

demonstrating dynamic changes of innate immune reactivity specific to age, stimulus and cytokine response. These data also provide the first suggestion of population-specific differences in innate immune development: Contrary to our findings presented here on innate immune ontogeny in South African infants, we had previously detected an increase rather than a decrease from birth up to 2 years of age in Th1 and pro-inflammatory cytokine production following TLR stimulation in infants born and raised in North America [38]. A recent study investigating TLR polymorphisms within a South African population, and the resulting heterogeneity of immune responsiveness to TLR stimulation [52], lends some support to our observation of population specific differences in innate immune responsiveness.

Our studies on North American infants had been conducted on peripheral blood mononuclear cells (PBMC) instead of whole blood (WB), which can lead to significant differences in responses as we have shown [83]. Furthermore, our previous studies did not include infant age groups between birth and 1 year of age, and thus are difficult to directly compare with this current study. Our previous results with North American infants were however in line with studies of European or Papa New Guinean infants that used whole blood and followed infants throughout the first year of life [36,37,40]. Specifically, both Nguyen et al. [36] and Belderbos et al. [37] detected an increase in LPS or CpG induced INFγ, IP-10 and IL-12p70 production over the first year of life in European infants. This stands in sharp contrast to our South African cohort, where we detected either no response or a decrease over time (Figure 3).

This striking difference between North American and European vs. South African infant innate immune ontogeny suggests that differences between resource-rich vs. resource-poor countries might play a role in development of the innate immune system. Comparing the results of our current study with South African infants to those of Burl et al. [39] who studied the TLR response of infants born and raised in The Gambia, we indeed found them to be mostly similar. For example, the trend for TNF-α responses over the first year of life following stimulation with TLR2/1, TLR4 and TLR7/8 was nearly identical to ours. However, the study by Burl et al. was cross-sectional in nature, and progressive changes in innate immunity over the first year of life could thus have been influenced by inter-individual variability [167]. A similar decline consistent with our findings and those of Burl et al. in TLR induced cytokine production in whole blood was also detected in children born and raised in Ecuador; this decline was observed between 1 and 2 years of age, with no information provided about the earlier time points [41]. However, such decline was not observed in a recent study of infants in Papa New Guinea [40]. Together, this data supports the notion that different developmental trajectories of innate immune ontogeny exist for infants from different regions of the world. Given the heterogeneity of innate immune development within a given population [52], and the many variables that can influence innate immune analysis [83], this hypothesis will have to be tested in a well-controlled, direct side-by-side comparison.

There are several possible mechanisms that might contribute to differences between European and North American infants on one side, and infants from South Africa, The Gambia, Ecuador and Papa New Guinea on the other. One possibility is related to the receipt of live vaccines around birth. For example, none of the infants in either Europe or North America received either BCG or OPV at birth, while children in both South Africa and The Gambia received both BCG and OPV at birth. The infants in the study from Papa New Guinea received BCG at birth, but were mixed with respect to a neonatal dose of oral polio vaccine; and infants in the study from Ecuador receive only BCG at birth. The similarities between our South African study and the Gambian study by Burl et al. or the Ecuadorian study by Teran et al. and the Papa New Guinean study by Lisciandro et al. also raise the possibility that differences in host genetics and/or differences in exposure to environmental stimuli other than vaccines might influence innate immune ontogeny. Differences in early life environmental exposure associated with atopic disease in Australian children have been shown to lead to an innate immune development more akin to the trajectories we detected in South African infants [121]. And differences in innate cytokine production have been described for the same ethnic groups living in different environment [44]. On the other hand, differences in ethnicity (and by extrapolation host genetics) are known to lead to different innate immune responses to malaria infection in children in West Africa [50], suggesting differences in host genetics between South African and e.g. North American or European infants could equally be important in our findings [111]. As such, evaluation of European-descent vs. African-descent or affluent vs. poor South African infants may generate

different results. The diversity of South African demographics provides a unique opportunity to investigate the precise role of genetics vs. early life live-exposures (including vaccines) on innate immune development. Irrespective of the role of impact of live vaccination, and/or genetics vs. environment, the currently available data strongly suggest that differences in innate immune ontogeny early in life between children born and raised in different regions of the world exist. How such differences impact protection from infection or response to vaccination represents an exceptionally important issue to address.

As we had done in our previous studies in Canada [38,161], we also included ethnically-matched adults as controls in our South African study, with the aim of providing a stable comparator for the developing innate immune ontogeny. Comparing results of WB responses following PRR stimulation between North American adults to South African adults, a strikingly lower response was noted for all stimuli and all cytokines in the South African compared to North American adults. European Caucasian adult controls similar to our Canadian adults also appear more responsive to PRR stimulation than South African adults [36,37]. It would possibly be informative to decipher how different innate immune trajectories relate to overall immune senescence [169]. While it is tempting to speculate that the trajectory we observed in South African infants to reach adult-like low responsiveness by 12 months of age could be a general difference between resource-rich vs. resource-poor settings affecting all age groups, and possibly relate to the higher infectious

morbidity and mortality across all age groups in resource-poor vs. resource-rich regions of the world, this hypothesis will need to be directly tested.

Infant morbidity and mortality from infectious disease are highest during the first year of life [170], identifying the first 12 months of life as the most critical period to induce protection from pathogens through vaccination [57]. Contrasting our findings to our previous studies in North America, others from Northern Europe, and those from Africa, South America or the Pacific Islands, it is likely that differences in innate immune ontogeny early in life exist in different populations. Based on our current findings, this may in fact apply not only to early but also to adult life. The underlying mechanisms for these striking observations are not yet known. Our data strongly argue that innate immune development is highly heterogenic across the globe, suggesting that strategies aimed at preventing infection through e.g. vaccination might benefit if tailored to the specific target population.

3.5 Chapter Three Figures

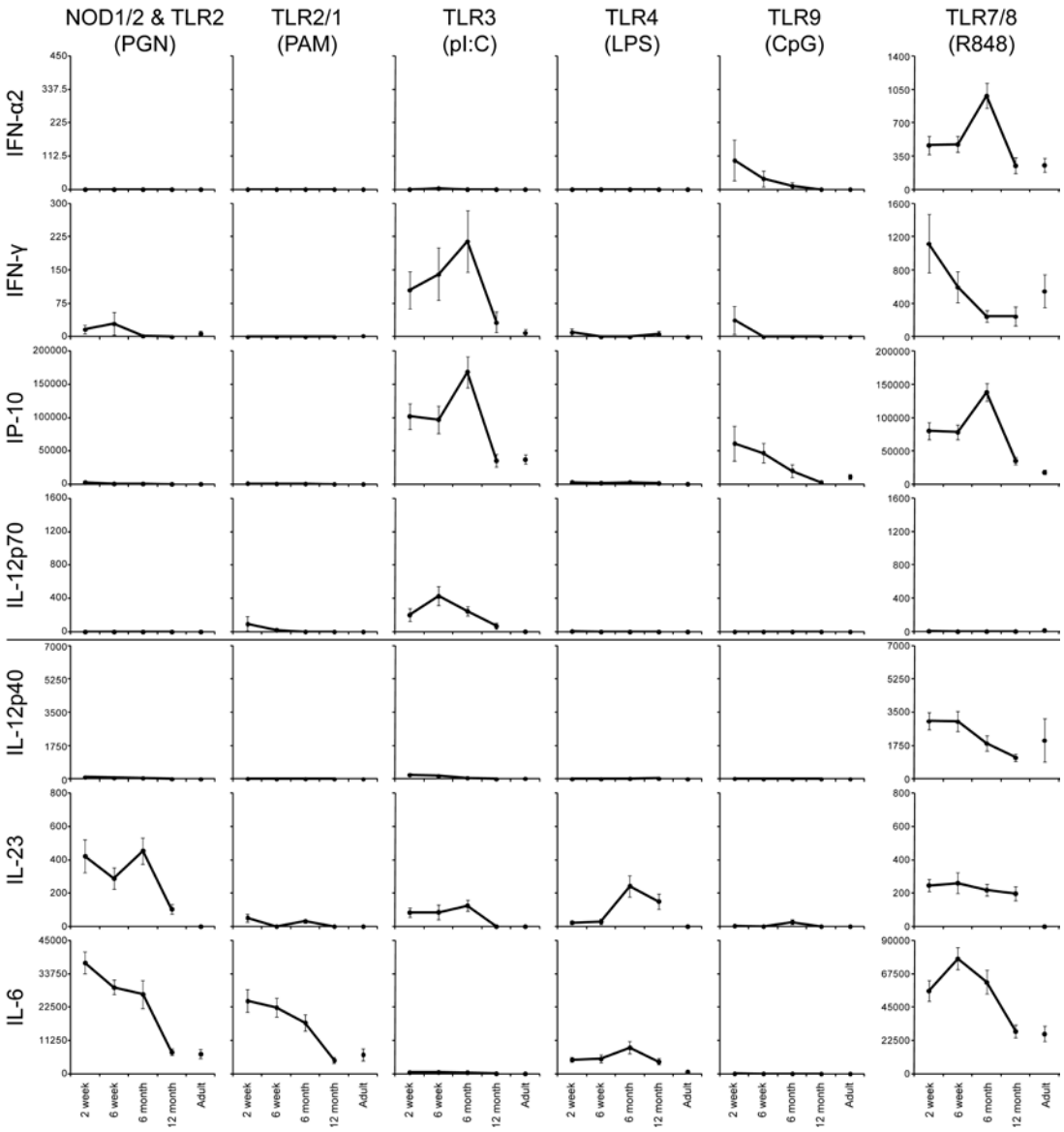


Figure 3: Decline in Secretion of Cytokines Promoting Either Th1 or Th17 Development in South African Infants' Whole Blood Within the First Year of Life

Whole blood from subjects enrolled in our longitudinal cohort and sampled at 2 weeks (2wk; n = 28), 6 weeks (6wk; n = 26), 6 months (6mo; n = 23) and 12 months (12mo; n = 20) of life, and 10 adults (Ad) was stimulated with the indicated TLR ligands and cytokine secretion into the culture supernatant was measured by Luminex xMAP cytokine assay or by ELISA (IL-23 only). Mean cytokine concentration (pg/ml) is indicated on the y-axis; error bars indicate SEM. Unstimulated samples displayed cytokine production at very low levels and were subtracted from the stimulated samples. Cytokines were grouped based on their major known functions, i.e. those promoting either Th1 (IFN-α, IFN-γ and IL-12p70 as well as IP-10), or Th17 development of CD4 T cells (IL-12p40, IL-23, and IL-6).

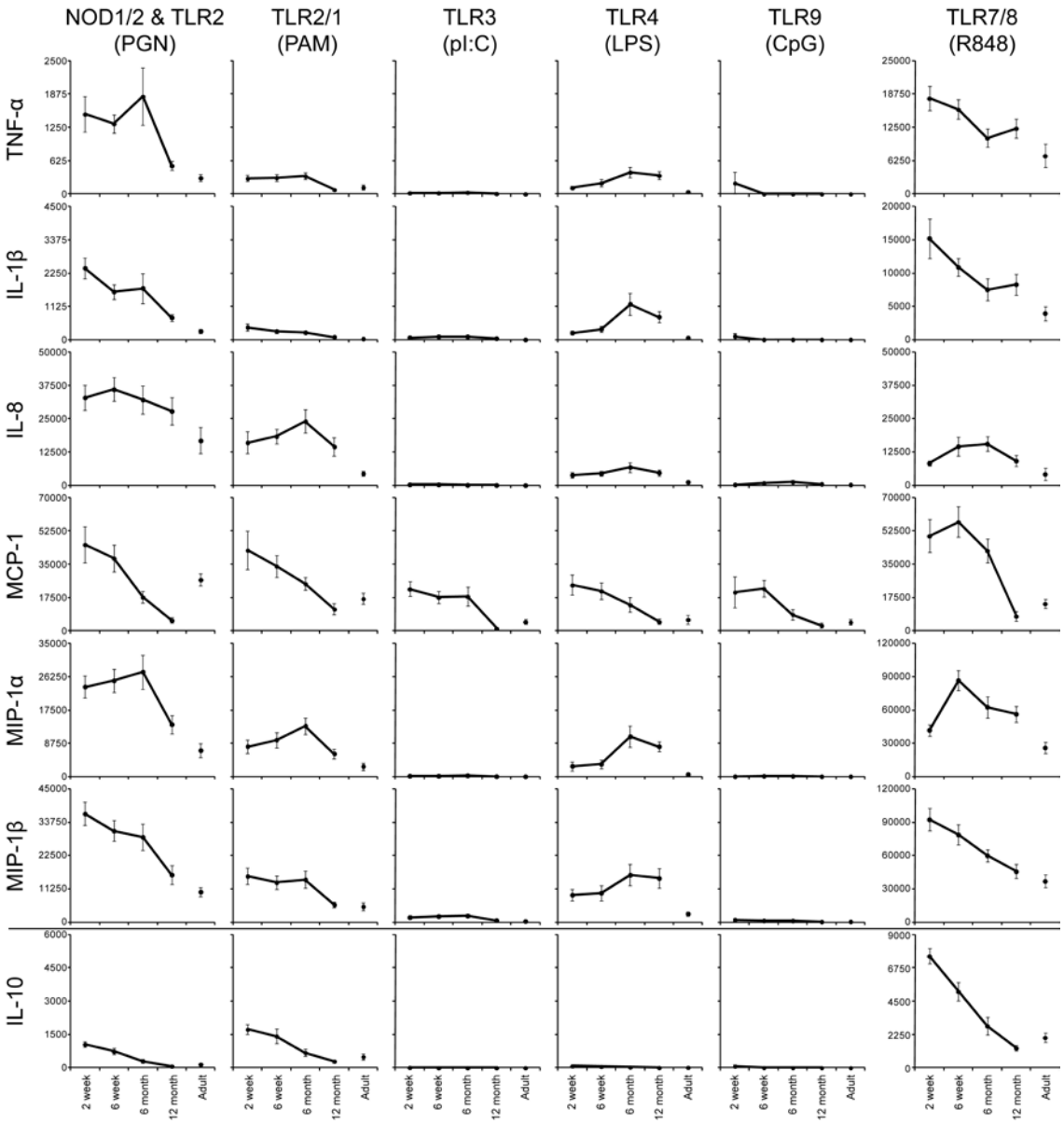


Figure 4: Decline of Secretion of Pro- and Anti-Inflammatory Cytokines Following PRR Stimulation in South African Infants' Whole Blood Within the First Year of Life

The same samples analyzed in Figure 3, collected at 2 weeks (2wk; n = 28), 6 weeks (6wk; n = 26), 6 months (6mo; n = 23) and 12 months (12mo; n = 20) of life, were also analyzed for production of cytokines considered to be general pro-inflammatory or chemoattractant (TNF-α, IL1-β, IL-8, MCP-1, MIP-1α, MIP-1β) vs. anti-inflammatory (IL-10).

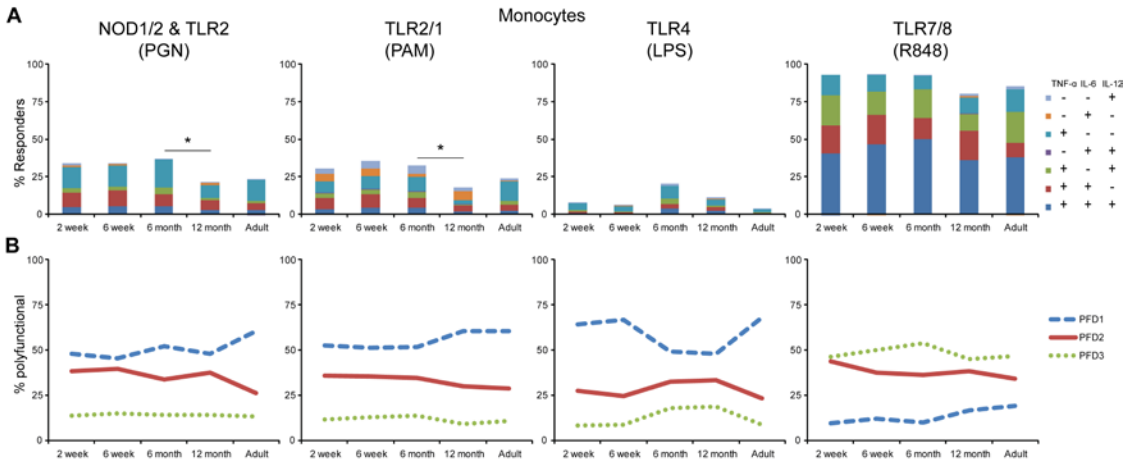


Figure 5: High Level of Monocyte Cytokine Response to PRR Stimulation Decreased Between 6 and 12 Months of Age.

Whole blood samples from neonate-infant subjects were followed longitudinally from 2 weeks (2wk; n = 28), 6 weeks (6wk; n = 26), 6 months (6mo; n = 23) and 12 months (12mo; n = 20) of life, and 10 adults (Ad) were stimulated with the indicated TLR ligands for 6h, and intracellular cytokine levels were measured by flow cytometry for TNF-α, IL-6 and IL12/23p40 gated on monocytes. For each cell type, the total percentage of cytokine-producing cells is represented by the overall height of bar graphs; color-coded segments constituting the bar allow differentiation of cells producing various cytokine combinations. Unstimulated samples displayed cytokine production at near 0% and were subtracted from the stimulated samples. (A) Overall stacked bar graph; statistically significant differences in percent responders are indicated by p-value * < 0.05. Cytokine profile – color combinations indicated in inset in identical order as in bar graphs from top to bottom. (B) Line graphs indicate polyfunctional degree (PFD) for monocytes, summarizing the percentage of cells producing 1 (PFD1), 2 (PFD2), or 3 (PFD3) cytokines for each age group.

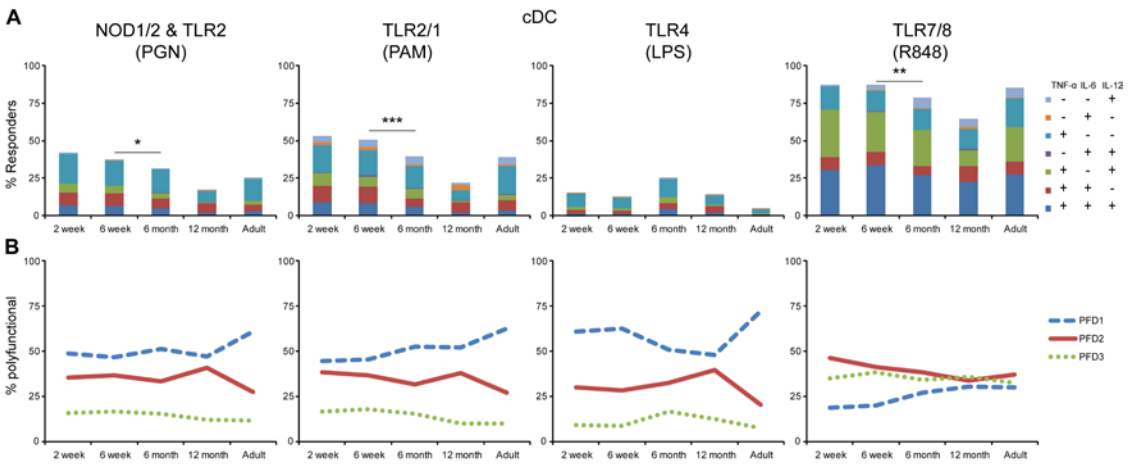


Figure 6: High Level of cDC Cytokine Response to PAMPs Decreased Over the First Year of Life for All Stimuli Except LPS

Whole blood samples from ~30 neonate-infant subjects were followed longitudinally from 2 weeks (2wk; n = 28), 6 weeks (6wk; n = 26), 6 months (6mo; n = 23) and 12 months (12mo; n = 20) of life, and 10 adults (Ad) were stimulated with the indicated TLR ligands for 6h, and intracellular cytokine levels were measured by flow cytometry for TNF-α, IL-6 and IL-12/23p40 gated on cDC. For each cell type, the total percentage of cytokine-producing cells is represented by the overall height of bar graphs; color-coded segments constituting the bar allow differentiation of cells producing various cytokine combinations. Unstimulated samples displayed cytokine production at near 0% and were subtracted from the stimulated samples. (A) Overall stacked bar graph height; statistically significant differences in percent responders are indicated by p-value * < 0.05, ** < 0.01, *** < 0.001. Cytokine profile – color combinations indicated in inset in identical order as in bar graphs from top to bottom. (B) Line graphs indicate polyfunctionality degree (PFD) for cDC, summarizing the percentage of cells producing 1 (PFD1), 2 (PFD2), or 3 (PFD3) cytokines for each age group.

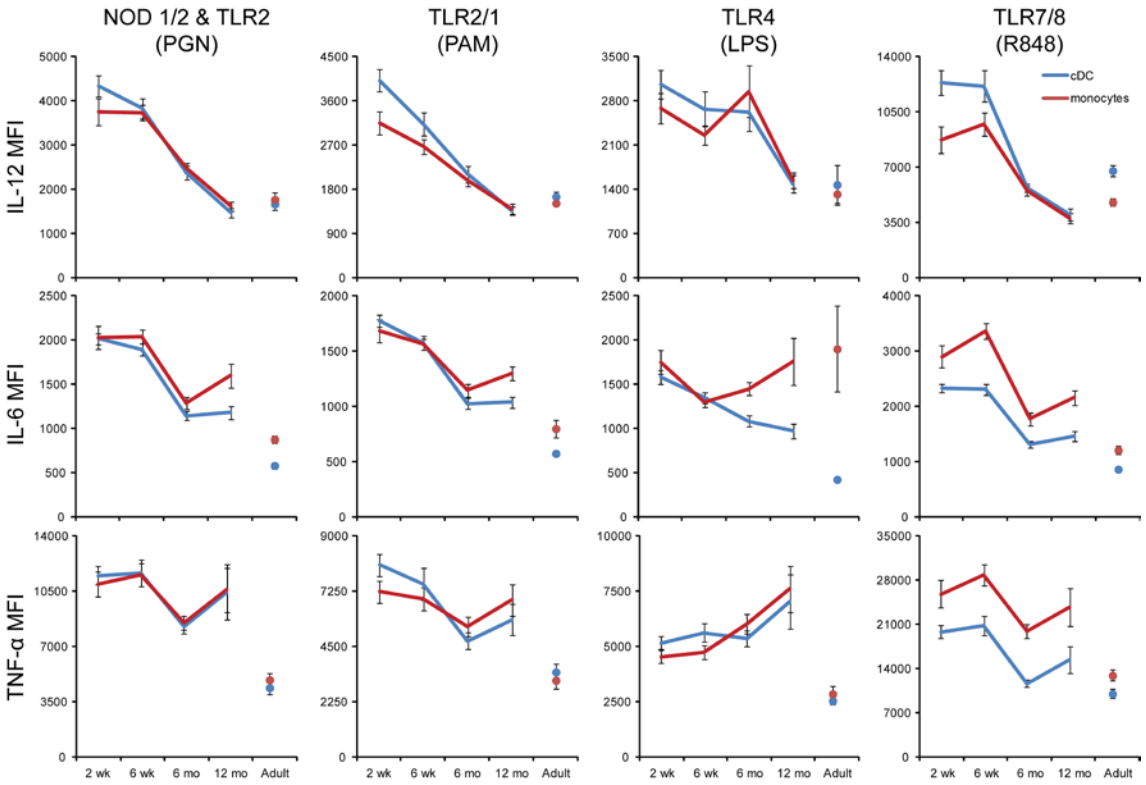


Figure 7: Mean Fluorescence Intensities (MFI) of Monocyte or cDC Cytokine Production Drastically Fell Over the First Year of Life for All Cytokines Except TNF-α and IL-6 In Response to LPS Stimulation

Whole blood samples from ~30 neonate-infant subjects were followed longitudinally from 2 weeks (2wk; n = 28), 6 weeks (6wk; n = 26), 6 months (6mo; n = 23) and 12 months (12mo; n = 20) of life, and 10 adults (Ad) were stimulated with the indicated TLR ligands for 6h, and intracellular cytokine levels were measured by flow cytometry for TNF-α, IL-6 and IL-12/23p40 gated on monocytes (blue line) and cDC (red line). The MFI values from all samples of each age group were averaged after first excluding the samples that had cytokine-positive percentage <4% of the APC subtype. Means for each population were derived from the FlowJo software; error bars indicate SEM.

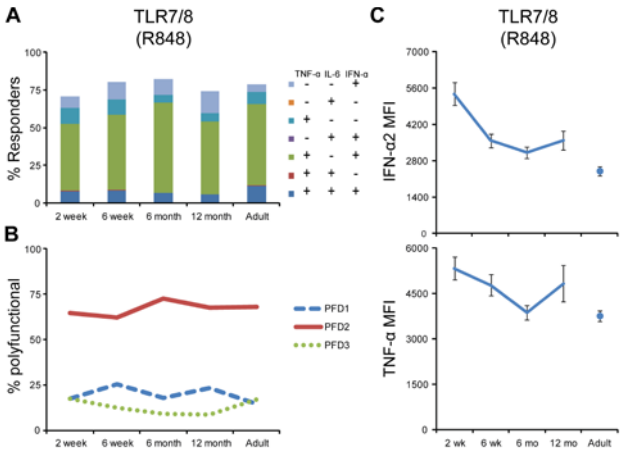


Figure 8: The Fraction of pDC Producing Cytokines in Response to PAMPs Remained High Throughout the First Year of Life, But the MFI for INF-α Decreased

Whole blood samples from ~30 neonate-infant subjects were followed longitudinally from 2 weeks (2wk; n = 28), 6 weeks (6wk; n = 26), 6 months (6mo; n = 23) and 12 months (12mo; n = 20) of life, and 10 adults (Ad) were stimulated with the TLR7/8 agonist R848 for 6h, and intracellular cytokine levels were measured by flow cytometry for TNF-α, IL-6 and INF-α gated on pDC. Unstimulated samples displayed cytokine production at near 0% and were subtracted from the stimulated samples. (A) Overall stacked bar graph height indicates the total percentage of pDC producing one of the measured cytokines. Overall responses were broken down by cytokine expression profile and were coded by color (cytokine profile – color combinations indicated in inset in identical order as in bar graphs from top to bottom). (B) Line graphs indicate polyfunctionality degree (PFD) for cDC, summarizing the percentage of cells producing 1 (PFD1), 2 (PFD2), or 3 (PFD3) cytokines for each age group. (C) Line graphs indicate the mean fluorescence intensity (MFI) in the respective sample for INF-α and TNF-α. The MFI values from all samples were averaged after first excluding the samples that have cytokine-positive percentage <4%. Means for each population are derived from the FlowJo software; error bars indicate SEM.

3.6 Chapter Three Supplemental Tables and Figures

Table 6: Increasing Number of Significant Differences in the Level of Cytokine Secretion Following PRR Stimulation With Increasing Age

Whole blood from subjects enrolled in our longitudinal cohort and sampled at 2 weeks (2wk; n = 28), 6 weeks (6wk; n = 26), 6 months (6mo; n = 23) and 12 months (12mo; n = 20) of life was stimulated with the indicated TLR ligands and cytokine secretion into the culture supernatant was measured by Luminex xMAP cytokine assay or by ELISA (IL-23 only). Differences in mean cytokine concentrations between 2 successive age groups (2 vs. 6 weeks; 6 weeks vs. 6 months; 6 vs. 12 months) were compared using the Wilcoxon matched-pair signed rank test, and corrected for multiple comparisons. A corrected p-value of less than 0.01 indicates a significant difference (highlighted in yellow).

Cytokine	TLR4 (LPS)			TLR9 (CpG)			TLR7/8 R848		
	2 week 6 week	6 week 6 month	6 month 12 month	2 week 6 week	6 week 6 month	6 month 12 month	2 week 6 week	6 week 6 month	6 month 12 month
IFN-α2	1	1	1	0.7500	0.2500	0.5000	0.2734	0.0046	0.0008
IFN-γ	1	1	1	1	1	1	0.9780	0.0649	0.0730
IL-10	0.5469	0.9375	0.2500	1	1	1	0.0554	0.0010	0.0332
IL-12p40	1	0.2500	0.3750	1	1	1	0.5614	0.0733	0.1183
IL-12p70	1	1	1	1	1	1	0.5000	1	1
IL-1β	0.1353	0.0283	0.6026	1	1	1	0.4887	0.0230	0.8129
Il-23	0.6875	0.0002	0.1787	0.2500	0.1250		0.8469	0.7510	0.6950
IL-6	0.8040	0.2514	0.0468	0.6523	0.7615	0.0039	0.0012	0.0872	0.0021
IL-8	0.7148	1	0.3438	0.0580	0.7022	0.0294	0.9515	0.3048	0.1183
IP-10	0.8469	0.0510	0.1183	0.8469	0.0075	0.0231	0.8040	0.0075	0.0003
MCP-1	0.5693	0.1650	0.0121	0.3054	0.0009	0.0523	0.1763	0.0255	0.0003
MIP-1α	0.2769	0.0075	0.4210	0.0391	0.3684	0.0017	0.0034	0.0674	0.9246
MIP-1β	0.3028	0.1776	0.5700	0.0067	0.9199	0.0060	0.2769	0.0230	0.0976
TNF-α	0.2412	0.0674	0.9794	1	1	1	0.4887	0.0510	0.3684
total #	0	3	2	2	2	5	2	6	5
Cytokine	TLR4 (LPS)			TLR9 (CpG)			TLR7/8 R848		
	2 week 6 week	6 week 6 month	6 month 12 month	2 week 6 week	6 week 6 month	6 month 12 month	2 week 6 week	6 week 6 month	6 month 12 month
IFN-α2	1	1	1	0.7500	0.2500	0.5000	0.2734	0.0046	0.0008
IFN-γ	1	1	1	1	1	1	0.9780	0.0649	0.0730
IL-10	0.5469	0.9375	0.2500	1	1	1	0.0554	0.0010	0.0332
IL-12p40	1	0.2500	0.3750	1	1	1	0.5614	0.0733	0.1183
IL-12p70	1	1	1	1	1	1	0.5000	1	1
IL-1β	0.1353	0.0283	0.6026	1	1	1	0.4887	0.0230	0.8129
Il-23	0.6875	0.0002	0.1787	0.2500	0.1250		0.8469	0.7510	0.6950
IL-6	0.8040	0.2514	0.0468	0.6523	0.7615	0.0039	0.0012	0.0872	0.0021
IL-8	0.7148	1	0.3438	0.0580	0.7022	0.0294	0.9515	0.3048	0.1183
IP-10	0.8469	0.0510	0.1183	0.8469	0.0075	0.0231	0.8040	0.0075	0.0003
MCP-1	0.5693	0.1650	0.0121	0.3054	0.0009	0.0523	0.1763	0.0255	0.0003
MIP-1α	0.2769	0.0075	0.4210	0.0391	0.3684	0.0017	0.0034	0.0674	0.9246
MIP-1β	0.3028	0.1776	0.5700	0.0067	0.9199	0.0060	0.2769	0.0230	0.0976
TNF-α	0.2412	0.0674	0.9794	1	1	1	0.4887	0.0510	0.3684
total #	0	3	2	2	2	5	2	6	5

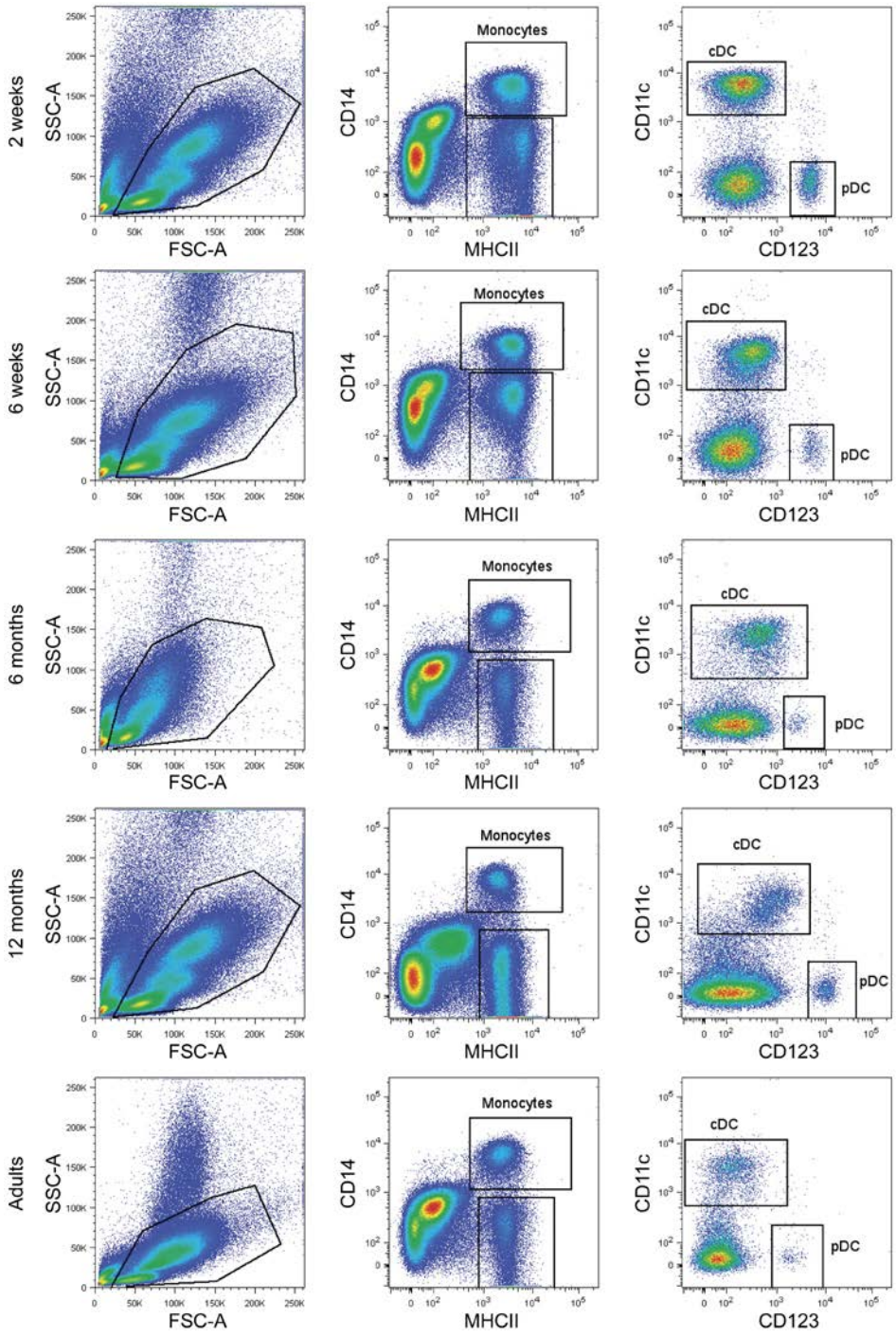


Figure 9: Gating Strategy for Antigen-Presenting Cell Subsets in WB

The gating strategy to identify innate immune cell subsets was as follows: monocytes ($MHCII^{+}$, $CD14^{+/high}$), conventional DCs ($MHCII^{+}$, $CD14^{-/low}$, $CD123^{-}$, $CD11c^{+}$), plasmacytoid DCs ($MHCII^{+}$, $CD14^{-}$, $CD11c^{-}$, $CD123^{+}$), and B cells ($MHCII^{+}$, $CD14^{-}$, $CD11c^{-}$, $CD123^{-}$).

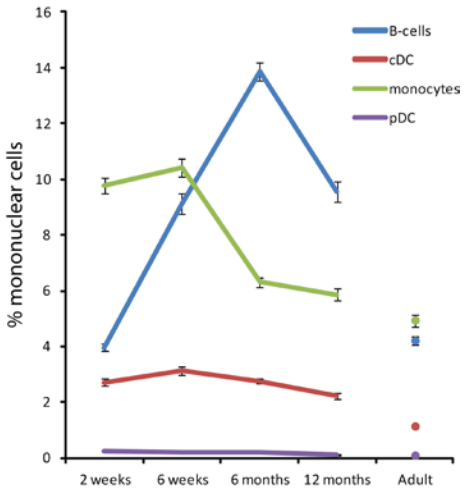


Figure 10: Antigen-Presenting Cell Subsets in WB

Relative percentage of WB APC populations gated on mononuclear cells in samples from 2 weeks (2wk; n = 28), 6 weeks (6wk; n = 26), 6 months (6mo; n = 23) and 12 months (12mo; n = 20) of life, and 10 adults. Error bars indicate SEM.

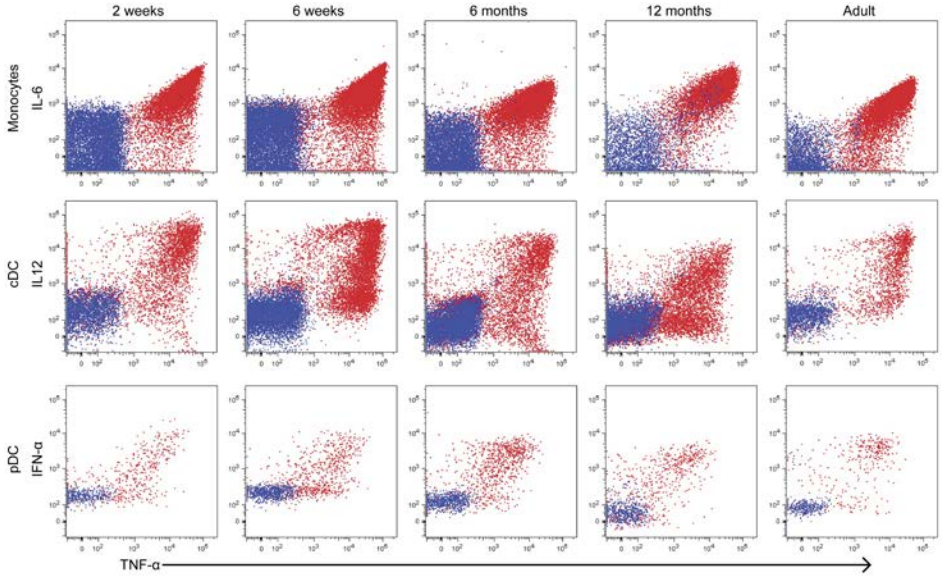


Figure 11: Flow Cytometric Analysis of Cytokine Producing Cell Subsets in WB

As an example to illustrate flow cytometry based cytokine assessment over the first year of life and contrasted to adult control, an overlay is used to compare the unstimulated sample (blue) with the sample stimulated (red) with the TLR7/8 ligand, R848.

4. ALTERED INNATE IMMUNE DEVELOPMENT IN HIV-EXPOSED UNINFECTED INFANTS

4.1 Introduction

More than 2 million babies are born to HIV-infected mothers every year [171]. With improved implementation of prevention of mother to child transmission programs, an increasing majority of these infants are HIV uninfected [172]. However, an increased risk of morbidity and mortality has been observed in HEU infants compared to HIV-unexposed infants [19,20,34,113-116]. The etiology of the increased infection risk in HEU infants is unknown.

Risk of severe infections is greatest early in life, especially for acute respiratory infections, which are the leading cause of mortality in HIV-exposed infants [1,21]. Specifically, the risk for severe LRTI is greatest during life's earliest months [19,22]. We previously reported a similar incidence of infectious episodes between UE and HEU infants but noted the frequency of severe infectious events was substantially higher in HEU infants [20] (using the DAIDS Table for Grading Severity of Adult and Pediatric Adverse Events [173]). The relative risk for severe infectious events in HEU compared to UE infants was 3.5 in the first 6 months of life; the majority being LRTIs**.** This strongly suggests an altered host response to pathogens in HEU vs. UE infants.

Innate immunity orchestrates the initial response to pathogens while shaping future adaptive responses [60], therefore differences in early life innate immunity between

HEU and UE infants may be associated with increased risk of infectious morbidity [117]. It is essential to understand how common exposures impact the trajectory of innate immune development in resource poor regions such as sub-Saharan Africa, where the risk of infant morbidity and mortality is the greatest [3]. This region carries the heaviest burden of HIV. For example, in South Africa the prevalence of HIV infection in pregnant women visiting antenatal clinics is 30% [174].

This study provides the most comprehensive functional analysis of APCs, and the most detailed description of innate immune development in HEU to date. Cytokine secretion was characterized in South African infants over the first year of life to determine if differences between HEU and UE innate immune development exist.

4.2 Methods

Study ethics are described in Chapter 2 Methods section. Cohort study design, blood sample processing, ICC staining and flow cytometric data collection are described in detail in Chapter 3 Methods section.

4.2.1 Statistical Analysis

This study was exploratory with the intent to generate, and not to test a particular hypothesis. The data were strongly interdependent, although the degree of correlation is not quantified between e.g. different cytokines produced in response

to particular PAMPs. Therefore, controlling specifically for familywise error rate due to multiple comparisons was inappropriate [175]. The null-hypothesis was that no differing trends in longitudinal data would be identified between HEU vs. UE innate immune development. The p-values from Mann-Whitney test comparisons between proportion of responder cells and cytokine produced per-cell in HEU vs. UE APC are reported (Supplementary Table 8/Table 10 and Supplementary Table 9/Table 11, respectively); p-values < 0.05 were considered significant for the purpose of trend identification. To assess polyfunctionality, the percentage of cells in a given cytokine-combination category was calculated (e.g. there are 7 possible cytokine-combination categories for 3 cytokines in which at least one cytokine is positive). Each of the 7 cytokine-combination categories was compared using Mann-Whitney test. Total response (shown as total bar height, Supplementary Figure 18) for each cell type was compared by Student's t-test. Bonferroni correction for multiple comparisons was applied to compare infant housing characteristics between HEU and UE, and $p < 0.01$ was considered significant.

4.3 Results

4.3.1 Racial Background and Housing Attributes Differed Between HIV-Exposed Uninfected and HIV-Unexposed Infants

No significant difference was identified between HEU and UE mothers' age, education, smoking or alcohol consumption during pregnancy. The mean antenatal

CD4 count of HIV positive mothers was 337 (range 131–673). A significant difference was identified in racial composition between HEU and UE groups. Eighty-one percent of HEU were of African origin, whereas 71% of UE had mixed racial backgrounds (p=0.001). HEU infants did not breastfeed, however failure to thrive was not identified by anthropometrics throughout the study period. The primary caregiver was the mother for 73% and 80% of HEU and UE infants respectively. The majority (58%) of HEU infants lived in informal housing, compared to 15% of UE infants (p=0.007), with the same proportion of HEU infants lacking access to running water relative to UE infants (p=0.007). More people occupied UE infant households (mean 5.8) versus HEU households (mean 3.9) (p=0.01), and UE infants also shared a room with more occupants (mean 3.4) relative to HEU infants who shared with an average of 2.3 (p=0.008) (Table 7).

4.3.2 Monocyte, cDC and pDC Cell Composition was Similar, and B-Cell Composition Differed Between HIV-Exposed Uninfected and HIV-Unexposed Infants

Monocytes, cDC, pDC and B-cells were identified by flow cytometry as illustrated in Figure 12A. The relative proportion of each cell type was compared between HEU and UE groups (Figure 12B) and between subjects of African and Mixed racial backgrounds (Figure 12C). Differences in proportion of B-cells were observed between HEU and UE groups at 2 weeks (UE>HEU) and between African and Mixed groups at 2 weeks and 6 months (Mixed>African). Differences were also observed

between African and Mixed racial groups in cDC proportions at 6 weeks (African>Mixed) and pDC proportions at 6 months (Mixed>African). No other differences were identified in HEU versus UE APC subsets.

4.3.3 Enhanced Pro-Inflammatory Response of Monocytes and Conventional Dendritic Cells in HIV-Exposed Uninfected vs. HIV-Unexposed Infants

Whole blood was stimulated with PAMPs prior to analysis of cytokine production (IL-6, IL-12, TNF-α and IFN-α) in monocytes, cDCs, pDCs, and B-cells. B-cells did not produce detectable amounts of cytokine in response to PAMP stimulation. The overall trend of reactivity of HEU and UE infant monocytes, cDC and pDCs was similar throughout the first year of life (Supplementary Figure 16), however when compared at individual time points several differences were observed between groups (Figure 13). HEU monocyte and cDC responses are illustrated in Figure 13A, and these cell types produced significantly more IL-6 and TNF-α in response to stimulation with the bacterial PAMPs, PAM and LPS. HEU cDCs also produced more IL-6 and IL-12 in response to PGN and the single stranded RNA (ssRNA)-like ligand R848 (IL-6 only). All ten significantly different responses detected at 2 weeks represented higher responses in HEU neonates compared to their UE counterparts. Blood samples collected at 6 weeks of age similarly demonstrated higher monocyte and cDC responses in HEU as compared to UE, but differences were restricted to bacterial LPS (TLR4) stimulation only. On the other hand, at 6 weeks UE monocytes produced more IL-12 in response to pI:C double stranded RNA (dsRNA). Only a

single difference was detected between HEU and UE pDC, with HEU pDC producing more IL-6 in response to R848, although the median response was similar between groups (Figure 13B). The only difference detected past 6 weeks was for UE monocytes producing more TNF-α in response to R848 stimulation at 6 months. By 12 months of age any difference in the reactivity between HEU and UE mononuclear cells had completely disappeared (Supplementary Table 8).

4.3.4 HIV-Exposed Uninfected Infant Mononuclear Cells Produced More Cytokine on a Per-Cell Basis Than Their HIV-Unexposed Counterparts

Measuring the proportion of cells responding to stimulation does not account for the strength of response per cell. We therefore quantified the difference in mean fluorescence intensity (MFI) between HEU and UE infants for monocyte and cDC (Figure 14A), and pDC (Figure 14B). The overall trend of evolving cytokine production per cell was similar between groups throughout the first year of life (Supplementary Figure 17), however, multiple differences were detected when MFI was compared between the two groups (Supplementary Table 9). At 2 weeks of life HEU monocytes produced more IL-12 per-cell for all PAMPs tested except for the viral stimuli R848. Similarly, HEU cDC produced more IL-12 per-cell for the bacterial PAMPs (PGN, LPS, PAM). HEU monocytes and cDC produced more IL-6 in response to TLR2/1 stimulation. HEU cDC displayed higher TNF-α production in response to stimulation of TLR4 and TLR7/8. By 6 weeks of age HEU and UE monocyte cytokine production was similar, with no significant difference detected except the UE

monocyte TLR3 response, which was the only instance where UE APCs mounted a higher per-cell response compared to the HEU group. HEU pDC produced more TNF-α and IFN-α per-cell in response to stimulation of TLR7/8 and TLR9, but only within the first 6 months of life.

4.3.5 Early-Life Variability Detected in Polyfunctional Mononuclear Cell Responses of HIV-Exposed Uninfected and HIV-Unexposed Infants

Total proportion of monocytes (Figure 18A (supplementary)), cDC (Figure 18B (supplementary)), and pDC (Figure 18C(supplementary)) that responded to PAMP stimulation was compared between HEU and UE infants (total bar height). Overall, a greater proportion of HEU monocytes and cDC responded to bacterial ligand stimulation (PAM, LPS) up to 6 weeks of age. Conversely, UE monocytes responded more strongly to viral ligand stimulation (pI:C and R848) at 6 months. No significant differences in total responders were detected at 12 months of age.

Mononuclear cell responses were subsequently differentiated into mono- and polyfunctional subtypes. For monocytes and cDC, 7 permutations were theoretically possible for cytokine expression of TNF-α and/or IL-6 and/or IL-12 (Figure 18A and Figure 18B, respectively (supplementary)). For pDC as well there were 7 possible permutations for TNF-α and/or IL-6 and/or IFN-α (Figure 18 (supplementary)). Comparisons between HEU and UE were performed for each subtype's percent responder cells. Overall twenty-seven differences were identified, sixteen at 2

weeks, nine at 6 weeks, none at 6 months, and only two at 12 months of age (Figure 15A). Of the differences identified between HEU and UE, 13 were found in response to LPS, 4 in response to PAM, 3 for PGN, 2 for CpG, 2 for pI:C and 3 for R848 (Figure 15B). At 2 weeks of age, when most differences between HEU and UE were observed, the majority of differences were detected in cytokine production in cDCs. For all statistical differences the HEU subset responses were higher than UE responses.

4.3.6 Comparisons of Antigen Presenting Cell Responses Between Groups Defined by Race Were Dissimilar to Comparisons Between Groups Defined by Maternal HIV Infection

Due to differences in racial composition between HEU and UE groups, additional analysis was performed to compare groups defined by race (African vs. Mixed descent). Contrasting the increased proportion of HEU vs. UE APCs responding primarily to bacterial stimuli at 2 weeks of age (Supplementary Table 8), less than a third of the differences were observed in African vs. Mixed race infants (Supplementary Table 10). No pattern was evident that would indicate a greater responsiveness of any cell type to a particular stimuli or at a particular time point. Differences in per-cell cytokine production were identified between infants of African and Mixed race. Infants of mixed race exhibited a pattern of stronger per-cell responses to PRR stimulation at 6 weeks of age (Supplementary Table 11). Specifically, Mixed race cDC produced more TNF-α, IL-6 and IL-12 in response to PGN. More TNF-α and/or IL-6 was produced by Mixed race cDC after LPS and PAM

stimulation at 6 weeks. African monocytes produced more TNF-α and IL-12 in response to R848 at 2 weeks and 6 months, respectively. R848 elicited more IL-12 from Mixed race cDC at 6 weeks, and IFN-α from African pDC at 2 weeks.

4.4 Discussion

HEU infants are at an increased risk of life-threatening infections [19,20,34,113-116] and differences in innate immunity in early life potentially contribute to their vulnerabilities [117]. This study examined early life HEU innate immune development, and contrasted it to UE infants using multiparameter flow cytometry to measure cytokine production in monocytes, cDC and pDC. Overall, HEU innate antigen presenting cells responded more strongly than UE to stimulation with PAMPs in both the proportion of responder cells and quantity of cytokine produced per cell. The majority of differences occurred at the earliest time points and in response to bacterial PAMPs.

Previous comparisons of early life innate immunity in HEU relative to UE demonstrate altered secretion of immune mediating cytokines, increased soluble indicators of inflammation [118,123] and cell surface receptor expression suggestive of APC activation in HEU [119]. Many of these observations have been in cord blood and these changes are no longer detected later in life. Functional comparison of natural killer (NK) cell activity at one month of age also demonstrates an increase of an intermediate NK phenotype for activation and perforin expression in HEU vs. UE,

which 'normalizes' by one year [120]. These findings are in line with our observations at the cellular level. Our single-cell focus now provides the sensitivity to detect differences of smaller magnitude (often less than 15% for any single parameter). Analysis focused at the single-cell level also offers the necessary resolution to observe trends when comparing HEU and UE innate immune development, which resulted in the most striking observations. These data delineate the time (≤6 weeks) and stimulus (bacterial PAMPs) restricted nature of differences in innate immune ontogeny between HEU and UE infants. Time-restricted differences in innate immune responses to PAMPs may be pertinent to corresponding periods of increased susceptibility of HEU infants to infectious diseases.

Additional resolution was provided by detecting APCs ability to produce more than one cytokine following stimulation (termed 'polyfunctionality'), which is a functional parameter previously correlated with clinical outcomes [176]. We subdivided innate responses into polyfunctional subgroups and observed heightened responses in HEU, which were almost exclusively induced by bacterial PAMPs in cDC and monocytes. In contrast, no consistent difference in polyfunctionality between HEU vs. UE was observed following stimulation with viral PAMPs, or in pDCs, which are instrumental in viral responses. These data suggest differences in polyfunctionality may be pathogen– and correspondingly also APC subtype–specific. However, our data do not allow for inference of causality for e.g. bacterial infections; they solely indicate that differences in innate response to these

PAMPs exist in a time frame closely associated with increased risk for severe infection.

The etiological factors driving the observed early life differences in innate immune ontogeny between HEU and UE are likely multifactorial[13,34]. Future analysis will be needed to evaluate how genetic and environmental differences between HEU and UE infants impact innate immune ontogeny. Just as direct comparisons would provide a better understanding of why there is variability between observations of innate immune development in resource rich settings [36-38] versus resource poor settings [39-42]. For example, lacking access to running water correlates positively with expression of IL-10 in childhood [44]. The majority (58%) of HEU infants in our study lacked access to running water, whereas only 15% of UE infants did. However, HEU APCs responded more strongly to PAMPs, suggesting that elevated IL-10 production associated with this basic measure of sanitation was unlikely a dominant factor influencing differences in innate immune development. ART exposure *in utero* and in the perinatal period may also impact immune development. In adults, ART is associated with anemia, neutropenia, relative lymphopenia, and down regulation of select pattern recognition receptor genes [46-49]. However, there are currently no data measuring the effect of ART exposure on functional innate immune ontogeny early in life.

Varied racial background was identified between groups (81% vs. 29% African in HEU vs. UE respectively) of our cohort. Different ethnic groups (with varied genetic

backgrounds by extrapolation) can exhibit varied innate immune responses to, and protection from, infectious challenge [50,51]. More specifically, TLR polymorphisms are associated with heterogeneity of innate responses [52]. In order to test the relative impact of race on our observations we redefined comparison groups by racial background (African vs. Mixed). Given the increased African composition in the HEU group, we expected to find a similar pro-inflammatory pattern in African vs. Mixed responses as was seen in HEU vs. UE. This was not observed. When proportion of responder APCs (to PAMP stimulation) was compared between African and Mixed groups, only 6 differences were detected, which was within the expected level of error using a p-value of <0.05. When the amount of cytokine produced per-cell was compared, a pattern emerged that may suggest pro-inflammatory cDC in Mixed race vs. African infants at 6 weeks. This pattern was weaker and distinct in its timing compared to changes in HEU vs. UE (and does not support a pro-inflammatory pattern in African vs. Mixed). Therefore, while genetic variability is a probable contributor, differences in racial composition between groups was likely not the cardinal factor determining the observed variability in innate immune responses between HEU and UE infants.

Differences in breastfeeding practices may have contributed to the differences in innate immune development between HEU and UE. Breast milk contains compounds that modulate PRR-mediated immune responses, including immunoglobulins, antimicrobial proteins/peptides, nucleotides and oligosaccharides [53,54]. Breast milk can also alter TLR responses to PRR specific agonists [126]. Clinical evidence indicate

that the time period of increased morbidity from diarrheal infections in HEU infants coincides with the average time of weaning [29]. The median duration of exclusive breastfeeding in UE infants of this cohort was 12 weeks, while HEU infants were not breastfed as recruitment occurred prior to the shift towards recommending breastfeeding for infants born to HIV positive mothers [20]. However, it is noteworthy that breastfeeding provides greatest protection from diarrheal disease as opposed to respiratory tract infections [12], which was the leading cause of severe illness and hospitalization of HEU in this study, and is the leading cause of infectious morbidity and mortality in HEU infants [19,22]. Lack of breastfeeding is thus likely to only partially explain increased morbidity and altered immune status in HEU vs. UE infants.

Our goal had not been to delineate precise etiological cause-effect relationships, but to first determine if differences in innate immunity between HEU and UE existed at all, and if so, for how long they persisted. Our data indicate that innate immune response to PRR stimulation differed between HEU and UE infants. Specifically, innate ontogeny initially followed a different trajectory in HEU compared to UE infants, but this difference became less apparent as the infant matured. No clear etiology for these differences can at this stage be assigned, as several factors may have contributed to our observation of significant differences in innate immune response between HEU and UE in the earliest postnatal period. Follow-up studies can now focus on examining the relative contribution of potential etiological factors (ranging from HIV-exposure to variables associated with host genetics) to

differences in innate immune ontogeny. Our data support the notion that early life represents a window of significant vulnerability for altered immune development [60,65]. Elucidating the mechanisms that drive these changes in innate immune development may move us closer to identifying targeted strategies to decrease infectious morbidity and mortality in HEU infants. This has potentially broad implications for the rapidly growing population of HIV-exposed uninfected infants.

4.5 Chapter Four Tables and Figures

Table 7: Maternal and Infant Demographic, Clinical and Housing Characteristics

Shown are maternal and infant characteristics adapted from Slogrove et al., 2012 [20], and data pertaining to housing and childcare conditions collected at the 6 month time point. Comparison between HEU and UE groups were performed by student's t-test.

Maternal characteristics *	**Total (55)**	**HEU (27)**	**UE (28)**	***p-value***
Age (in years)–mean (SD)	26.6 (6.3)	25.9 (6.8)	27.3 (5.7)	0.4
Completed secondary education (%)	15 (27)	6 (22)	9 (32)	0.6
Smoked during pregnancy (%)	14 (25)	3 (11)	11 (39)	0.17
Consumed alcohol during pregnancy (%)	4 (7)	0 (0)	4 (14)	0.11
Antenatal CD4 count in cells μl^{-1}–median (range)		337 (131–673)		
Infant Characteristics *	**Total (55)**	**HEU (27)**	**UE (28)**	***p-value***
Antiretroviral exposure[a] (%)		23 (85)		
Male (%)	22 (40)	7 (26)	15 (54)	0.35
Ethnicity				0.001
African[b] (%)	30 (55)	22 (81)	8 (29)	
Mixed or Caucasian[c] (%)	25 (45)	5 (19)	20 (71)	
Birthweight–mean (95% CI) (g)	2966 (2857–3075)	2945 (2866–3024)	2986 (2830–3142)	0.7
Breastfeeding (%)	29 (53)	1 (4)	28 (100)	<0.001
Growth at 6months–*n*	**Total (47)**	**HEU (25)**	**UE (22)**	
WAZ (SD)	–0.25 (1.07)	+0.17 (0.95)	–0.73 (1.16)	0.03
LAZ (SD)	–0.64 (1.26)	–0.44 (1.05)	–0.87 (1.61)	0.42
Growth at 12 months	**Total (44)**	**HEU (23)**	**UE (21)**	
WAZ (SD)	–0.09 (1.16)	+0.26 (1.13)	–0.47 (1.09)	0.18
LAZ (SD)	–0.20 (1.29)	+0.10 (1.09)	–0.53 (1.43)	0.22

Continued on the next page

Infant Care and Housing Conditions	**Total (46)**	**HEU (26)**	**UE (20)**	***p-value***

Mother as primary caregiver (%)	35 (76)	19 (73)	16 (80)	0.812
Formal housing (%)	28 (61)	11 (42)	17 (85)	0.007
Number of people in the household–mean (SD)	4.76 (2.47)	3.92 (2.25)	5.76 (2.39)	0.010
Number of people sharing infants room–mean (SD)	2.76 (1.37)	2.28 (1.14)	3.35 (1.42)	0.008
Access to running water (%)	28 (61)	11 (42)	17 (85)	0.007

*****Maternal and Infant Characteristics adapted from Slogrove et al. [20]

[a]ARV exposure as PMTCT prophylaxis (*n*=19) or from mother with lifelong combination ARV therapy (*n*=4)

[b]African included infants of Xhosa speaking South African (*n* =27), Malawian (*n*=2) and Zimbabwean (*n*=1) descent

[c]All mixed ethnicity except 1 Caucasian UE

SD, standard deviation; WAZ, weight-for-age *Z*-score; LAZ, length-for-age *Z*-score

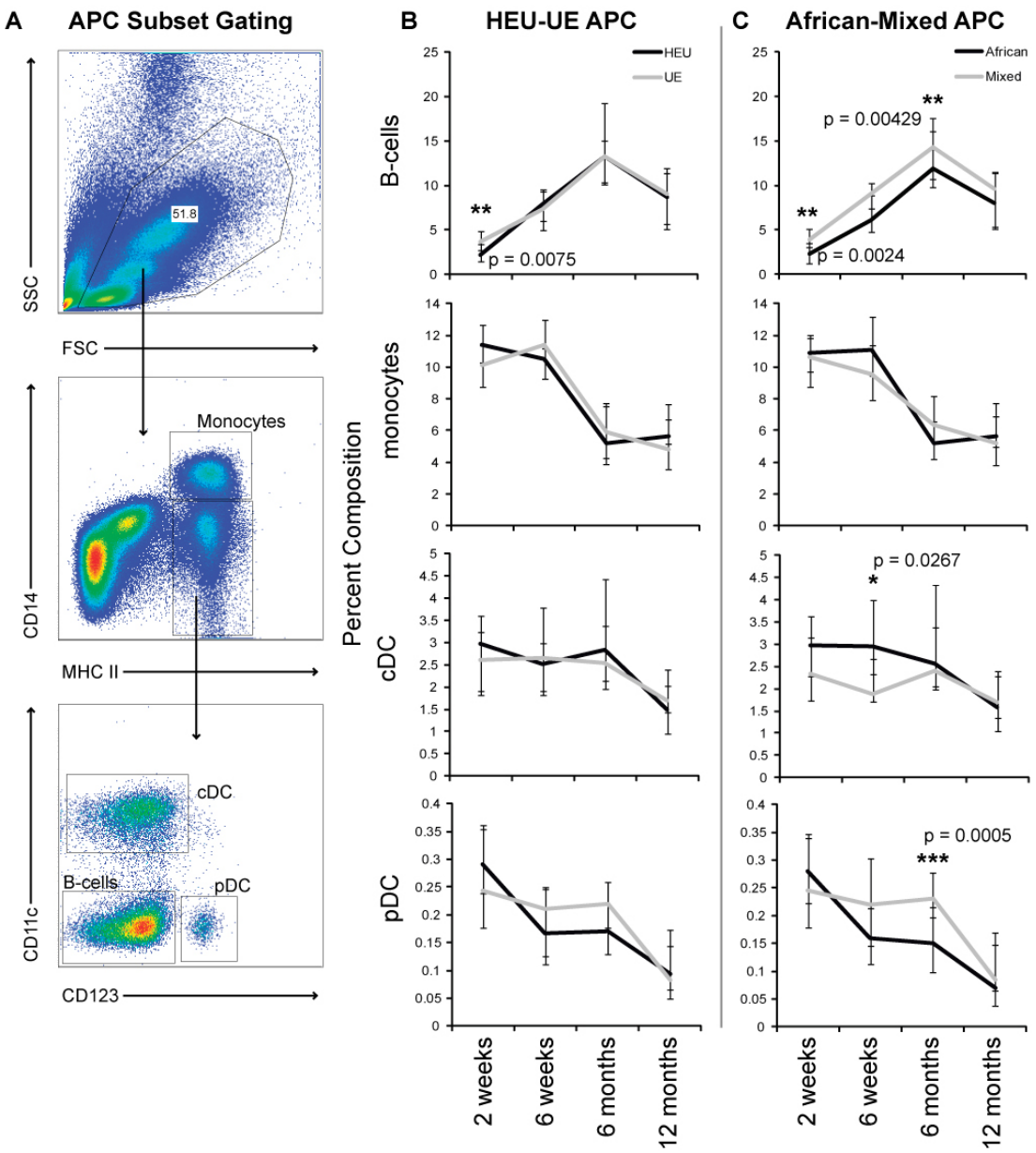


Figure 12: **Similar Proportions of Monocytes, cDC and pDC, but Different Proportions of B-cells in HIV-Exposed Uninfected Versus HIV-Unexposed Infants**

A) Example of differentiation of antigen presenting cells by flow cytometry. B) Shown are the relative proportions of live-gated B-cells, monocytes, cDC and pDC between HEU (black) and UE (grey) subjects at 2 weeks, 6 weeks, 6 months and 12 months of life. C) The relative proportion of antigen presenting cell sub-types is also shown for subjects of Mixed (grey) or African (black) descent at the respective time points. Number of subjects at 2 weeks (UE = 28 [29% African], HEU = 27 [81% African]), 6 weeks (UE = 27, HEU = 25), 6 months (UE = 23, HEU = 25) and 12 months (UE = 21, HEU = 23) of life. Racial distribution did not significantly change between HEU and UE groups throughout the first year. Y-axis represents the median proportion of each cell type; error bars indicate interquartile range. Mann-Whitney test was used to compare groups and p-value is shown at time points where $p<0.05$.

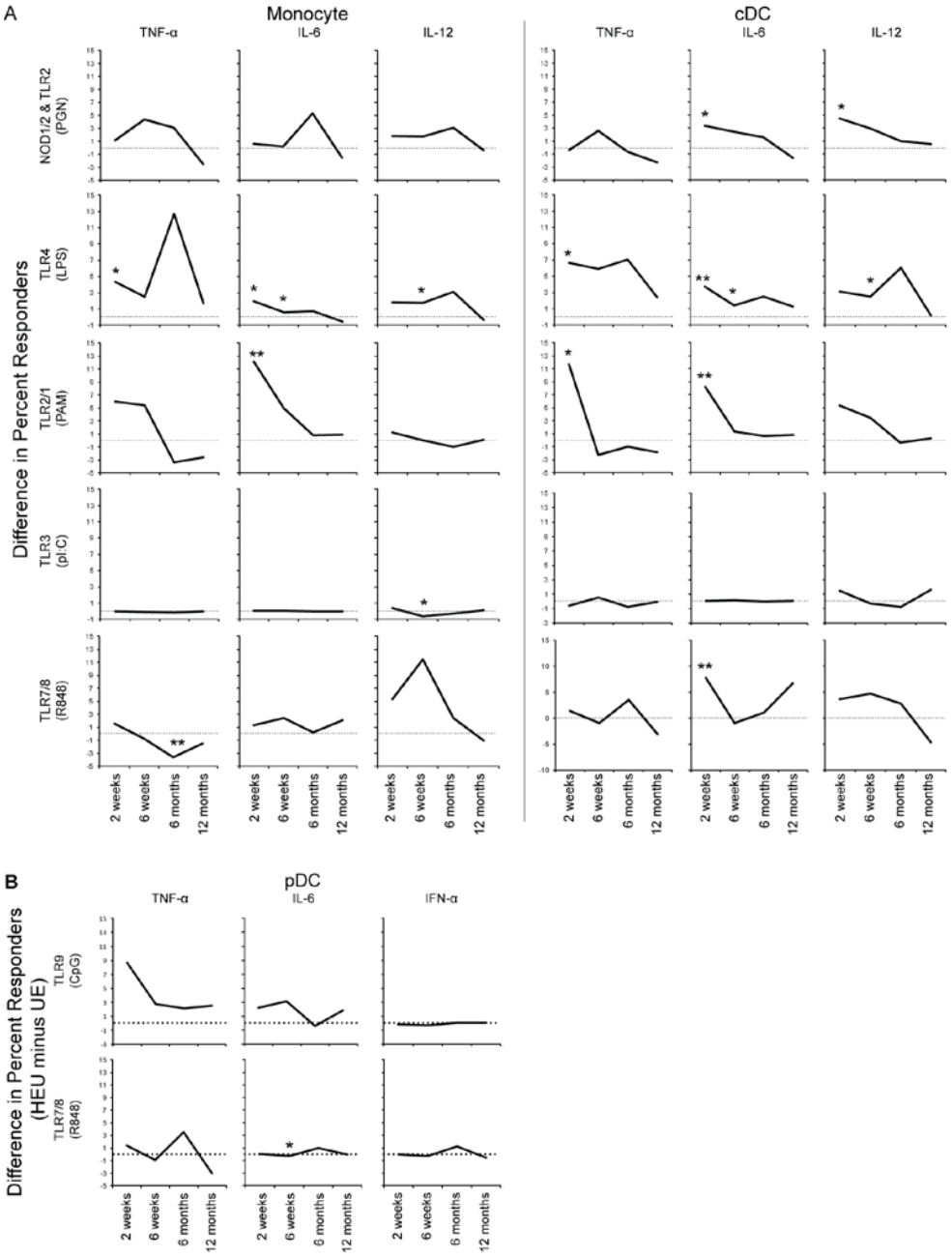


Figure 13: Elevated Pro-Inflammatory Cytokine Response of Antigen Presenting Cells in HIV-Exposed Uninfected Versus HIV-Unexposed Infants

Whole blood from HEU and UE subjects was stimulated with the indicated TLR ligands at 2 weeks, 6 weeks, 6 months and 12 months of life. Multiparameter flow cytometry was used to detect production of TNF-α, IL-6 or IL-12/23p40 in A) monocytes and cDC, and B) TNF-α, IL-6 or IFN-α was detected in pDC. Y-axis represents difference between the median HEU–minus–median–UE proportion of cytokine-producing cells. Number of subjects at 2 weeks (UE = 28, HEU = 27), 6 weeks (UE = 27, HEU = 25), 6 months (UE = 23, HEU = 25) and 12 months (UE = 21, HEU = 23) of life. Mann-Whitney test was used to compare HEU and UE response at each time point and differences are signified by * ($p<0.05$) and ** ($p<0.01$). Unstimulated samples (near 0% of cytokine producing cells) were subtracted from stimulated samples.

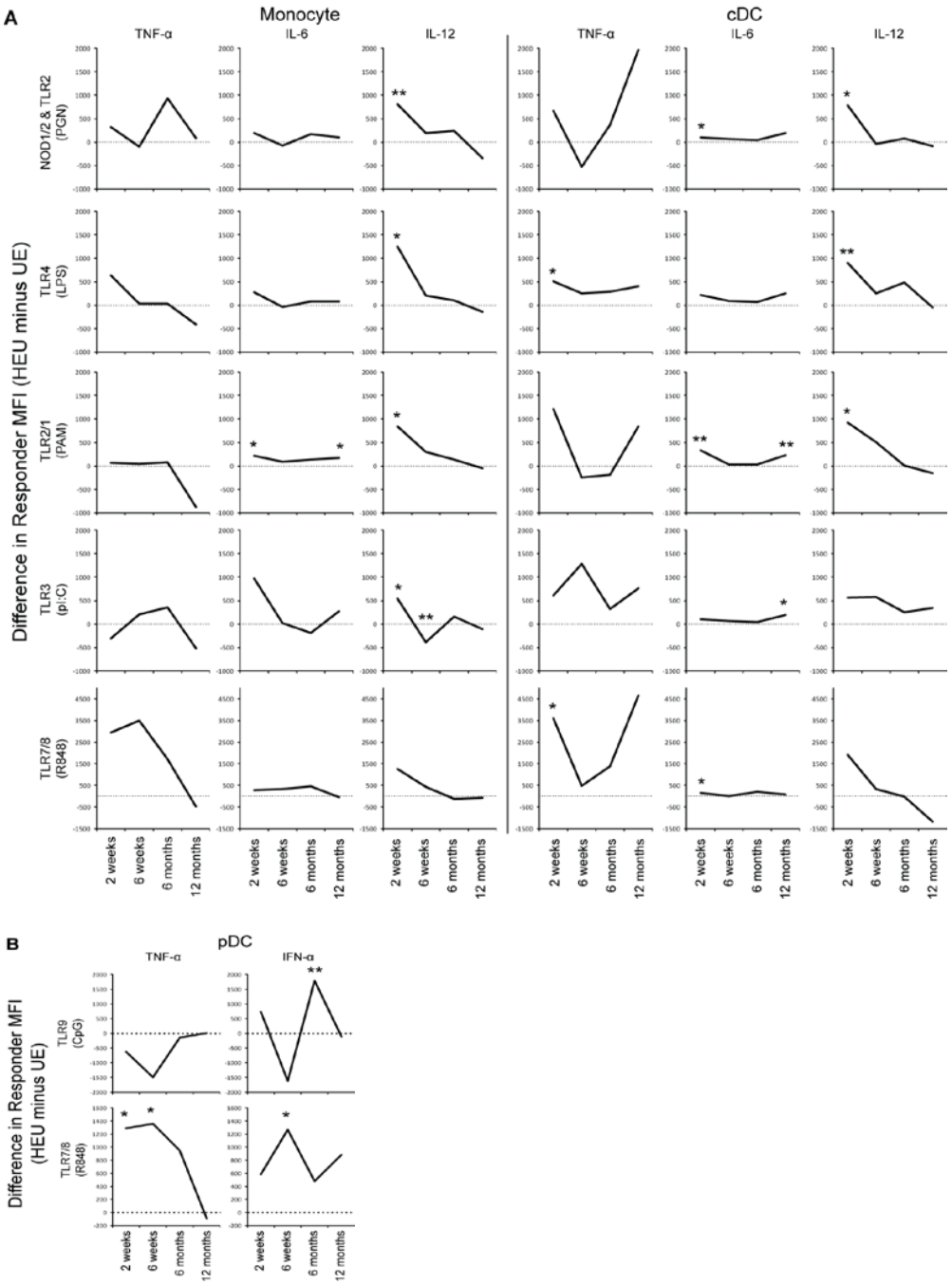
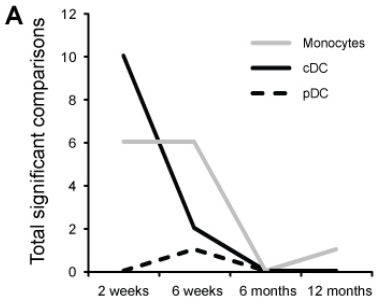


Figure 14: Increased Cytokine Production on a Per-Cell Basis in HIV-Exposed Uninfected Infant Versus HIV-Unexposed Infant Antigen Presenting Cells

Shown are the same samples as in Figure 13, but with the difference in mean fluorescent intensity (MFI) graphed for median HEU MFI minus the median UE MFI in the respective samples for A) monocytes and cDC, for which we measured TNF-α, IL-6 and IL-12/23p40, and B) pDC, with TNF-α, IL-6 and IFN-α measured. Number of subjects at 2 weeks (UE = 28, HEU = 27), 6 weeks (UE = 27, HEU = 25), 6 months (UE = 23, HEU = 25) and 12 months (UE = 21, HEU = 23) of life. Means for each population (y-axis) are derived from FlowJo software. Differences in MFI between HEU and UE groups as detected by Mann-Whitney test are indicated by * ($p<0.05$) and ** ($p<0.01$).

A



B

Monocytes

Cytokines expressed				p-values <0.05			
TNF-a	IL-6	IL-12	PAMP	2wk	6wk	6mo	12mo
–	–	+	LPS		0.0104		
			pIC		0.0188		
–	+	–	pIC				0.0149
+	–	–					
–	+	+	PAM	0.0439			
			LPS	0.0128	0.0245		
			PGN	0.0209			
+	–	+	LPS		0.0211		
+	+	–	LPS		0.0306		
			CpG	0.0326			
+	+	+	PAM	0.0252			
			LPS	0.0313	0.0176		

cDC

Cytokines expressed				p-values <0.05			
TNF-a	IL-6	IL-12	PAMP	2wk	6wk	6mo	12mo
–	–	+	CpG		0.0256		
–	+	–					
+	–	–	LPS	0.0481			
–	+	+	PAM	0.0332			
			LPS	0.0213	0.0459		
+	–	+	LPS	0.0163			
			PGN	0.0215			
+	+	–	LPS	0.0181			
+	+	+	PAM	0.002			
			LPS	0.021			
			R848	0.0118			
			PGN	0.0068			

pDC

Cytokines expressed				p-values <0.05			
TNF-a	IL-6	IL-12	PAMP	2wk	6wk	6mo	12mo
–	–	+	R848				0.0257
–	+	–					
+	–	–					
–	+	+					
+	–	+					
+	+	–					
+	+	+	R848		0.0241		

Figure 15: Summary of Differences in Functional Responses Between HIV-Exposed Uninfected vs. HIV-Unexposed Infants

Whole blood samples were stimulated with TLR ligands at 2 weeks, 6 weeks, 6 months and 12 months of life. Multiparameter flow cytometry was used to detect production of TNF-α, IL-6 and IL-12/23p40 in monocytes, and cDC. TNF-α, IL-6 and IFN-α was detected in pDC. Cell groups were subdivided based on expression of 1, 2 or 3 cytokines in various permutations, and each functional group was compared between HEU and UE APC cell types for percent responder cells. A) Summary of the number of comparisons for monocytes (grey), cDC (black) and pDC (dashed) that were statistically different between HEU and UE groups ($p<0.05$). For every comparison, the HEU functional group responded more strongly than their UE counterparts. B) Specific functional subgroups with significantly different responses ($p<0.05$) between HEU and UE infants. Specific p-values from Mann-Whitney test are shown. Number of subjects at 2 weeks (UE = 28, HEU = 27), 6 weeks (UE = 27, HEU = 25), 6 months (UE = 23, HEU = 25) and 12 months (UE = 21, HEU = 23) of life.

4.6 Chapter Four Supplemental Tables and Figures

Table 8: Stronger Pro-Inflammatory Antigen Presenting Cell Responses in HIV-Exposed Uninfected Infants Versus HIV-Unexposed Infants

Shown are the p-values from Mann-Whitney test of the same samples shown in Figure 13. Number of subjects at 2 weeks (UE = 28, HEU = 27), 6 weeks (UE = 27, HEU = 25), 6 months (UE = 23, HEU = 25) and 12 months (UE = 21, HEU = 23) of life. Significantly different responses ($p<0.05$) are highlighted in blue when HEU > UE, and in yellow when HEU < UE.

Monocytes

		2 week	6 week	6 month	12 month
TNF-α	PGN	0.570	0.546	0.902	0.601
	LPS	0.034	0.064	0.496	0.626
	PAM	0.096	0.194	0.789	0.913
	pIC	0.174	0.134	0.081	0.445
	R848	0.522	0.770	0.008	0.652
IL-6	PGN	0.194	0.453	0.522	0.324
	LPS	0.013	0.048	0.577	0.761
	PAM	0.006	0.096	0.757	0.893
	pIC	0.630	0.849	0.448	0.472
	R848	0.249	0.634	0.877	0.706
IL-12	PGN	0.256	0.170	0.773	0.458
	LPS	0.067	0.007	0.584	0.961
	PAM	0.469	0.227	0.820	0.913
	pIC	0.726	0.011	0.676	0.537
	R848	0.826	0.187	0.781	0.855

cDC

TNF-α	PGN	0.153	0.534	0.951	0.817
	LPS	0.011	0.055	0.536	0.864
	PAM	0.023	0.956	0.635	0.893
	pIC	0.110	0.262	0.118	0.680
	R848	0.055	0.898	0.635	0.733
IL-6	PGN	0.044	0.534	0.509	0.817
	LPS	0.002	0.035	0.516	0.990
	PAM	0.004	0.701	0.926	0.724
	pIC	0.755	0.755	0.230	0.732
	R848	0.005	0.770	0.409	0.884
IL-12	PGN	0.032	0.234	0.353	0.779
	LPS	0.065	0.019	0.458	0.951
	PAM	0.059	0.595	1.000	0.894
	pIC	0.814	0.528	0.216	0.741
	R848	0.400	0.595	0.445	0.635

pDC

TNF-α	R848	0.153	0.087	0.789	0.817
	CpG	0.426	0.280	0.601	0.483
IL-6	R848	0.107	0.028	0.427	0.942
	CpG	0.398	0.099	0.491	0.461
IFN-α	R848	0.714	0.138	0.734	0.894
	CpG	0.909	0.177	0.248	0.476

Table 9: More Cytokine Detected on a Per-Cell Basis in Stimulated HIV-Exposed Uninfected Infant Antigen Presenting Cells Compared to HIV-Unexposed Antigen Presenting Cells

Shown are p-values from Mann-Whitney test of the same samples shown in Figure 14. Number of subjects at 2 weeks (UE = 28, HEU = 27), 6 weeks (UE = 27, HEU = 25), 6 months (UE = 23, HEU = 25) and 12 months (UE = 21, HEU = 23) of life. Significant differences between groups ($p<0.05$) are highlighted in blue when HEU > UE, and in yellow when HEU < UE.

Monocytes

		2 weeks	6 weeks	6 months	12 months
TNF-α	PGN	0.680	0.833	0.375	0.825
	LPS	0.184	0.652	0.967	0.611
	PAM	0.522	0.804	0.975	0.907
	pIC	0.977	0.942	0.556	0.328
	R848	0.050	0.422	0.665	0.990
IL-6	PGN	0.051	0.855	0.265	0.246
	LPS	0.405	0.683	0.796	0.136
	PAM	0.023	0.204	0.256	0.018
	pIC	0.053	0.087	0.248	0.197
	R848	0.076	0.360	0.223	0.787
IL-12	PGN	0.008	0.833	0.934	0.192
	LPS	0.010	0.642	0.837	0.171
	PAM	0.016	0.286	0.570	0.969
	pIC	0.034	0.005	0.522	0.611
	R848	0.143	0.704	0.726	0.473

cDC

TNF-α	PGN	0.400	0.622	0.375	0.648
	LPS	0.044	0.583	0.288	0.686
	PAM	0.115	0.672	0.726	0.611
	pIC	0.401	0.988	1.000	0.506
	R848	0.028	0.490	0.375	0.667
IL-6	PGN	0.038	0.662	0.063	0.419
	LPS	0.052	0.360	0.409	0.171
	PAM	0.008	0.715	0.127	0.005
	pIC	0.078	0.609	0.483	0.020
	R848	0.017	0.735	0.124	0.629
IL-12	PGN	0.028	0.833	0.885	0.735
	LPS	0.004	0.622	1.000	0.764
	PAM	0.017	0.214	0.606	0.303
	pIC	0.604	0.965	0.853	0.348
	R848	0.138	0.683	0.563	0.442

pDC

TNF-α	CpG	0.665	0.095	0.641	0.290
	R848	0.035	0.010	0.055	0.825
IFN-α	CpG	0.578	0.403	0.006	0.883
	R848	0.111	0.014	0.536	0.540

Table 10: Similar Antigen Presenting Cell Responses in Infants with African and Mixed Race Backgrounds

Whole blood from infants of African and Mixed racial background was stimulated with the indicated TLR ligands at 2 weeks, 6 weeks, 6 months and 12 months of life. Multiparameter flow cytometry was used to detect production of TNF-α, IL-6 or IL-12/23p40 in monocytes and cDC, and TNF-α, IL-6 or IFN-α in pDC. Number of subjects at 2 weeks (UE = 28, HEU = 27), 6 weeks (UE = 27, HEU = 25), 6 months (UE = 23, HEU = 25) and 12 months (UE = 21, HEU = 23) of life. Shown are p-values from Mann-Whitney test. Significant differences between groups ($p<0.05$) are highlighted when African > Mixed, and in yellow when Mixed > African.

Monocytes

		2 wk.	6 wk.	6 mo.	12 mo.
TNF-α	PGN	0.3835	0.6652	0.6887	0.1155
	LPS	0.2146	0.7026	0.0986	0.1204
	PAM	0.0099	0.0631	0.1202	0.9908
	pIC	0.718	0.623	0.0676	0.3639
	R848	0.2489	0.1828	0.0279	0.2366
IL-6	PGN	0.6791	0.5135	0.2695	0.0912
	LPS	0.1192	0.7535	0.0909	0.3011
	PAM	0.0006	0.2663	0.0769	0.8182
	pIC	0.7359	0.2978	0.5342	0.5395
	R848	0.1072	0.7665	0.7619	0.3065
IL-12	PGN	0.465	0.6529	0.1682	0.0643
	LPS	0.3368	0.44	0.1067	0.2852
	PAM	0.8533	0.6776	0.1871	0.8813
	pIC	0.8699	0.0461	0.6846	0.6678
	R848	0.8396	0.7665	0.4756	0.6051

cDC

TNF-α	PGN	0.5672	0.1565	0.8374	0.2853
	LPS	0.5091	0.8453	0.4401	0.3225
	PAM	0.3373	0.9932	0.5126	0.6875
	pIC	0.1706	0.8473	0.0796	0.4075
	R848	0.5913	0.9391	0.8835	0.5502
IL-6	PGN	0.6662	0.1126	0.5775	0.3065
	LPS	0.2347	0.9797	0.3583	0.1573
	PAM	0.037	0.5245	0.4756	0.6541
	pIC	0.2594	0.3315	0.214	0.5263
	R848	0.0927	0.225	0.807	0.5656
IL-12	PGN	0.6035	0.085	0.2695	0.0289
	LPS	0.7576	0.9662	0.2145	0.6945
	PAM	0.7051	0.2591	0.8527	0.9359
	pIC	0.8628	0.0833	0.237	0.8624
	R848	0.6283	0.05	0.4878	0.352

pDC

TNF-α	R848	0.3646	0.786	0.9766	0.8093
	CpG	0.7539	0.5395	0.273	0.7947
IL-6	R848	0.3286	0.0946	0.6321	0.5734
	CpG	0.3335	0.1707	0.5535	0.9096
IFN-α	R848	0.7985	0.1376	0.7397	0.9542
	CpG	0.7631	0.2147	0.1201	0.4414

Table 11: More Cytokine Detected on a Per-Cell Basis in Stimulated Antigen Presenting Cells in African Infants Compared to Infants with Mixed Racial Backgrounds

Whole blood from infants of African and Mixed racial background was stimulated with the indicated TLR ligands at 2 weeks, 6 weeks, 6 months and 12 months of life. Multiparameter flow cytometry was used to detect production of TNF-α, IL-6 or IL-12/23p40 in monocytes and cDC, and TNF-α or IFN-α in pDC. Number of subjects at 2 weeks (UE = 28, HEU = 27), 6 weeks (UE = 27, HEU = 25), 6 months (UE = 23, HEU = 25) and 12 months (UE = 21, HEU = 23) of life. Shown are p-values from Mann-Whitney test. Significant differences between groups ($p<0.05$) are highlighted when African > Mixed, and in yellow when Mixed > African.

Monocytes

		2 wk.	6 wk.	6 mo.	12 mo.
TNF-α	PGN	0.4189	0.0527	0.1122	0.7045
	LPS	0.1739	0.022	0.1691	0.9137
	PAM	0.5056	0.0833	0.4685	0.1714
	pIC	0.1628	0.9755	0.3267	0.8496
	R848	0.0046	0.1676	0.254	0.8496
IL-6	PGN	0.1959	0.0167	0.1691	0.4482
	LPS	0.3765	0.0527	0.6467	0.3038
	PAM	0.6481	0.3042	0.2864	0.7865
	pIC	0.5166	0.0446	0.0861	0.7761
	R848	0.1483	0.0782	0.332	0.1902
IL-12	PGN	0.3227	0.0886	0.2367	0.0527
	LPS	0.9621	0.8602	0.0901	0.4811
	PAM	0.4027	0.3475	0.0606	0.4811
	pIC	0.4915	0.0594	0.5434	0.3569
	R848	0.27	0.1129	0.0397	0.1843

cDC

TNF-α	PGN	0.1565	0.0116	0.1328	0.8496
	LPS	0.4876	0.0069	0.0606	0.6648
	PAM	0.151	0.0345	0.5721	0.11
	pIC	0.3067	0.8421	0.3011	0.2847
	R848	0.0637	0.0886	0.4491	0.6259
IL-6	PGN	0.1959	0.0161	0.0305	0.3431
	LPS	0.5944	0.1268	0.1222	0.4322
	PAM	0.4189	0.0459	0.075	0.839
	pIC	0.8059	0.5706	0.2816	0.6746
	R848	0.2272	0.071	0.3373	0.5695
IL-12	PGN	0.1679	0.0161	0.5019	0.2178
	LPS	0.3274	0.4995	0.0942	0.8284
	PAM	0.2129	0.0687	0.2453	0.9352
	pIC	1	0.679	0.424	0.8285
	R848	0.464	0.0063	0.2367	0.1672

pDC

TNF-α	R848	0.1305	0.7027	0.332	0.4645
	CpG	0.2344	0.4951	0.2538	0.783
IFN-α	R848	0.0254	0.3628	0.5019	0.8074
	CpG	0.5423	0.5693	0.0046	0.3523

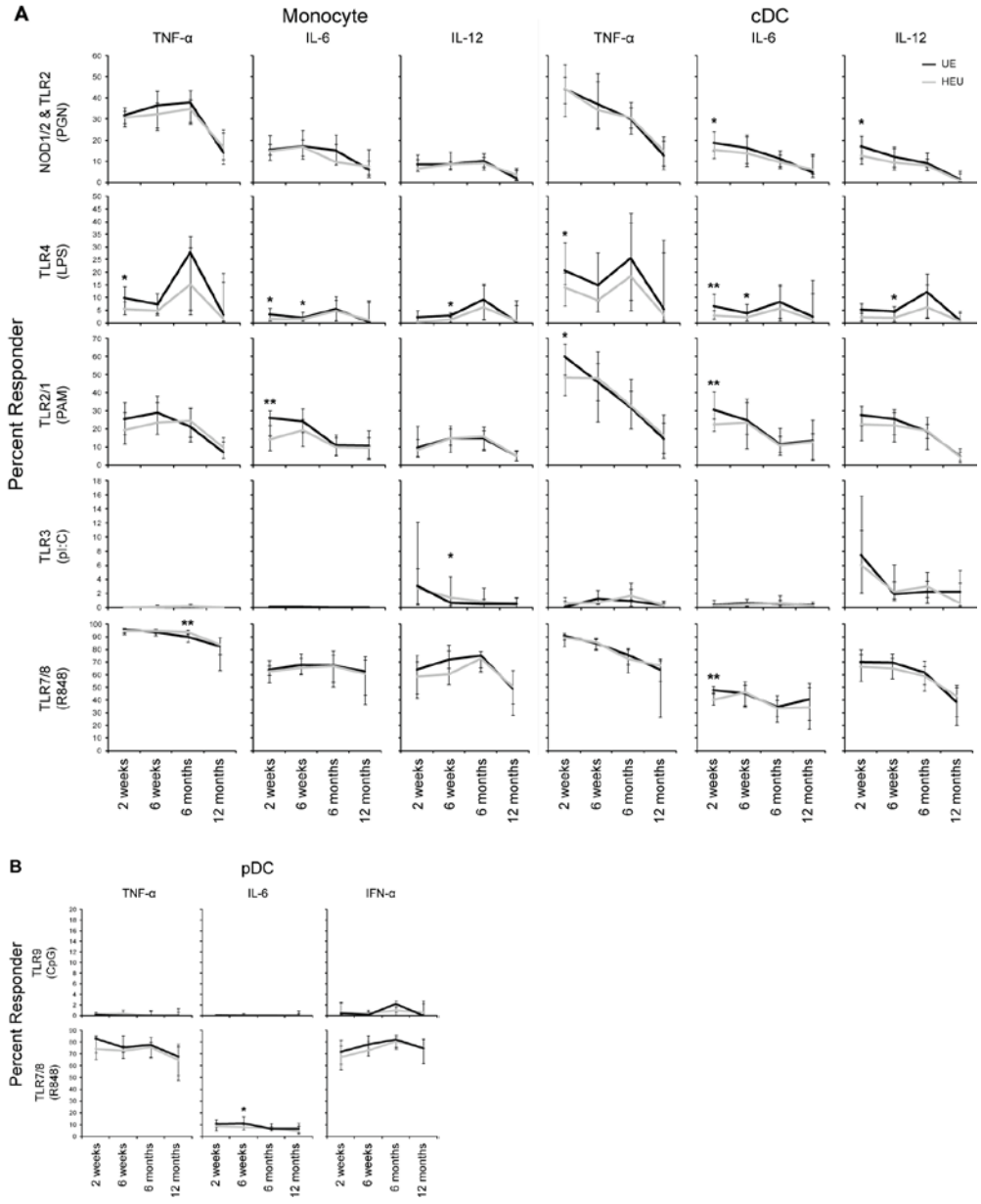


Figure 16: Elevated Proportion of Antigen Presenting Cells Responsive to PAMP Stimulation in HIV-Exposed Uninfected Versus HIV-unexposed infants

Shown are the proportions of cytokine producing cells for HEU (black) and UE (grey) subjects. The cytokines detected were TNF-α, IL-6 or IL-12/23p40 in A) monocytes and cDC, and B) TNF-α, IL-6 or IFN-α in pDC. Y-axis represents total percentage of cytokine-producing cells; error bars indicate interquartile range. Figure 13 was generated by subtracting the median UE response from the median HEU response for each time point as shown. Number of subjects at 2 weeks (UE = 28, HEU = 27), 6 weeks (UE = 27, HEU = 25), 6 months (UE = 23, HEU = 25) and 12 months (UE = 21, HEU = 23) of life. Mann-Whitney test was used to compare HEU and UE response at each time point and differences are signified by * ($p<0.05$) and ** ($p<0.01$). Unstimulated samples (near 0% of cytokine producing cells) were subtracted from stimulated samples.

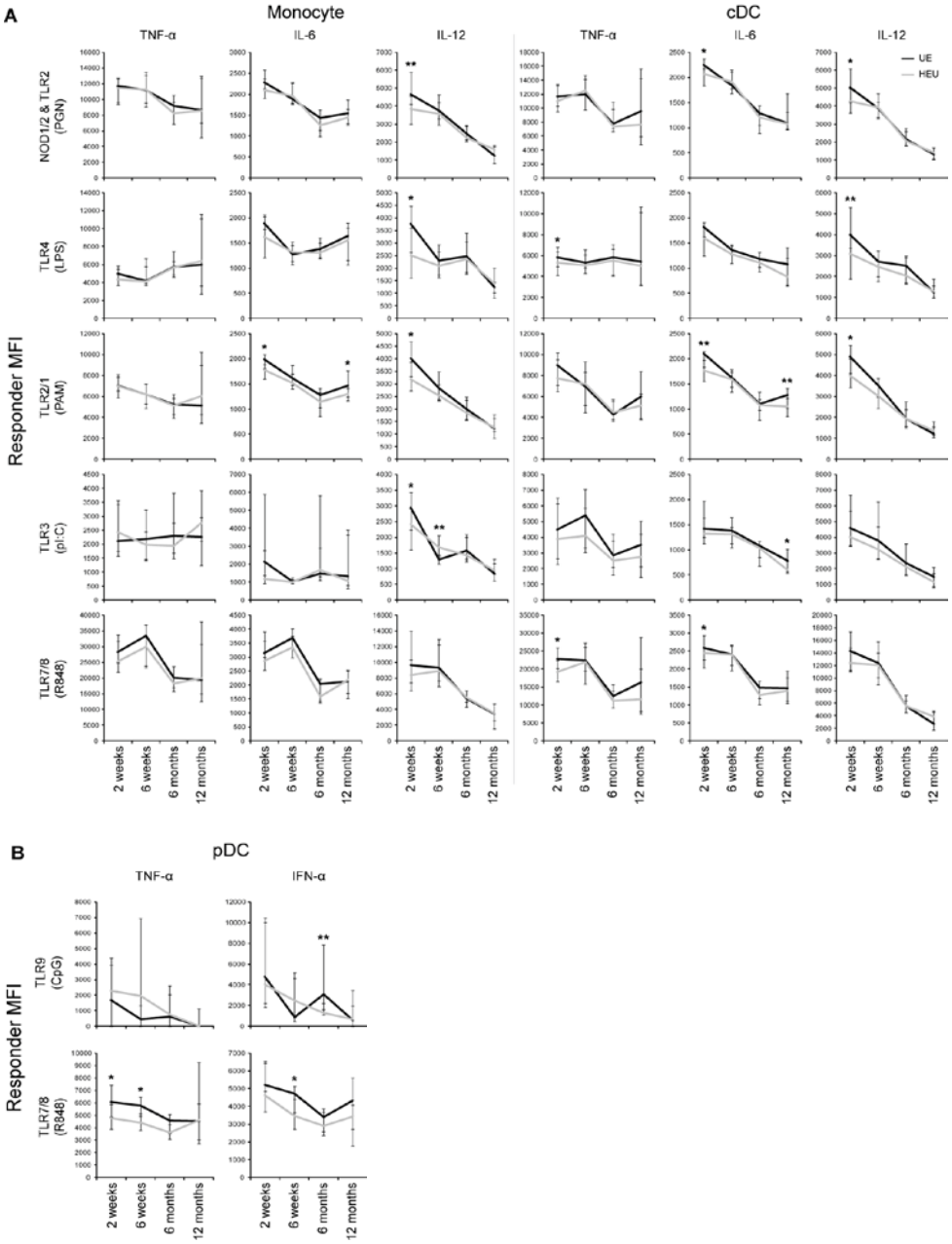


Figure 17: HIV-Exposed Uninfected Infant Antigen Presenting Cells Produce More Cytokine Than in HIV-Unexposed Infant Antigen Presenting Cells After PAMP Stimulation

Shown are the same samples as in Figure 14, but with mean fluorescent intensity (MFI) graphed for HEU (black) and UE (grey) in the respective samples for A) monocytes and cDC, for which we measured TNF-α, IL-6 and IL-12/23p40, and B) pDC, with TNF-α, IL-6 and IFN-α measured. Medians for each population's MFI (y-axis) are derived from FlowJo software; error bars indicate interquartile range. Figure 14 was generated by subtracting the median UE response from the median HEU response. Number of subjects at 2 weeks (UE = 28, HEU = 27), 6 weeks (UE = 27, HEU = 25), 6 months (UE = 23, HEU = 25) and 12 months (UE = 21, HEU = 23) of life. Differences in MFI between HEU and UE groups as detected by Mann-Whitney test are indicated by * ($p<0.05$) and ** ($p<0.01$).

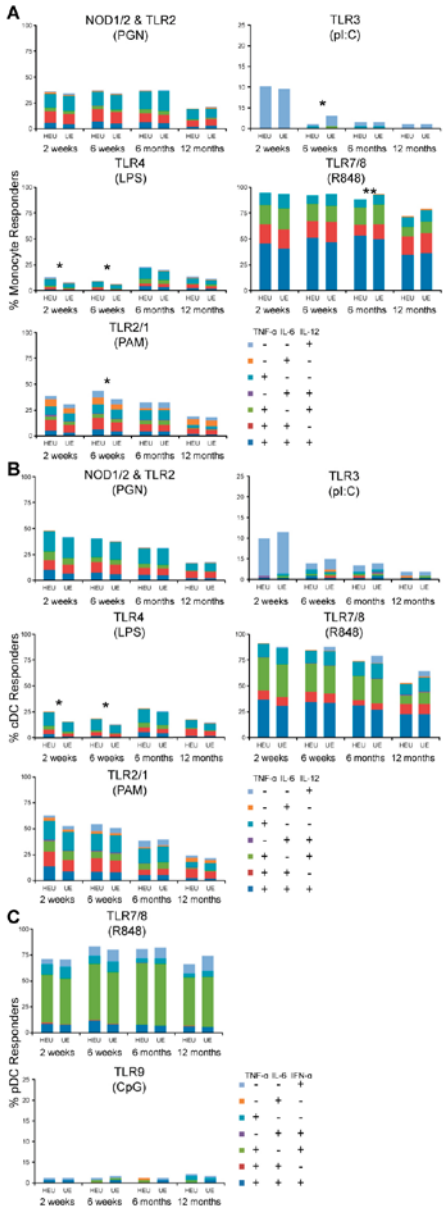


Figure 18: HIV-Exposed Uninfected Infant Poly- and Mono-Functional Antigen Presenting Cells Respond Stronger Than HIV-Unexposed Infants, but Few Differences Detected in Total Fraction of Responder Cells

Whole blood samples stimulated with the indicated TLR ligands were followed at 2 weeks, 6 weeks, 6 months and 12 months of life. Multiparameter flow cytometry was used to detect production of TNF-α, IL-6 and IL-12/23p40 in A) monocytes, and B) cDC; C) TNF-α, IL-6 and IFN-α was detected in pDC. Number of subjects at 2 weeks (UE = 28, HEU = 27), 6 weeks (UE = 27, HEU = 25), 6 months (UE = 23, HEU = 25) and 12 months (UE = 21, HEU = 23) of life. Total percentage of cytokine-producing cells is represented as total bar height, with differences measured by Student's t-test indicated by * ($p<0.05$) or ** ($p<0.01$). Color-coded segments allow differentiation of monocytes producing various combinations of the 3 cytokines analyzed. Cytokine profile – color combinations are indicated in the key in identical order from top to bottom as shown in bar graphs. Unstimulated samples, with near 0% of cytokine producing cells, were subtracted from stimulated samples.

5. ANTIBODY RESPONSES TO VACCINATION AMONG SOUTH AFRICAN HIV-EXPOSED AND UNEXPOSED UNINFECTED INFANTS OVER THE FIRST YEAR OF LIFE

5.1 Introduction

Vaccination is essential to combat infectious mortality and morbidity in children under 5 years of age [177]. Despite the existence of effective vaccines, 6 million children die from infectious diseases annually, mainly in low-middle income countries, where HIV is often prevalent [9,178]. While access to vaccines surely is the most important contributor to the high number of vaccine preventable deaths in these regions, it is unclear if vaccines are equally protective in all children. HEU infants are at a higher risk of infectious morbidity and mortality compared to their UE couterparts, and multiple factors likely contribute to this phenomenon (as posited in previous chapters). Suboptimal response to vaccination has also been suggested to contribute to the increased infectious burden of HEU. This notion has been supported by several studies, which documented low vaccine specific antibody titers in HIV-infected mothers and altered vaccine-specific antibody levels in HEU compared to UE infants [55,137-139]. These differences have been ascribed to compromise in maternally transferred antibodies, differing antibody half lives [139], and altered responses to vaccination [137] [55]. To date, however, studies have only investigated vaccine responses in either short-term cohorts or cross-sectional studies [55,137-139], thereby not addressing long-term vaccine induced immunity in HEU [143]. A longitudinal analysis of HEU responses to vaccination is required to

better understand how the rapidly expanding population of HEU infants responds to childhood vaccination. To this end we established a birth cohort study in South Africa, a country with an antenatal HIV prevalence of 30% [179], and followed HEU and UE infants from 2 weeks up to 2 years of life, evaluating their vaccine specific immune responses. Based on the above cited published literature [55,137,139], we expected to find lower pre-vaccine specific antibody titers in the HEU than in the UE infants pre-vaccination, followed by a higher level of response to certain vaccines in HEU vs. UE infants following vaccination. However, we were uncertain of the HEU infants' immune response following subsequent booster doses and the longevity of the resulting immune response.

5.2 Methods

Study ethics are described in Chapter 2 Methods section. Cohort study design and blood sample processing are described in detail in Chapter 3 Methods section.

5.2.1 Quantification of Serum Antibody Titers

Quantitative determination of specific IgG levels to *Bordetella pertussis* (Bp), TT, Hib, HepB and Measles were performed by standard commercial ELISAs conducted according to the manufacturers' instructions. Specific IgG levels to Bp, TT, and Measles were evaluated using SERION ELISA *classic* kits (ESR120G, ESR108G and ESR 102G; Serion Immunodiagnostica GmbH, Würzburg, Germany). Hib specific IgG

was measured using VaccZyme™ Human Anti-Hib ELISA kits (MK016 and MK012; The Binding Site Ltd, Birmingham, England). Hepatitis B surface antigen (HBsAg) specific IgG was measured using Human Anti-HBsAg Quantitative ELISA kit (4220-AHB; Alpha Diagnostic International Inc., San Antonio, Texas, USA). As recommended by the manufacturer, vaccine specific IgG levels were expressed using the following units: anti-Bp IgG in FDA units per milliliter, anti-TT IgG in international units per milliliter, anti-Measles IgG and anti-HBsAg IgG in milli-international units per milliliter, and anti-Hib IgG in milligrams per milliliter. Quantitative determination of total IgG was evaluated at each visit using GenWay Human IgG ELISA kits (GWB-A04A50 GenWay Biotech, San Diego, CA); total IgG levels were measured in milligrams per milliliter. Antibody titers of 30 FDA Units/mL were deemed protective for Pertussis, as defined by the manufacturer. For Measles, 200 mIU/mL was the cut-off for protection established by the manufacturer. Minimum protective antibody titers for Tetanus are 0.1 IU/mL [180,181], for HepB 10 mIU/mL [180,182], and for Hib 1 ug/mL [180,183] was considered protective.

5.2.2 Statistical Analysis

Statistical analysis of the antibody levels was performed using Microsoft Excel software (2007). Antibody responses were log transformed, and subjected to unpaired, two-tailed Student's *t* tests for comparison between HEU and UE of mean specific antibody level at each time point; Bonferroni adjustment for multiple comparisons was applied to each individual antibody, resulting in a $p<0.071$

considered significant for anti-Hib, pertussis, tetanus, HepB and measles. Statistical analysis of the baseline cohort characteristics was performed using R version 2.13.1; continuous variables were analyzed using the Student's t-test and categorical variables the Chi-square, and two-sided alpha was set at 0.05.

5.3 Results

5.3.1 Participant Characteristics

Fifty-nine infants remained enrolled at 2 weeks; four were identified as HIV-infected by HIV DNA PCR testing at 2 weeks of age and excluded from analysis. Thus 28 UE infants and 27 HEU infants were retained at the first clinical visit; substantial attrition occurred past 12 months and 17 HEU and 20 UE were retained throughout the 2 year study. All HEU infants were confirmed to remain HIV-uninfected by HIV DNA PCR at 1.5, 3 months and 24 months of age. There was no significant difference in sex, birth weight, gestational age or number retained at follow-up at 2 years between the two groups (Table 12). All HEU infants but one were exclusively formula fed; all but one UE initiated breastfeeding at birth and continued breastfeeding with a median duration of exclusive breastfeeding of 12 weeks. Three infants developed moderately acute malnutrition (2 UE at 6 months and 1 HEU at 12 months). There was no difference in the number of caregiver-reported infectious events between HEU and UE [184]. Etiologies of infectious events were only confirmed for cases that resulted in hospitalization. Amongst those hospitalized, no clinically

or microbiologically identified cases of pertussis, Hib disease or tetanus were identified; one case of measles infection occurred in an UE before the first vaccine was administered, and one measles infection occurred in a fully vaccinated HEU infant. Detailed descriptions of participant characteristics and infectious morbidity for this cohort are provided by us elsewhere [184].

5.3.2 Specific Antibody Levels

All HEU and UE subjects were vaccinated within the same time relative to blood draws for serological analysis. None of the infants received DTaP/Hib or HepB vaccination prior to the 0.5 and 1.5 month blood draw, thus antibody levels at these time points are reflective of transplacental maternal antibody transfer. Infants at the 6 month blood draw were also still measles vaccine naïve, as this vaccine was not administered until 9 months of age.

Pre-vaccination, at 0.5 and 1.5 months, HEU had overall lower levels of antibody specific for tetanus, pertussis and Hib specific antibodies (Figure 19A, C, E, G). However, pre-vaccine, only anti-tetanus levels were statistically higher in UE ($P<0.025$). Conversely, HepB antibody levels were higher in HEU prior to vaccination ($p<0.025$), though only ¼ had levels considered to be protective.

Vaccine specific titers of HEU and UE infants reached similar levels after 2 vaccine doses as measured at the 3-month blood draw. However, at that time point (3

months) the average antibody titers reached protective levels in > 50% of recipients only for tetanus, Hib and HepB, while < 20% of infants had protective anti-pertussis antibody levels (Figure 19B, D, F, H). Of note, HEU demonstrated significantly higher titers of anti-pertussis antibody after the 3rd dose (by 6 months) already. As a result, a higher proportion of HEU vs. UE reached protective levels at 6 months of age; protective anti-pertussis titers in UE were not reached until 18months of age (after the 4th vaccine dose). Notably, for either group, once protective levels were reached, antibodies were maintained at protective titers through 2 years of age.

Individual titers were analyzed within each group to determine the proportion of subjects with protective antibody titers. Prior to vaccination greater than 25% of infants in both HEU and UE groups exhibited protective levels of anti-pertussis antibodies (Figure 19B). Greater than ¼ of HEU subjects were also protected from HepB before vaccination, whereas no UE had protective anti-HepB titers prior to vaccination (Figure 19H). In contrast, the proportion of UE protected from Hib was greater than in the HEU group (Figure 19F). Very few infants in either group demonstrated protective titers of anti-tetanus antibodies prior to their first vaccination (Figure 19D).

Protective levels of anti-measles antibody were on average not detected per group in either the HEU or UE groups until after the first vaccine dose had been given at 9 months (Figure 20A). When measured at 2 years, anti-measles antibody titers remained unchanged following administration of the second dose (administered at

18 months). The average levels of anti-measles antibody detected were nearly indiscernible between the HEU and UE groups at all time points in time through the first 2 years of life. Analyzing the individual subjects' anti-measles antibody revealed fewer than ¼ of subjects with protective pre-vaccination titers in both groups at 2 weeks, which as expected further dropped by 6 weeks. The highest proportion of protected subjects was found at 12 months in UE and at 18 months in HEU, with ~¾ of subjects protected. Interestingly, the number of subjects deemed protected decreased by 2 years only in the UE group, to only ~½ maintaining protective measles specific antibody titers (Figure 20B).

5.3.3 Total Immunoglobulin

Total IgG levels were measured at all time points in order to determine whether changes in vaccine specific IgG were due to non-specific differences in total IgG (e.g. hypergammaglobulinemia) [185]. Equivalent total IgG levels, with age-appropriate physiological hypogammaglobulinemia at 1.5 and 3 months were observed in HEU and UE (Figure 21).

5.4 Discussion

In this prospective, longitudinal comparison of antibody response in HEU and UE infants to vaccines administered over 24 months, we identified robust serological responses to the EPI vaccines. With the exception earlier strong responses to

pertussis vaccine and higher anti-tetanus titers at 24 months in HEU vs. UE infants, the vaccine responses were comparable overall in magnitude. This suggests HEU infants mount at least as good a response to EPI vaccines as UE infants.

The diminished baseline antibody levels for tetanus (significant) and Hib (not significant) we detected in HEU could be due to lower vaccine specific antibodies in the mother, or placental dysfunction resulting in decreased antibody transfer, or due to decreased half-life of transferred maternal antibodies [55,137,139,140]. On the other hand the higher pre-vaccine HepB antibodies observed in HEU infants likely reflected maternal HepB infection in HIV-infected mothers; indeed, high variability in the pre-vaccine HepB antibody levels were observed in our HEU cohort, suggesting individual differences such as maternal HepB infection, to be involved. This however, was not formally evaluated.

HEU infants developed significantly higher anti-pertussis titers than UE at 6 and 12 months of age, extending the previous observation of Jones *et al,* who showed increased response at 4 months of age. Anti-Bp titers reached protective levels in HEU already by 6 months, after receiving only 3 doses in total. HEU may therefore be protected from pertussis infection sooner than UE infants, who only exceeded levels of anti-Bp antibody considered to be protective after an additional booster at 18 months of age. This could possibly relate to decreased antibody interference during priming secondary to lower level of transferred maternal antibodies [141].

Overall, the period of suboptimal protection from pertussis in infancy was unexpectedly long for both HEU as well as UE, namely 6-12 months, requiring 3 to 4 doses of vaccine. Since the vaccine antibody response to Hib, Tetanus, Hepatitis B and Measles reached protective levels after 1 or at most 2 doses, an overall lower immune response to vaccination in general in our subjects seems unlikely to be the cause for the suboptimal response to pertussis vaccination. The most critical period for severe pertussis is early in life; the prolonged gap in protection of South African infants thus appears worthy of further investigation.

Our study suffers a number of limitations. First and foremost, our sample size was small. However, our results confirm previous cross-sectional reports on vaccine responses in HEU infants [55,137], and in their aggregate strongly support our overall conclusion. More importantly, the prospective, longitudinal nature of our study, and especially the extension up to 24 months of age, provide the much-needed insight into long-term vaccine induced protection in this highly vulnerable population. Secondly, feeding mode differed significantly between UE and HEU, as formula feeding of HEU was prevalent in the Western Cape during our study. Our goal however, was to define the long-term vaccine response of HEU in comparison to UE, not why they differed (if there were a difference). And while breast- vs. bottle feeding is known to influence vaccine responses, the differences in feeding preference was the reality HEU infants in South Africa (and elsewhere) were facing. For comparison of antibody responses we did not apply a correction for multiple comparisons, as these comparisons were not independent of one another, so this

must be considered when interpreting results. For example, antibody responses are interrelated between time points for reasons such as interference from pre-existing serum antibody levels and pre-established B cell memory largely dictating the strength of booster responses. Furthermore, immune attributes that impact an individual's vaccine response likely apply to other vaccines. Lastly, we did not perform functional studies regarding antibody responses following immunization. This is important, as for example a study comparing response to the 7-valent pneumococcal vaccine in HEU and UE infants showed similar responses in HEU and UE infants for 6 of 7 serotypes, and even better response than UE to one serotype (6B). However, upon evaluation of qualitative function by the opsonophagocytic killing assay, HEU infants in fact required higher concentrations of antibody for 50% killing activity against one of the serotypes (19F) [142]. We can thus not exclude qualitative differences between HEU and UE to the EPI vaccines we tested.

In conclusion, outside of selected differences of pre-vaccination antibody levels, HEU infants responded quantitatively at least as well as UE to current standard EPI vaccination.

5.5 Chapter Five Tables and Figures

Table 12: Baseline Infant Characteristics by HIV Exposure Status

	Infant exposure type			
Parameter[a]	Total (n = 55)	HEU (n = 27)	UE (n = 28)	P value
No. (%) male	22 (40)	7 (26)	15 (54)	P =0.35
Mean birth weight (95% CI), in g	2,966 (2,857-3,075)	2,945 (2,866-3,024)	2,986 (2,830-3,142)	P = 0.7
Mean gestational age (95% CI), in wk	37.8 (37.1-38.4)	37.7 (36.7-38.7)	37.9 (37.0-38.7)	P = 0.8
No. (%) of infants in follow-up at 24 mo	37 (67)	17 (63)	20 (71)	P = 0.7

[a] CI, confidence interval

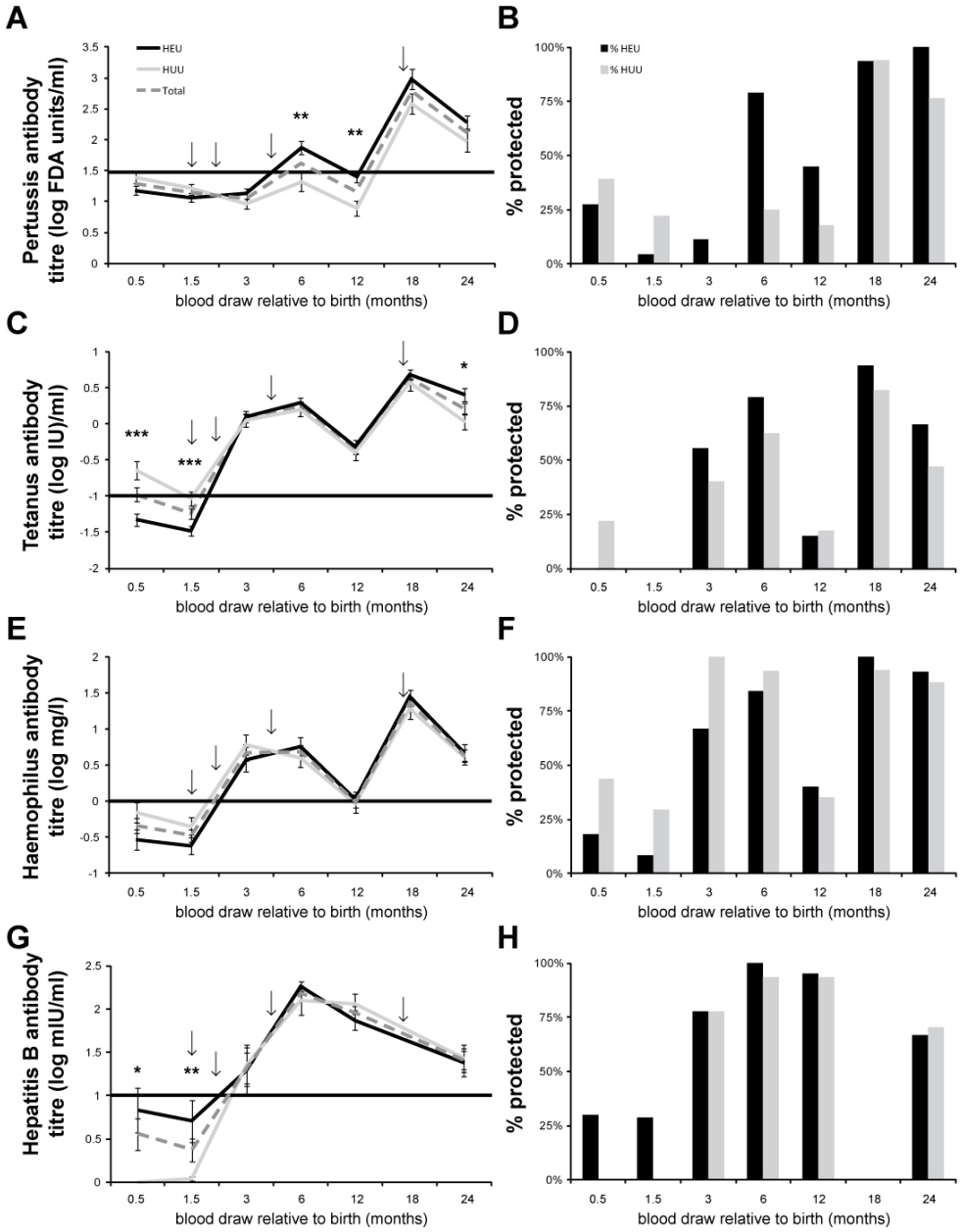


Figure 19: HEU and UE Pertussis, Tetanus Toxoid, Haemophilus Influenza B and Hepatitis B Specific Antibody Levels Over the First Two Years of Life

(A, C, E, G) Specific antibody levels were measured in HEU (black line) and UE (grey line) infants by enzyme linked immunosorbent assay at 0.5, 1.5, 3, 6, 12, 18 and 24 months of age. Total response of the two groups combined is shown with a dashed line. Vaccine antibody level is indicated on the y-axis. Error bars, Standard Error of the Mean (SEM). * = $p<0.05$, **=$p<0.01$, ***=$p<0.001$. (b, d, f, h) Individual antibody titers were compared to minimum protective antibody levels. The proportion of subjects whose titers exceeded the minimum level for protection was shown for HEU (black bar) and UE (grey bar). Number of subjects at 0.5 months (UE = 22, HEU = 25), 1.5 months (UE = 27, HEU = 28), 3 months (UE = 27, HEU = 28), 6 mo (UE = 21, HEU = 29), 12 months (UE = 20, HEU = 23), 18 months (UE = 20, HEU =17) and 24 months (UE = 20, HEU = 17) of life.

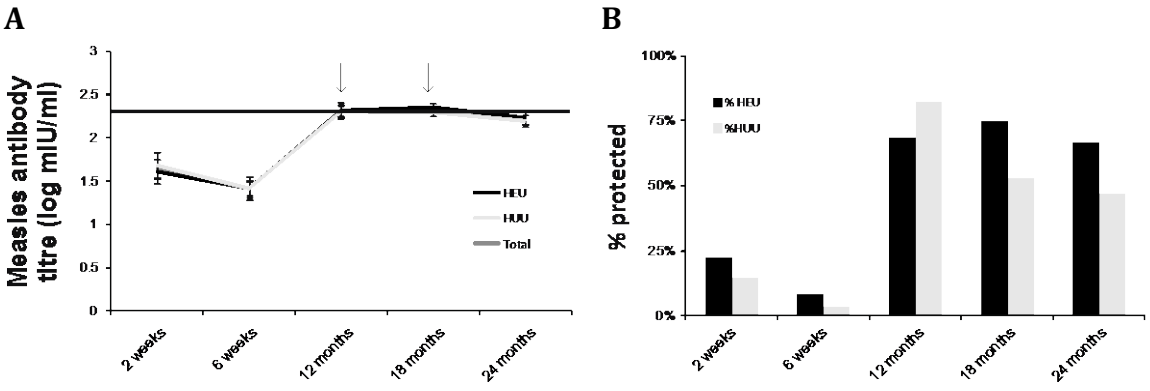


Figure 20: HEU and UE Measles Specific Antibody Levels Over the First Two Years of Life

(A) Specific antibody levels were measured in HEU (black line) and UE (grey line) infants by enzyme linked immunosorbent assay at 0.5, 1.5, 3, 6, 12, 18 and 24 months of age. Total response of the two groups combined is shown with a dashed line. Vaccine antibody level is indicated on the y-axis. Error bars, Standard Error of the Mean (SEM). * = $p<0.05$, **=$p<0.01$, ***=$p<0.001$. (B) Individual antibody titers were compared to minimum protective antibody levels. The proportion of subjects whose titers exceeded the minimum level for protection was shown for HEU (black bar) and UE (grey bar). Number of subjects at 0.5 months (UE = 22, HEU = 25), 1.5 months (UE = 27, HEU = 28), 3 months (UE = 27, HEU = 28), 6 mo (UE = 21, HEU = 29), 12 months (UE = 20, HEU = 23), 18 months (UE = 20, HEU =17) and 24 months (UE = 20, HEU = 17) of life.

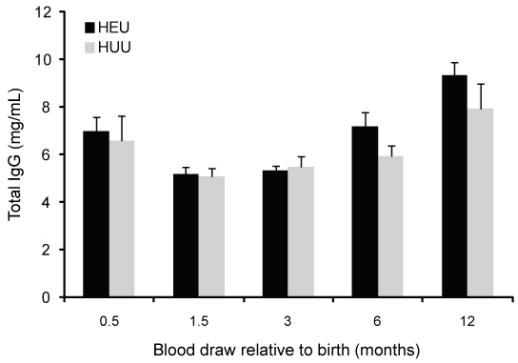


Figure 21: HEU and UE Total IgG Levels Over the First Two Years of Life

Total IgG levels were measured in HEU (grey bar) and UE (black bar) infants by enzyme linked immunosorbent assay at 0.5, 1.5, 3, 6 and 12 months of age. Total IgG level is indicated on the y-axis. Error bars, SEM. Significance, $p<0.05$. Number of subjects at 0.5 months (UE = 22, HEU = 25), 1.5 months (UE = 27, HEU = 28), 3 months (UE = 27, HEU = 28), 6 mo (UE = 21, HEU = 29), 12 months (UE = 20, HEU = 23), 18 months (UE = 20, HEU =17) and 24 months (UE = 20, HEU = 17) of life.

6. GENERAL DISCUSSION

6.1 Summary

6.1.1 Study Aims Summary

The work comprising this thesis aimed to assess immune development and clinical outcomes over the first year of life in infants born in a developing nation's setting, Specifically, this work was intended as a 'pre-pilot' study, as we first had to establish feasibility of implementing a birth cohort, in order to interrogate immunologic variability between UE and HEU infants in South Africa. Our goal was to use pre-pilot data to facilitate logical and directed design of future studies of etiological factors underlying increased risk of infectious morbidity and mortality early in life. We therefore powered this study to serve as a tool for broad discovery, ie. this study was powered for hypothesis generation and not hypothesis testing.

A prospective cohort study was established, which consisted of HEU and UE newborns in South Africa. Specifically, to accomplish the first aim of this study, *the frequency and severity of clinical outcomes were described in our cohort of South African infants*; novel data was generated through the comparison of infection severity that HEU and UE infants experienced over the first year of life. A common focus of pediatric infectious disease research has been to identify correlates of disease (e.g. HIV infected mothers, or lack of childhood vaccination). The next challenge was to identify subgroups of infants who are at the greatest risk of

infection, and precisely when increased rates of infectious morbidity and mortality occur.

An intimate understanding of early life immune development is essential to investigate etiological mechanisms that underpin increased risk of severe infections in highly vulnerable populations. Tremendous efforts have been made in recent years to better understand changes in the early-life immune system as it goes through profound transitions from a tolerogenic intrauterine phase in the fetus, to a phase of controlled responses with a plethora of new environmental, antigenic or pathogen exposures early in the postnatal period. By a child's first birthday the immune system has gone through the majority of its maturation into an 'adult-like' immune system [61,186], therefore the first year marks a period of rapid change. Section 1.3 (early-life innate immune ontogeny) outlines a plethora of changes to the innate immune system early in life. Early-life changes include increases in complement functionality with concurrent decreases in maternal immunoglobulin, neutrophils and natural killer cells. Other known early-life changes to some of our first lines of defenses include altered integumentary and mucosal barriers, as well as waning passive immunity with increases in active humoral immunity. Section 1.3.2.1 details the importance of PRRs for APCs to recognize and respond to pathogens. PRRs are paramount to containment of initial infection and for the orchestration of subsequent adaptive responses to clear the infection and rapidly respond to future challenges. Figure 23 summarizes developmental changes in APC responses to PRR stimulation through early life. Data for PRR mediated APC

responses in the first year of life was previously lacking. The immune system's successful navigation of the first year is crucial for both early life protection and to set the stage for healthy and protective immunity later in life. For these reasons, the second broad aim of this thesis was established *to provide a comprehensive description of innate immune development in a cohort of South African infants over the first year of life*. Previous studies analyzing early life immune development have been either cross-sectional in nature, or have not focused on changes that occur within the first year of life, therefore this body of work was carried out to provide detailed information on immune ontogeny throughout this time period.

Children in Sub-Saharan Africa are among those at the highest risk of infectious morbidity and mortality early in life, and HEU are particularly vulnerable. HEU infants appear to be transiently immunocompromised, and there is currently no consensus precisely when or why this occurs. Evidence suggestive of altered immunity in HEU infants lead us to the third broad aim of this thesis, which was *to compare innate immune development and adaptive immune development between HEU and UE infants throughout the first year of life*. Despite the markedly increased incidence of morbidity and mortality in the ever-expanding HEU infant population, there was a paucity of data describing HEU immune development throughout this critical time period. Prior to the work comprising this thesis, few differences had been detected between HEU and UE infant innate immune responses, and the majority of immune analysis excluded single cell functional analysis. This study's high fidelity, multiparameter, single-cell analysis, applied over several time points

throughout the first year of life, allowed for increased detection of differences in immune development between HEU and UE infants. This work uncovered age– and pathogen–specific aberrancies in the early life HEU infant immune responses.

6.1.2 Summary of Study Design

In collaboration with Stellenbosch University and the University of British Columbia we developed a birth cohort of South African infants starting in 2009. Newborns and their mothers were recruited in the postnatal period, while on the maternity ward at TAH, in the Western Cape of South Africa. Study subjects were from surrounding communities in the TAH catchment area. Power calculations were based on analysis of similar immunological outcome measures in a cohort of Canadian infants, and we estimated that 17 subjects would be required per group to detect approximately 50% difference in cytokine production, with a power of 80% ($p<0.01$). Over 100 patients were recruited, with 31 UE and 29 HEU patients retained at the first clinic visit. Clinical data, social data and whole blood was collected at 2, 6 and 12 weeks, 6, 12, 18 and 24 months of age. Multiple time points were selected throughout the first year of life in order to describe early life innate immune development in a cohort of infants, and canonical pathogen moieties, associated with broad groups of bacteria or viruses, were used to assess innate immune development. Passive and adaptive immunity were also assessed by measuring antibody levels to commonly used vaccines at each of the time points mentioned above.

6.2 Burden of Disease in HIV-Exposed Uninfected and HIV Unexposed Infants in the Developing Nation Setting: a Critical Summary

Clinical outcomes were reported for our cohort of HEU and UE infants over the initial 18 months of life. Parent reported and clinically documented infections were recorded at each study interval. A physician also performed Child health assessments at each interval. In this cohort there was no increase in frequency of infections, but the frequency of severe infections was greater in the HEU group (Figure 24, summarized in Table 13). This was an important observation as increased infectious disease exposure in the HIV affected household has been proposed as a source for increased infectious morbidity [10]. This theory is not supported by our observations, rather, our results demonstrated that, despite potential differences in infectious exposures at home, HIV-exposed and unexposed infants in our cohort got sick from a similar number of illnesses. HIV infected mothers in our study were not severely immunocompromised (based on absolute CD4 count (Table 5)), and therefore were less likely to be a significant source for increased infectious exposures in HEU. Even if differences in infectious disease exposure existed, increased disease severity in the context of similar infection type and frequency suggested impaired ability to contain infection in HEU infants. Since the immune system is responsible for containing and clearing infections, this

observation was strongly in support of our hypothesis that HEU infants are at least transiently immunocompromised.

Our study did not assess pathogenic etiologies for the observed illnesses experienced by infants, instead we recorded clinical manifestation of disease. Future studies aimed at evaluating specific microbiological causes for increased morbidity and mortality in HEU infants would be invaluable. While our immune response comparison between HEU and UE infants demonstrated altered immune responses in HEU infants, we still do not know if these altered immune responses cause changes in immune mediated protection from pathogens. We provided data on responses to canonical molecular moieties for various pathogen groups, e.g. Gram negative versus Gram positive bacteria versus single stranded RNA viruses, but the clinical significance of these findings can only be determined with future analysis of microbiological etiologies for severe diseases in HEU infants. We also have no evidence to indicate if altered innate immune responses in HEU infants has a cause or effect relationship, i.e. do the detected variabilities in immune response confer susceptibility to disease, or do they indicate protective responses mounted by HEU infants? These are essential questions to address to better understand the multifactorial driving factors influencing morbidity and mortality in HEU infants.

Several factors contribute to decreases in ability to contain infections in young infants, such as malnutrition, prematurity, maternal immunosuppression or missed immunizations [10,187]. There was no difference detected for these factors between

groups in our cohort, however, an important difference was a lack of breastfeeding in HEU infants. There is an abundance of data indicating lack of breastfeeding as a risk factor for diarrheal disease [12,29] and recently a study suggested a decreased incidence of upper and lower respiratory tract infections in breastfed infants [188]. Although there was no difference detected in severity of diarrheal disease in our cohort, more severe LRTIs were detected. Studies comparing HEU and UE morbidity and mortality demonstrate an association between breast milk weaning and increased infectious morbidity [189]. However, the increased morbidity associated with breast milk weaning is from diarrheal disease and not LRTIs. LRTIs are the leading cause of increased morbidity in HEU infants worldwide. Furthermore, changes in breastfeeding result in much less than the 3-fold difference in morbidity observed in HEU vs. UE infants. Therefore increased morbidity and mortality in HEU infants should not be attributed solely to altered breastfeeding practices.

There are larger studies that have compared infectious disease in HEU and UE infants [19,115]. Unlike studies conducted in Zimbabwe, Uganda, Melawi and Zambia [114,115,189,190], we did not observe significantly elevated mortality rates in HEU infants, however, our analysis involved stratification of disease severity, which demonstrated increased susceptibility to LRTIs in HEU vs. UE infants (clinical categories of severe infections are summarized in Table 14). While a weakness of our study was the limited cohort size, presumably a stronger signal was required to demonstrate significant differences in a smaller group. We would therefore expect similar observations in larger follow up studies. Ours is the first comparison of

infection frequency and severity of HEU and UE infant infections, and lends further support for the hypothesis of altered early life immune development in HEU infants.

6.3 Functional Analysis Of Infant Immune Responses

Understanding defense mechanisms from infectious disease, and how those defense mechanisms can fail, is essential for optimizing preventative medicine interventions. With efforts to decrease child morbidity and mortality, there has thus been concerted efforts in recent years to better understand development of immune defense mechanisms in early life [60-62,87,97]. In an effort to better describe innate immune responses, our group established high throughput immune assays that could be used to detail immune responses to several pathogen types [82]. We utilized innate immune stimulation assays for a variety of pathogen associated molecular patterns present on bacterial and viral pathogens, and built them into a single standardized immunoassay (Appendix A Table 1 and Appendix A Figure 5). Our whole blood stimulation assays were developed to accommodate freezing and storage of biological samples for batch analysis at a centralized location (Appendix A Figure 9), which allowed a high degree of standardization. This approach also expanded our capability to perform immunoassays on site, where blood was collected, rather than only at locations where technological resources for the complex analysis of biological samples were available. This was pivotal because

APCs are highly sensitive to environmental changes, and therefore handling, or blood draw– to– assay times must be minimized to decrease artifact. In order to provide a more comprehensive representation of innate immune development we optimized multiparameter flow cytometry technologies to detect several intracellular cytokines and cell surface receptors concurrently [82]. We also utilized multiplex bead array technologies to detect multiple secreted cytokines, which serve various functions, such as proinflammatory or promotion of Th1 or Th17 immune responses (Appendix A Figure 9) [82,97].

Single cell analysis enabled detection of subtle changes in innate immune function between comparison groups. Some cell types may increase in function over time, while others may decrease, thus changes at the cellular level are likely missed when only secreted cytokines are analyzed. An example of this was demonstrated in our analysis of a Canadian cohort, where both supernatant and cellular analysis were performed on same biological samples, yet several changes in cytokine production over the first year were detected at the cellular level that were not as evident in culture supernatant [97]. Furthermore, we demonstrated that subdividing cells into functional groups based on level of polyfunctionality uncovered differences in cytokine production (between polyfunctional subgroups) that were not evident at a less differentiated level [97]. By applying this robust, high throughput, high fidelity immune assay, we were able to provide the most detailed and comprehensive analysis of UE and HEU immune development to date.

6.4 Innate Immune Ontogeny Throughout the First Year of Life: a Critical Summary

There is very little data on innate immune development in early life, and previous data describing innate immune development in resource poor settings was obtained with cross-sectional studies. The majority of these cross-sectioned data were phenotypic as opposed to functional. We aimed to provide an understanding of functional innate immune development in a cohort of HIV unexposed infants in South Africa throughout the first year of life. Single cell analysis focused on monocytes, cDC, pDC and B cells, which are key players in the containment of initial infection, and for the development of subsequent adaptive immunity.

pDC are instrumental in anti-viral responses, and in our study pDC exhibited approximately equivalent functional responses between the initial weeks of life and one year of age. cDC and monocytes demonstrated a decrease in production of Th1 and Th17 cytokines over 12 months in response to Gram positive bacterial and viral antigens (PGN/PAM/pIC/R848). Responses to Gram negative bacteria derived LPS did not change over this time period. Our observations of adult-like responses to LPS already at 2 weeks of life were in sharp contrast to previously established data. Both Nguyen et al. [36] and Belderbos et al. [37] detected an increase in LPS or CpG induced IFN-γ, IP-10 and IL-12p70 production over the first year of life in European infants. Altered expression of these cytokines is significant as they are important modulators of immune function. IFN-γ is a potent inducer of macrophages, antigen

processing and MHC expression, it is central to signaling for Th1 differentiation and regulates B-cell isotype switching. IL-12p70 is also a potent inducer of Th1 differentiation, and of T cell and NK cell activation. IP-10 is secreted in response to IFN-γ and is a potent chemoattractant for the aforementioned cell types. Elevated IFN-γ, IL12p70 and IP-10 secretion may therefore enhance responses to viruses and intracellular bacteria throughout the first year by promoting Th1 immunity, and for the same reason may be protective from development of allergy and atopy (which can result from unopposed Th2 responses early in life). Alternatively, by maintaining Th1-promoting responses throughout the first year of life, infants may exhibit decreased responses to parasites or extracellular bacteria, which are primarily mediated by Th2 immune responses.

Contrasting results from European infants, South African infant Th1-promoting responses to LPS did not increase over the first year of life. With our study design, responses were not measured prior to 2 weeks of life. It is therefore possible that the lack of increase of cytokine production in response to LPS between 2 weeks and 1 year is a result of an early maturation of the TLR4 mediated response. Future studies that incorporate immune analysis of cord blood would thus be invaluable. Levy et al. (2004) demonstrated lower responsiveness of cord blood versus adult blood to LPS stimulation in infants born by cesarean section in Boston, USA [67]. Our contrasting observations may have been due to the time of collection (birth vs. 2 weeks), or could be attributed to different environmental exposures and genetic backgrounds in infants born in South Africa vs. the United States. LPS-induced

tolerance is also a potential mechanism behind the lack of increased LPS responsiveness in South African infants. Exposure to LPS has been demonstrated to induce an epigenetically mediated state innate immune tolerance [191]. Regardless of the timing of etiological factors driving the observed differences, we have demonstrated that differences do exist between South African and non-South African infant innate immune development. A potential causative factor for the observed variability in LPS responses is elevated exposure to Gram negative bacteria in infants living in lower income households. Indeed, increased exposure to Gram negative bacteria, such as E. coli, would be consistent with observations of decreased measures of sanitation, such as lack of running water in households involved in our cohort. Studies have shown environmental enteropathy to be associated with poor sanitation [192], and have detected increased levels of LPS in the blood of inhabitants living in households with poor measures of sanitation, such as lacking running water or toilets [193,194]. Our study was designed to develop such hypotheses by determining if and when differences occur in immune development of South African infants. As a first step, providing a detailed description of immune ontogeny in various global populations is essential to better understand clinical manifestation of disease, and to logically investigate preventative measures for infant morbidity and mortality.

6.5 Altered Immune Development in HIV-Exposed Uninfected Infants: a Critical Summary

HEU infants are a large group, increasing in size, who suffer from significantly increased infectious morbidity and mortality relative to their UE counterparts. We therefore set out to compare and contrast HIV-exposed infant immune development to HIV unexposed infant immune development. We recruited HEU and UE infants born to mothers who resided in the same hospital catchment area, and were presumed to be from similar communities, with similar environmental exposures, and similar distribution of genetic background. We accomplished this case-control comparison only in part. Some important environmental and genetic factors were significantly different between HEU and UE groups. Our study design involved recruiting all willing, competent, and informed participants who met selection criteria and presented to the maternity ward at TAH between March and June 2009. This recruitment strategy resulted in representative populations of HEU and UE infants for the TAH catchment area surrounding Cape Town, South Africa. Our study was designed to determine if and when immune development differed between these groups, and this data would inform future studies, which could subsequently address why differences exist, or how altered innate immune development impacts clinical outcome. Overall, we found HEU infant antigen presenting cells to be more responsive to stimulation with PAMPs than their UE counterparts. Both the proportion of responder cells and the amount of cytokine produced per cell were

greater in the HEU group. Furthermore, we demonstrated that the majority of differences occurred at the earliest time points, and in a pathogen specific manner.

Transient differences detected in HEU infant innate immune development are summarized in Figure 25, and these changes occur during and preceding the transiently increased rates of morbidity and mortality previously reported by groups in Zimbabwe, Uganda and Melawi [19,114,115,190]. The number of severe infectious events and the timing of altered innate immune function with respect to severe infections in our cohort are summarized in Table 13 and Figure 25, respectively. Determining if these innate immune aberrancies contribute directly or indirectly to increased susceptibility to infectious disease is an area of ongoing research interest.

One of the greatest interventions for prevention of early life infectious morbidity and mortality is vaccination. Innate immunity guides adaptive immune responses to vaccines, therefore the quality of vaccine induced immunity depends on appropriate innate immune responses to antigenic stimulation. For example, strong inflammatory immune responses may be effective for protection from initial pathogenic challenge, but overactive inflammatory pathways are shown to hinder effective immune memory responses [195,196]. To establish whether aberrancies in HEU innate immune development may have impacted vaccine responses, we compared and contrasted HEU and UE responses to vaccines over the first year of life. Vaccine responses were comparable in magnitude, except for higher titers

detected in the HEU response to the pertussis vaccine. Decreased baseline titers (pre-vaccination) in HEU infants indicated differences in passively acquired maternal antibodies, and may correlate with decreased immunity in HIV infected mothers, placental dysfunction resulting in decreased antibody transfer, or even decreased half-life of transferred maternal antibodies [55,137,139,140]. Our study was not designed to decipher relationships between infant and mother adaptive immune responses, antibody quality or placental function. In HEU infants HepB antibody titers were higher pre-vaccination and anti-pertussis titers reached protective levels earlier than in UE infants, and levels in HEU were higher overall post-vaccination. Anti-pertussis titers in HEU infants reached protective levels much earlier (12 months earlier), requiring one less vaccine dose than UE infants to produce protective titers. This may have important clinical consequences since, by far, most morbidity and mortality from pertussis occurs during the first year of life. However, we did not assess the functional capacity of the antibodies produced. This would be an important next step to understand responses to vaccination in HEU infants.

There is evidence to suggest that the strength of adaptive memory responses may be inversely proportional to the magnitude of initial effector responses [195]. The quality of a memory response can be diminished when priming takes place in an immune milieu that is hyperinflammatory [195]. Antibody avidity and affinity analysis in HEU vs. UE would therefore help us understand the clinical significance of our findings of altered antibody titer in HEU infants. Functional immune memory could also be examined with *ex vivo* challenge of memory B-cells with pertussis after

primary responses have subsided. Although we observed differences in frequency of severe infectious morbidity our cohort, we did not observe differences in frequency of vaccine preventable diseases (based on clinical presentation). In order to assess protection from vaccine preventable illnesses, a study would have to obtain data on microbiological diagnosis of disease, and then compare those results with antibody responses to the cognate vaccine. Our study did not obtain microbiological diagnosis for disease, nor was our cohort powered to compare incidence of relatively rare vaccine preventable illnesses. Commonly used vaccines included in the EPI were initially developed in a 'one size fits all' fashion, i.e. not taking into account the heterogeneity of immune responses between the World's populations. Our data [43], and others' strongly suggests that population-specific vaccine response analysis is warranted, and that vaccines may be more optimally designed if they are specifically tailored for the unique environmental and immune qualities characteristic of populations at the greatest need [197]. Indeed, the NIH has recently modified their guidelines for vaccination of HIV-infected infants to include customized immunization recommendations for HIV-exposed infants as well [198].

Several potential mechanisms have been proposed to explain increased morbidity and mortality in HEU infants. Proposed mechanisms include, but are not limited to, sub-optimal breastfeeding practices for HEU infants, lower quality breast milk with respect to immune factors from HIV infected mothers, higher number of infectious disease exposures, atypical infectious disease exposures in HIV affected households, poorer sanitation in HIV affected households, and even differing genetic

backgrounds between populations. A more in-depth discussion of potential factors leading to the increased susceptibility to infectious disease that is observed in HEU infants is provided in the Chapter 4 discussion (Section 4.4). ART is associated with altered cytokine levels in the newborn. HIV-infected women who received ART-treatment have shown greatly increased placental production of inflammatory cytokines. TNF-α and IL-8 is detected in much higher levels in placentas from HIV infected, ART-treated women, as opposed to placentas from HIV uninfected, ART-Naïve women [112,199]. High maternal viral load is associated with decreased CD4 counts in newborns, suggesting that immune cell populations shift in response to intrauterine exposure to HIV or maternal response to HIV [200]. This pro-inflammatory, immune activated intra-uterine state may provide insight into the etiology of altered immune responses in HEU vs. UE infants. Future studies correlating maternal viral load and/or ART exposure would thus be invaluable.

Recently there has been a focus on vaccine induced non-specific immunity. In areas such as Sub Saharan Africa, with exceptionally high incidence of infectious morbidity and mortality, determining differences in non-specific immunity of HEU infants may improve our understanding of altered infectious disease risk. In South Africa, and in many other developing nations, newborns are vaccinated with BCG as a TB preventative strategy. Although BCG demonstrates limited efficacy for protecting from respiratory TB, it is still used for protection from disseminated TB. Small studies in Guinea-Bissau and Brazil have shown lower rates of pneumonia in BCG immunized children [201,202], and a large study utilizing demographic and health

surveys in 37 countries showed that BCG vaccination was associated with 17 – 37% risk reduction for acquiring LRTIs [203]. These reductions are far in excess of clinical pulmonary TB infection incidence in infants. Therefore BCG vaccination likely confers protection from disease in a non-specific manner. BCG vaccination may also enhance the immune system's capacity to contain infections as shown in a study among 405 malnourished children with radiologically-confirmed pneumonia. Infants with pneumonia who were not previously vaccinated with BCG demonstrated a 3-times greater risk of bacteremia or mortality secondary to their LRTI [204]. The non-specific immunity induced by BCG is thought to be due in part to induction of adaptive type responses from innate immune cells, which is evidenced by the rapidity of the non-specific immune responses [205]. The phenomenon of innate immune cells demonstrating adaptive characteristics in the response to subsequent infectious exposures has been termed "trained immunity" [206]. A study examining trained immunity demonstrated epigenetic programming of monocytes by H3K4 trimethylation, and these effects were by and large transient, with diminished effects at 12 months post BCG exposure [207]. If the innate immune response can be 'trained' by previous exposures, such as BCG, to increase defenses against pathogens that cause LRTIs, then equally likely is that other exposures may decondition innate immune responses in a non-specific manner. Altered *intra uterine* or early-life exposures could therefore 'untrain immunity', leaving infants more vulnerable to infections. Infants in the HEU group demonstrated a transient increase in risk by greater than 3-fold to severe infections. Determining if the transient diminished capacity to contain infection is mediated at least in part by non-specific exposure,

e.g. HIV, ART, or environmental exposures, is an important next step towards understanding morbidity and mortality in HEU infants.

6.6 General Impact and Future Directions

Preliminary analysis for the work comprising this thesis has already lead to work extending our findings to a cross-sectional comparison at 2 years, of innate immune development between infants across 4 continents. Our early life data suggested changes between South African innate immune development and Canadian innate immune development, and our subsequent multicenter study provided a direct comparison, which supported these findings [43]. This study demonstrated significant differences in South African infant innate immune development, between our study's infants, and the other 3 centers (in North America, South America, and Europe), such as significantly reduced responses to extracellular and endosomal PRR stimulation.

In recent years there has been a greater awareness that increased vulnerability to infectious morbidity and mortality in HEU infants is a significant public health problem [208,209]. With increased awareness there has been a growing focus on intrinsic differences or altered environmental exposures in HEU versus UE infants. The work comprising this thesis establishes that altered innate immune development exists in HEU infants, and the changes were detected primarily between 2 weeks and 6 months of life, and largely in response to bacterial PAMP

stimulation. HEU infants experience altered exposures early in life, such as HIV, ART, breast milk, and exposure to PAMPs such as LPS from increased exposure to pathogens; any of these altered exposures may influence the innate immune system's ability to contain infections within the first year of life. Given the primary limitation of our study, which was lack of sample size, we were not powered to address these questions. Rather, our data now provides information that will be used to formulate focused investigations of etiological mechanisms underlying HEU vulnerability to infectious morbidity and mortality.

We demonstrated transient changes in innate immunity between HEU and UE that occurred early in the first year of life, and preceded the onset of increased susceptibility to severe infections (innate immune changes and severe infection incidence is summarized in Figure 25). When we compared South African UE innate immune ontogeny data to similar studies around the globe, we found altered immune development in South African infants that persisted through at least the first 2 years of life. Examples of innate immune exposures that induce transient or long-lasting changes in innate immune function include BCG and early-life allergens, respectively. BCG, can induce transient non-specific changes in the immune system through 'trained immunity', and in some instances these changes were shown to be largely resolved by 12 months of age [210]. Early life exposures can also result in long lasting changes as demonstrated by research supporting the 'developmental origins of health and disease' (DOHaD) hypothesis. DOHaD suggests that environmental (including *intra uterine*) exposures can induce epigenetic changes that set the

course for certain health outcomes much earlier in life. For example, multiple studies have demonstrated that poor *in utero* nutritional status in the first trimester is associated with increased risk of coronary artery disease later in life [211], and in animal models, folic acid supplementation *in utero* influenced epigenetic changes leading to allergic airway disease [212]. Figure 26 summarizes the multitude of exposures that work together to shape early and long-lasting changes in immune function. These alterations in immune function may be mediated by epigenetic changes via DNA methylation, histone modification, nucleosome position and non-coding RNA expression [207]. Future research will examine proposed etiological environmental and genetic factors for their effect on innate immune development between populations. A particular focus will be made to identify mechanisms underlying these changes in high-risk populations, such as HEU infants. Valuable and broadly applicable data would be provided by elucidating what qualities of early life exposures are associated with transient, versus long lasting changes to innate immune development. Ultimately, we aim to better understand the qualities of early-life immune exposures that alter immune mediated protection, how changes in immune development impact susceptibility to disease, and which of those factors are modifiable.

6.7 Chapter 6 Tables and Figures

Table 13: Infants with Infectious Events Between 0 and 18 Months of Age

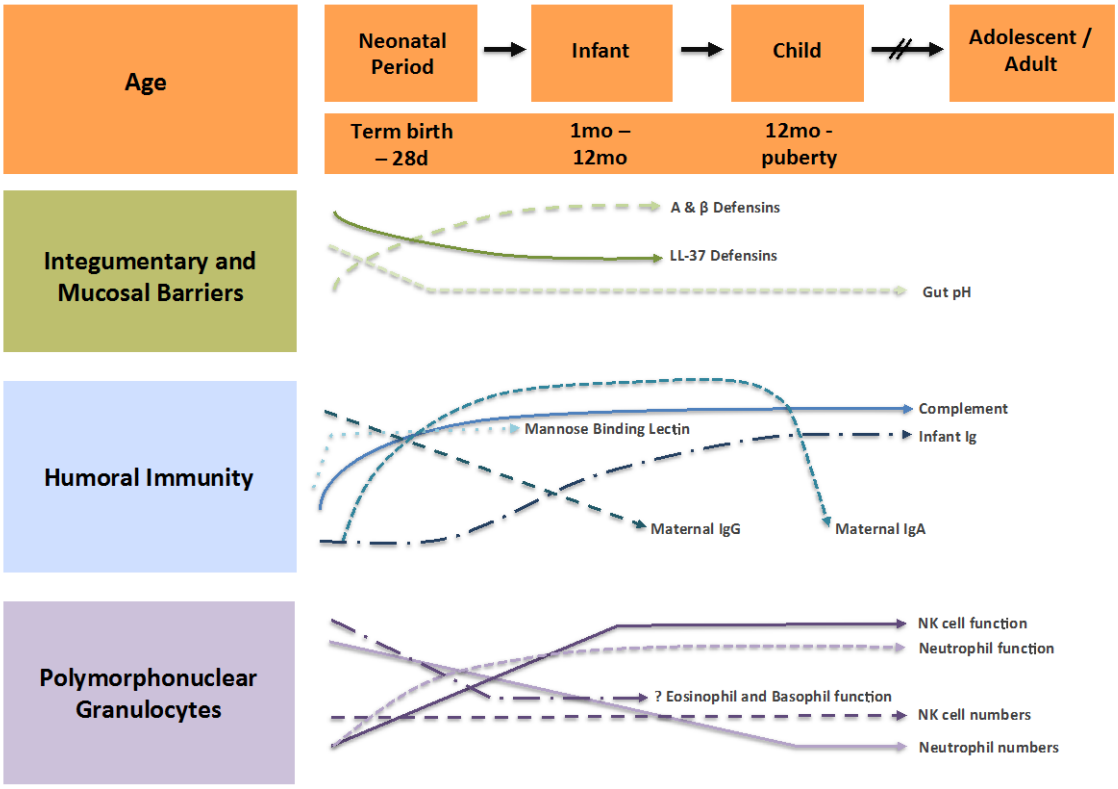
	Infants with any infectious event (0 – 18 months)			**Infants with severe* infectious events (0 – 18 months)**		
	Number of infants	Percent	Number of infections	Number of infants	Percent	Number of infections
Total (n=55)	54	98%	178	19	35%	28
HEU (n=27)	27	100%	85	13	48%#	19#
UE (n=28)	27	96%	95	6	21%#	9#

#Significant differences between HEU and UE detected with Student's t test
*Severe events were determined using the criteria outlined in the Division of AIDS Table for Grading Severity of Adult and Pediatric Adverse Events [173]

Table 14: Number and Type of Severe Infectious Events in HEU and UE Infants Between 0 and 18 Months

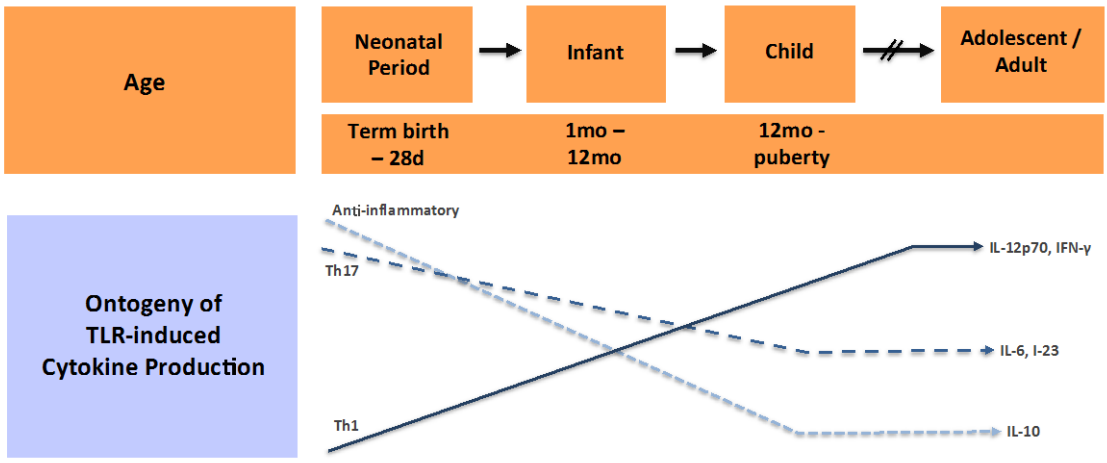
	Total (25)	HEU (19)	UE (6)
LRTI	14	11	3
Gastroenteritis	3	3	0
Systemic Infection	2	0	2
Urinary Tract Infection	3	2	1
Viral Exanthem	3	3	0

Severe events were determined using the criteria outlined in the Division of AIDS Table for Grading Severity of Adult and Pediatric Adverse Events [173]



Adapted from Goenka and Kollmann, 2015 [186]

Figure 22: Early-Life Changes in First Line Innate Immune Defenses



Adapted from Goenka and Kollmann, 2015 [186]

Figure 23: Ontogeny of TLR-Induced Cytokine Production

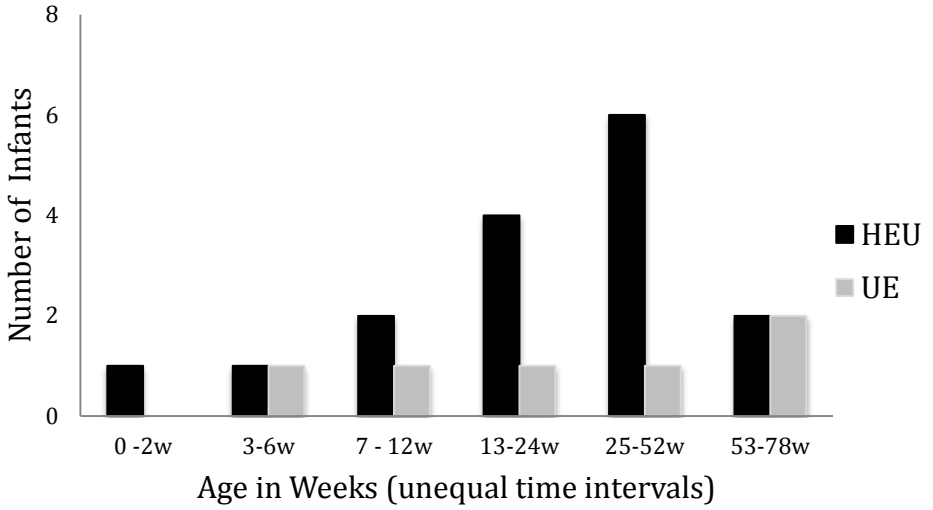


Figure 24: Age at Occurance of Severe Infectious Events in HEU and UE infants

Infectious events were recorded in the perinatal period, and then at patient visits 2, 6, 12, 24, 52 and 78 weeks of age. The number of severe infectious events, as described using the Division of AIDS Table for Grading Severity of Adult and Pediatric Adverse Events [173], that took place between the interval of patient visits (x-axis), for HEU (black) and UE (grey) infants are recorded on the y-axis. Number of subjects at 2 weeks (UE = 28, HEU = 27), 6 weeks (UE = 26, HEU = 25), 12 weeks (UE = 23, HEU = 25), 24 weeks (UE = 23, HEU = 25), 52 weeks (UE = 20, HEU = 23), 78 weeks (UE = 20, HEU = 20).

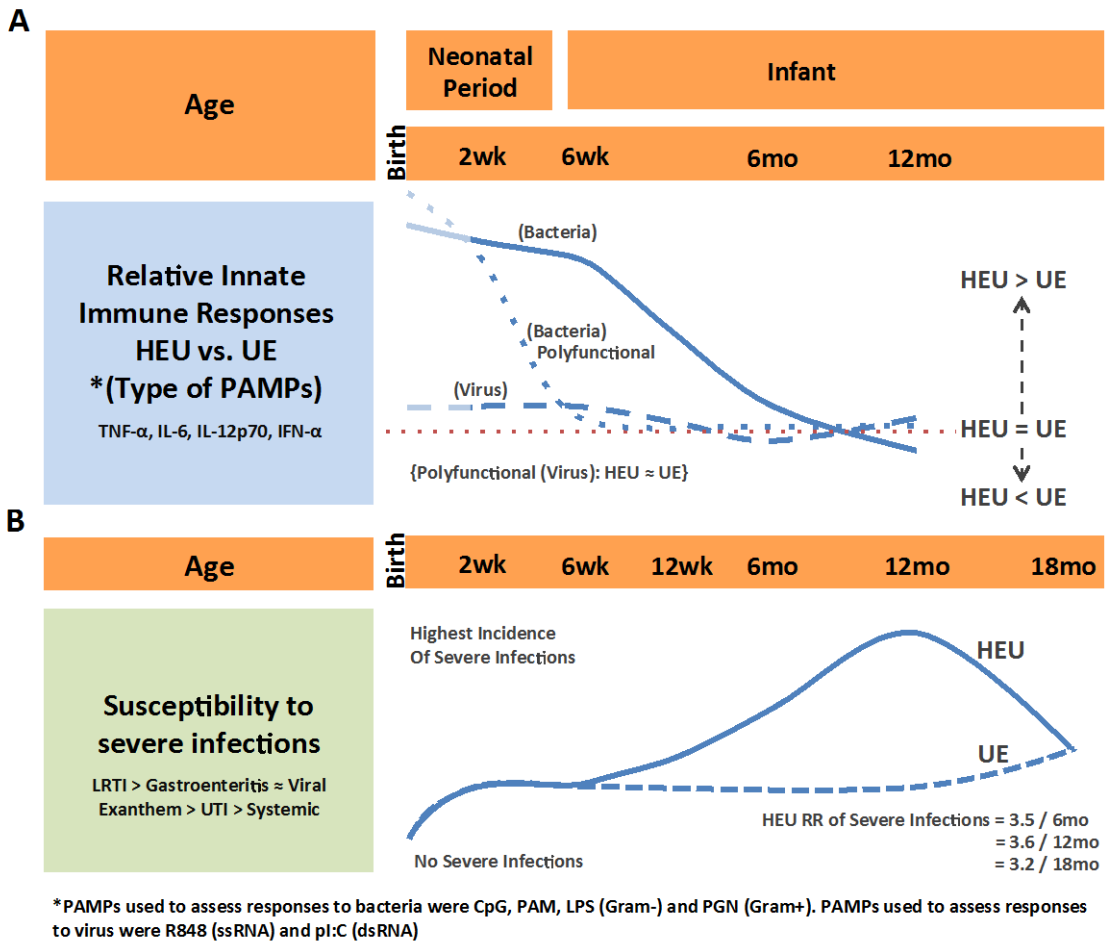
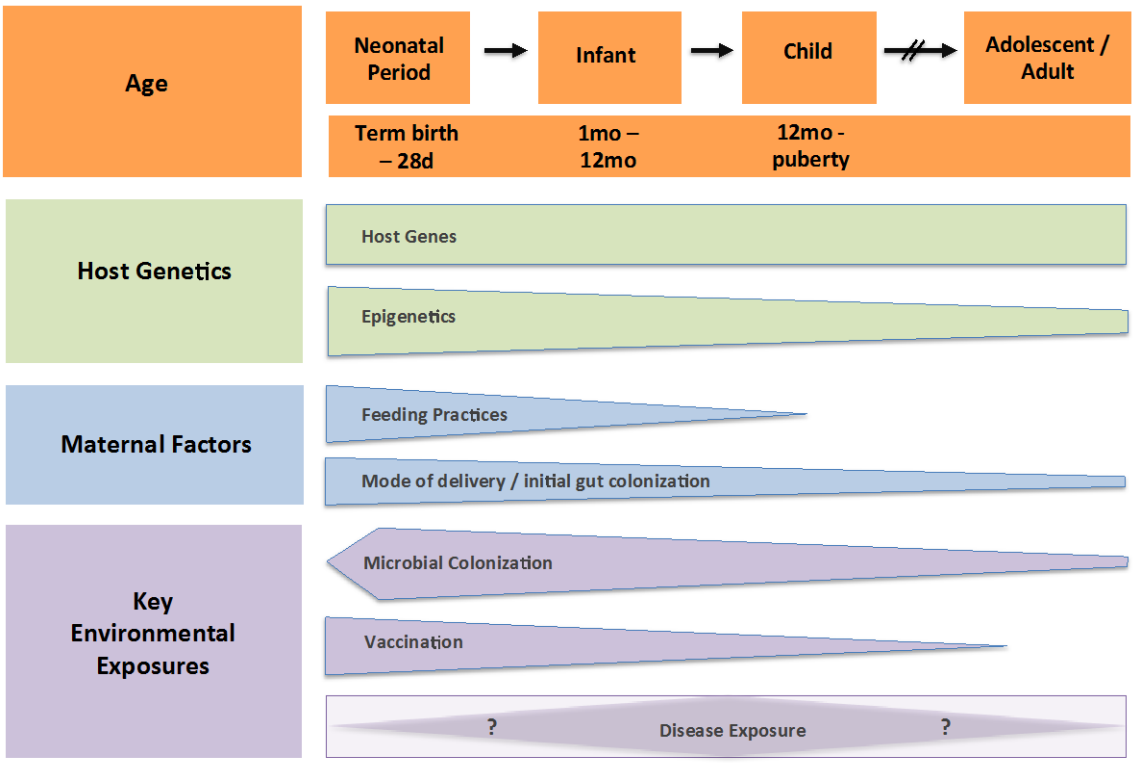


Figure 25: Summary of Innate Immune Cellular Responses in HEU vs. UE Infants Over the First Year of Life

A) Illustrated summary responses of monocytes and cDC (TNF-α, IL-6 and IL-12p70 measured) and pDC (TNF-α, IL-12p70 and IFN-α measured) in HEU relative to UE infants over the first year of life. APC responses are separated based on viral or bacterial stimuli, and based on APC polyfunctionality. Faded lines indicate extrapolated values as responses were not measured prior to 2 weeks of life. B) Summary of severe infection incidence in HEU and UE infants over the first 18 months of life. RR, Relative Risk compared to UE.



Adapted from Goenka and Kollmann, 2015 [186]

Figure 26: Environmental, Maternal and Host Factors Impact Innate Immune Ontogeny

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APPENDIX A: POLYCHROMATIC FLOW CYTOMETRIC HIGH-THROUGHPUT ASSAY TO ANALYZE THE INNATE INNUME RESPONSE TO TOLL-LIKE RECEPTOR STIMULATION

The innate immune system represents a highly sophisticated system for recognition of specific pathogen associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs), of which Toll-like receptors (TLRs) are the best studied example. In the course of a normal host response to infection, antigen presenting cells (APCs) take up the invading microbe, process and present parts of it to the adaptive immune system (T and B cells) in the context of costimulatory surface molecules and cytokines, which in turn leads to a pathogen-specific adaptive immune response efficient at eliminating the offending invader. The magnitude and quality of APC responses is largely directed by PAMPs binding to PRRs on these cells. Stimulation with purified TLR ligands has been shown to change antigen-processing and to induce the maturation, migration and the production of cytokines and costimulatory markers on professional APCs. The APCs integrate information from multiple PRRs, resulting in a finely-tuned signal that instructs the subsequent adaptive immune response [213,214].

While this highly complex system appears to work well in most individuals most of the time, changes in any of the essential components of the PRR signaling pathways increase the risk for several disease processes. For example, alteration of essential signaling PRR components leads to a state of heightened susceptibility to infection in both mice and humans. Even subtle single nucleotide changes in the sequence of

some of the molecules involved in this orchestrated response to PAMPs have potentially lethal consequences if the individual is exposed to the right microbe at the wrong time [167]. The response of APCs to TLR stimulation also appears to be developmentally regulated, with important clinical implications for pre-term labor and heightened risk for infection early in life [87]. Several TLRs have been identified as potentially major contributors to autoimmune disease [215], chronic inflammation and cancer [216]. TLR activity has also been proposed to change the function of T regulatory cells [217] and with that allograft rejection [218]. All of the above emphasizes the centrality of the innate immune system to our health and well-being. Yet, our understanding of the cause-effect relationships is very rudimentary. A reliable yet versatile assay of innate immune function would allow scientists to unravel the mechanisms at work, providing the foundation for rational therapeutic interventions.

Despite this lack of knowledge, the realization that innate immune responses can be altered through TLR stimulation has propelled TLR ligands onto the stage of clinical trials as immune modulators [219]. For example, TLR agonists are under consideration as therapies for asthma and allergy [220]. Most advanced is the concept of coupling TLR ligands as adjuvants to vaccine antigens [221]. A high-throughput assay allowing the rapid screening of hundreds of potential innate immune modulators and adjuvants would speed up the discovery phase of these promising applications [222].

Previously assays for innate immune analysis were limited in scope and speed, analyzing either only innate immune cell numbers [223], or cytokines secreted into culture supernatants [224]. While this global assessment of innate immune function has its advantages, it does not provide the necessary precise single cell-specific information to accurately assess an innate response to TLR ligation. One previously described flow cytometric assay allowed assessment of specific innate cell types using four-color flow cytometry, with only two colors as a response read-out [225]. The complexity of the innate response to TLR stimulation far exceeds the capacity of this assay. We reasoned that recent developments of polychromatic (>5 colors) flow cytometry [226] should allow the inherent complexity of the innate immune response to be captured more fully. We strived to design an automated flow cytometry-based assay to provide a widely usable innate immune analysis platform. Bulk cytokine information as done previously can easily be gathered in parallel with the more detailed cell specific information from this flow cytometry platform to create a detailed picture of innate immune function. Here we outline the optimization and validation of these multiplex assays to analyze complex innate immune responses to TLR stimulation.

Method for Set Up of TLR Stimulation Plates

Deep-96-well (VWR) source plates containing 1.3 mL of various TLR ligands at 10x the desired concentration were prepared using sterile procedures under a laminar air-flow hood. Ten TLR ligands were used, each diluted in RPMI-1640 medium

(Invitrogen) containing Glutamax (RPMI) over a 5 $\log_{10}$ concentration range for a total of 50 different stimuli (Appendix A Table 1). Two unstimulated wells were included as a negative control, using the same medium. Brefeldin A (BFA, Sigma) was added at a concentration of 100 μg/mL (10x the desired final concentration of 10 μg/mL) to all wells except those wells containing TLR3 and TLR9 ligands (see below). Compounds containing the TLR ligands PAM_3CSK_4, R-FSL-1 or LPS were sonicated at least 10 min and vortexed thoroughly before each initial dilution and between serial dilutions, while the other ligands were not sonicated but vortexed initially and between serial dilutions. Source plates were sealed with sterile plate sealers (USA Scientific), frozen at -80°C and thawed prior to use. 20μl from each well of the source plate was dispensed into each well of recipient 96-well round bottom polystyrene plates (Corning) using the Evolution™ P3 Precision Pipetting Platform (Perkin Elmer), under a laminar air-flow hood using sterile procedures. Recipient plates were sealed with sterile aluminum sealers (USA Scientific) and frozen at -80°C until use.

Method for Blood Sample Processing and in vitro Stimulation

All studies were approved by the Institutional Ethics Review Board at both the University of Washington and the University of British Columbia. We obtained blood from healthy adult individuals via sterile venipuncture into vacutainers containing Na-Heparin (Becton Dickinson). Blood and all reagents were kept at room temperature. PBMC were isolated by density gradient centrifugation; whole blood

(WB) was mixed 1:1 with sterile RPMI. Peripheral blood mononuclear cells (PBMC) were cultured in RPMI supplemented with 100 units of penicillin/mL, 100μg streptomycin/mL (Invitrogen) and 10% human AB serum (Gemini Bio-Products). 200 μl/well of cell suspension (either PBMC or WB mixed 1:1 with RPMI) was added to pre-made 96 well plates containing the specific TLR ligands at the desired concentrations. Cells were incubated for 6 hours at 37°C in 5% CO2. For TLR3 andTLR9 ligands BFA was added for the final 3 hours at a final concentration of 10μg/mL. An identical set of plates was incubated in parallel for 18hrs without BFA; at 18hrs, these plates were spun and 100μl of supernatant was removed and frozen at -80°C for later Luminex analysis. Then EDTA was added to a final of 2mM, and plates were incubated for 10 min at 37°C to detach adherent cells. Plates containing PBMC were spun and resuspended in 100 μl FACSLyse (Becton Dickinson); for plates containing WB, the entire mixture was added to 1400 μl FACSLyse in deep 96-well plates (VWR). Both plates were then frozen at -80˚C until staining.

Method for Cell Staining, Acquisition and Analysis

Antibodies (clones and source) for either intracellular cytokine cytometry at 6hr or activation-induced cell surface molecule expression at 18 hr are shown in Appendix A Table 2A and 2B, respectively. The concentrations of all antibodies were titrated prior to use, and the optimal dilutions for each are shown in Appendix A Table 2. Frozen plates were thawed, either in a 37˚C incubator for 15 minutes for PBMC (100μl per well in microwell plates) or for 1.5 hours in a 37˚C water bath for WB

(1.6ml per well in deep well plates) and spun, then pellets were resuspended in 200 μl FACS Permeabilizing Solution (BD) and incubated at room temperature for 10 min; permeabilizing solution was not added to 18 hr plates. After one wash in PBS containing 0.5% bovine serum albumin and 0.1% sodium azide (PBSAN), cells were stained in a final volume 100 μl PBSAN for 30-60 minutes at room temperature. After two further washes with PBSAN, cells were resuspended in PBS containing 1% paraformaldehyde and immediately analyzed on an LSRII Flow Cytometer (Becton Dickinson) set up according to published guidelines [226]. Compensation beads (CompBeads, Becton Dickinson) were used to standardize voltage settings and were used as single stain controls as described [165,227] 3 μl of anti-mouse Ig CompBeads and 3 μl of anti-FBS negative control CompBeads were added to 94μl of PBSAN in the same plate as the cells, and stained with 3μl of antibody for each single stain control at the same time as cells were stained. For the rest of the protocol, CompBeads were treated in the same way as the cells. For PBMC at least 200,000 and for whole blood at least 1,000,000 events were acquired uncompensated through a high-throughput sampler (set to mix at medium and to acquire at no more than 2μl/sec). Extra volume of at least 50μl beyond what the 96-well sampler uses was added to the wells to prevent introduction of air into the system. Compensation was set in FlowJo (TreeStar, OR) or Labkey Flow (Labkey, WA), and samples were analyzed compensated. Cytometer optimization and calibration were performed according to published guidelines [226].

Statistical Analysis

Graphs were prepared using Excel software (Microsoft, WA). For statistical analysis, the Prism (GraphPad Software) software analysis program was used.

Optimization of Reagents and Equipment

Through initial pilot experiments we realized that all materials, including plastic hardware (96 well plates, pipettes etc.), blood-draw equipment (e.g. Vacutainer tubes, heparin, etc.), that came into contact with the innate immune cells, had to be tested and shown to be free of innate immune stimulating activity in order to avoid potential artifacts resulting from unintended activation of the cells. This testing was performed not only via commercially available systems (e.g., Limulus amebocyte lysate test), which only screen for one specific contaminant, but more effectively through exposure of fresh PBMC from healthy adult subjects to the materials to be tested, followed by analysis for activation of innate immune cells. For example, we plated unstimulated PBMC with different batches of human AB serum to identify the source and batch that provides the highest signal-to-noise ratio (lowest background activation, yet the highest TNF-α production in mDC and macrophages if stimulated with 10ng/ml of LPS). Once optimal reagents were identified, we procured sufficient quantities to complete the ongoing study. Of note, there was no difference between fetal calf serum (FCS) and human AB serum as a culture additive when responses were assessed by flow cytometry (data not shown). TLR ligands were tested in reporter cell lines to confirm that each of those used in our assays were specific for

the appropriate TLR and lacked activity for other TLRs [228]. The source of each TLR ligand that satisfied this stringent assessment is shown in Appendix A Table 1. The optimal antibody and fluorochrome combination used for the polychromatic innate immune evaluation were chosen according to published guidelines [165]. Neither positive interference (antibodies added to our cocktail inhibiting each other's binding) nor negative interference (reagents contained in culture medium or its additives inhibiting the binding of antibodies present in our cocktail) was detected for the final clones chosen [229]. This required testing of over 70 clone/fluorochrome combinations to assemble the satisfactory panel of antibodies shown in Appendix A Table 2, with Table 2A listing the optimal panel for ICC, and Table 2B those optimal for staining of surface markers of activation in the innate target cells.

Optimization of Stimulation and Analysis

Gating Strategy

The gating strategy for PBMC and whole blood (WB) from a representative healthy adult subject that were stimulated with the TLR7/8 ligand 3M-003 is shown in Appendix A Figure 1 A and B respectively. Our assay allows the simultaneous analysis of monocytes (identified based on FSC/SSC, MHCII^{hi}, CD14^{hi}), myeloid DC (mDC; identified based on FSC/SSC, MHCII^{hi}, CD14-/low, CD123-, CD11c^{hi}), plasmacytoid DC (pDC; identified based on FSC/SSC, MHCII^{hi}, CD14-/low, CD11c-, CD123^{hi}), and B cells (identified based on FSC/SSC, MHCII^{hi}, CD14-, CD19^{hi}). This approach allows specific identification of the target cell populations (gate 'in'), and

stands in contrast to other widely used gating strategies that rely on stable expression of certain lineage-markers to exclude (i.e. gate 'out') the "undesired" target cells [225]. The Golgi-secretion inhibitor BFA was added to the culture to permit detection of intracellular cytokines by flow cytometry (ICC) on permeabilized cells [230,231] as shown in Appendix A Figure 1C. Cultures stimulated for 18 hrs in the absence of BFA can be used to concomitantly assess changes of cell surface (e.g. costimulatory and homing receptors) markers (Appendix A Figure 1D) and to detect cytokines secreted into the supernatant (not shown) as parallel functional read-outs. It is important to recognize, that certain surface molecules such as costimulatory molecule CD40 as well as the surface marker CD19 were sensitive to membrane permeabilization (Appendix A Figure 2), while all other surface antibodies listed in Appendix A Table 2B were not affected by permeabilization.

Choice of Golgi-Secretion Inhibitors

The addition of the Golgi-inhibitors BFA or Monensin concomitantly with TLR stimulation yielded optimal results for all TLR ligands except for CpG and pI:C. For these two ligands, a measurable response was obtained only if BFA (or Monensin) addition was delayed, as illustrated by the pDC response to CpG (Appendix A Figure 3); for these ligands, addition of BFA at 3-4 hrs after addition of the CpG or pI:C appeared to be optimal for detection of intracellular cytokines. We directly compared Monensin and BFA and found no substantial difference between them except for TNF-α and to a lesser extent IL-6 (data not shown), for which BFA performed better than Monensin after CpG stimulation (Appendix A Figure 4).

Dose-Response Titration & Kinetics

Performing similar analysis for each of the TLR ligands listed in Appendix A Table 1, across a wide range of concentrations, we found that different APC subsets have different concentration optimums for different cytokines. Furthermore, difference in dose-response curves also exist between PBMC and WB responses, as illustrated for the TLR7/8 ligand 3M-003 and the TLR4 ligand LPS (Appendix A Figure 5). Importantly, very high concentrations of TLR7/8 (>10 μM) or TLR4 (>100 ng/ml) ligands altered the expression of the lineage markers CD14 and CD11c (Appendix A Figure 6). We were able to solve this problem by reducing the concentrations of these ligands and by sequentially gating, first on MHCII+ and $CD14^{high}$ for monocytes, then on MHCII+ and $CD11c^{high}$ /CD123 negative for mDCs (see Appendix A Figure 1), rather than trying to use CD14 vs. CD11c to differentiate between monocytes and mDCs as shown in Appendix A Figure 6. For detection of intracellular cytokines by flow cytometry, a total incubation period of ~5-8 hours appeared optimal (Appendix A Figure 7), with incubation periods of less than 5 hours resulting in substantial reduction of the response, and incubation periods of over 8 hours producing a steady increase in dead cells (data not shown).

Precision

Precision refers to the ability of the assay to generate reproducible results as operational parameters change [232]. We looked at replicate variability between same plate vs. separate plate experimental set-ups. Well-to-well variability was assessed

by comparing triplicate wells stimulated within the same plate, while plate-to-plate variability was assessed for the same individuals by comparing triplicates across 3 separate plates. As expected, we found the standard deviation to be somewhat higher across separate plates (Appendix A Figure 8), but the mean of the 'same-plate' triplicates still fell within the standard deviation of the triplicates measured across three different plates.

Summary for Development of a Polychromatic Flow Cytometric High-Throughput Assay to Analyze the Innate Immune Response to Toll-Like Receptor Stimulation

The innate immune system plays an important role in health and disease, and presents potentially important therapeutic and diagnostic targets. We describe here a high-throughput polychromatic flow cytometry based platform that produces reliable and reproducible data that can easily be used in parallel with a multiplexed bulk cytokine assay. Given that this platform is in strict compliance with published guidelines for polychromatic flow cytometry, we anticipate this protocol will provide a common platform for innate immune evaluation.

Previous innate immune assays lack the speed and scope provided by our assay. For example, Deering and Orange describe a clinical assay to evaluate TLR function, but limit their analysis to cytokines present in culture supernatants of PBMC stimulated with TLR ligands [224]. Ida et al. describe a flow cytometric method to assess DC

function in response to TLR stimulation, but only employ 4 colors, 2 of which are used to identify the DC subsets, leaving only 2 for a functional read out [225]. Neither of these assays provides the detail necessary to adequately evaluate the complex innate immune response, and neither has been developed into a rigorously standardized, high-throughput platform.

Based on our data, there are several interesting points to emphasize:

1. The sensitivity of CD40 to permeabilization procedures precluded the simultaneous detection of the CD40 surface marker alongside intracellular cytokines. Detection of intracellular cytokines in B-cells requires the use of other B cell surface markers than CD19 for subset identification, as CD19 detection is reduced in permeabilized cells.

2. The lack of response to TLR3 and TLR9 ligands if Golgi blockers were added at the start of culture (Appendix A Figure 3) might be related to transport requirements for these ligands or their receptors from the endoplasmic reticulum to and from the endosome [233-235]. This effect was observed with both BFA and Monensin. The optimal delay in the addition of BFA to cells stimulated with TLR3 or TLR9 ligands was 3-4 hours, as this delay allowed assessment of the maximal direct TLR stimulation response (TNF-α and INF-α double positive cells) [236].

3. While optimal time and dose response kinetics varied between TLR ligand and cytokine read-out (Appendix A Figure 5 and 7), we chose the 6 hour time point for total length of stimulation for ICC, as it captured most of the cytokines within their maximum response. We chose the range of concentrations listed in Appendix A Table 1 based on providing a range of low to high response for most cytokines in most APC subsets in WB as well as PBMC (Appendix A Figure 5). As indicated in Appendix A Figure 5, we did note potential differences between WB and PBMC in some TLR response patterns. This has been described previously [87]. We are currently in the process of identifying the precise mechanisms underlying this phenomenon.

4. The down-regulation of CD14 and CD11c after stimulation with a high concentration of TLR7/8 and TLR4 ligand (Appendix A Figure 6) was surprising, but similar observations in response to Histamine [237] and LPS [238] have been made before. Whereas the mechanisms responsible for this phenomenon are not clear, it is important to note that flow cytometric strategies relying on 'dump-gates' (e.g. excluding T-, B-, NK-cells and CD14+ monocytes all in one channel), as described in the one previously published flow cytometric assessment of innate immune function [225], would fail to detect the loss of these TLR ligand-responsive cells, producing an artifact that would likely underestimate the response to this ligand in the affected cell populations. Our assay allows one to capture these cells, since we specifically include ('gate-in') our target population through the use of the polychromatic antibody cocktails described above. To avoid this problem, one can also chose

concentrations of TLR7/8 and TLR4 ligands at which this phenomenon is minimal such that the relevant cell populations can still be discriminated from each other.

5. Appendix A Figure 8 suggests that our assay delivers precise measurements of an individual's response to TLR stimulation. Two different aspects are presented in Appendix A Figure 8 simultaneously: A. The standard deviation (SDEV) of blood samples from 3 individuals stimulated on the same plate vs. those stimulated on different plates were compared. The SDEV was clearly larger if samples were stimulated on different plates. While this is not surprising, it is important to keep in mind, when serial samples of an individual are compared over time (e.g. the length of a given study) since by definition they would be stimulated on different plates. B. To test whether an individual's innate immune response changes over time, one needs to control for potential artifacts due to the use of different stimulation plates. Our data indicate that a given subject's response appears relatively stable over at least 3 months, as indicated by the fact that the mean of the first time point, which was derived from three replicates stimulated on the same plate, still falls within the SDEV of three replicates of PBMC obtained 3 months later, even though these 3 replicates were each stimulated in a different plate. This stability has important practical ramifications, as population-wide searches for changes in TLR response might otherwise be handicapped by variation over time. To adequately evaluate if potentially significant differences in a subject's response over time occur, we are currently in the process of investigating longer time periods with many more subjects.

While cryopreservation of innate cells is known to alter their response profile [224], and we thus had to use fresh samples for stimulation, it is important to point out that our assay is compatible with cryopreservation and shipment on dry ice after stimulation and fixation in FACS lysing solution of cells, and thus can easily be employed in multi-center studies with only one central analysis facility. Furthermore, the assay described here has been optimized for whole blood (WB) and peripheral blood mononuclear cells (PBMC), allowing one to compare responses in the presence or absence of soluble factors contained in an individual's plasma and thus to assess the potential impact of such factors on the innate immune response [87]. This platform is easily coupled to other multiplex assays, such as cytokine bead arrays, without increasing the necessary sample volume. Our polychromatic flow cytometric assay platform described here is clearly amenable to study not only TLR ligands, but of any innate immune modulator, and thus should be of general applicability to studies of human innate immune responses.

Appendix A Tables and Figures

Appendix A Table 1: TLR Ligand Panel Used to Stimulate Innate Immune Cells

TLR	Ligand	Source	Catalog #	Concentration Range
TLR 2/1	PAM3CSK4	EMC microcollections	L2000	0.01 - 10 ug/mL
TLR 2/6	R-FSL-1	EMC microcollections	L7022	0.01 - 10 ug/mL
TLR 3	poly I:C	Amersham	27-4729-01	6.25 - 100 ug/mL
TLR 4	0111:B4 LPS	InVivogen	tlrl-pelps	0.1 - 1000 ng/mL
TLR 7	3M-013	3M	MTA	0.01 - 100 uM
TLR 7/8	3M-003	3M	MTA	0.01 - 100 uM
TLR 8	3M-002	3M	MTA	0.01 - 100 uM
TLR 9	CpG A ODN 2336	Coley	N/A contact Coley	6.25 - 100 ug/mL
TLR9	CpG B ODN 10103	Coley	N/A contact Coley	6.25 - 100 ug/mL
TLR 9	CpG C ODN 2395	Coley	N/A contact Coley	6.25 - 100 ug/mL

Appendix A Table 2: Staining Panel Used for Polychromatic Flow Cytometry

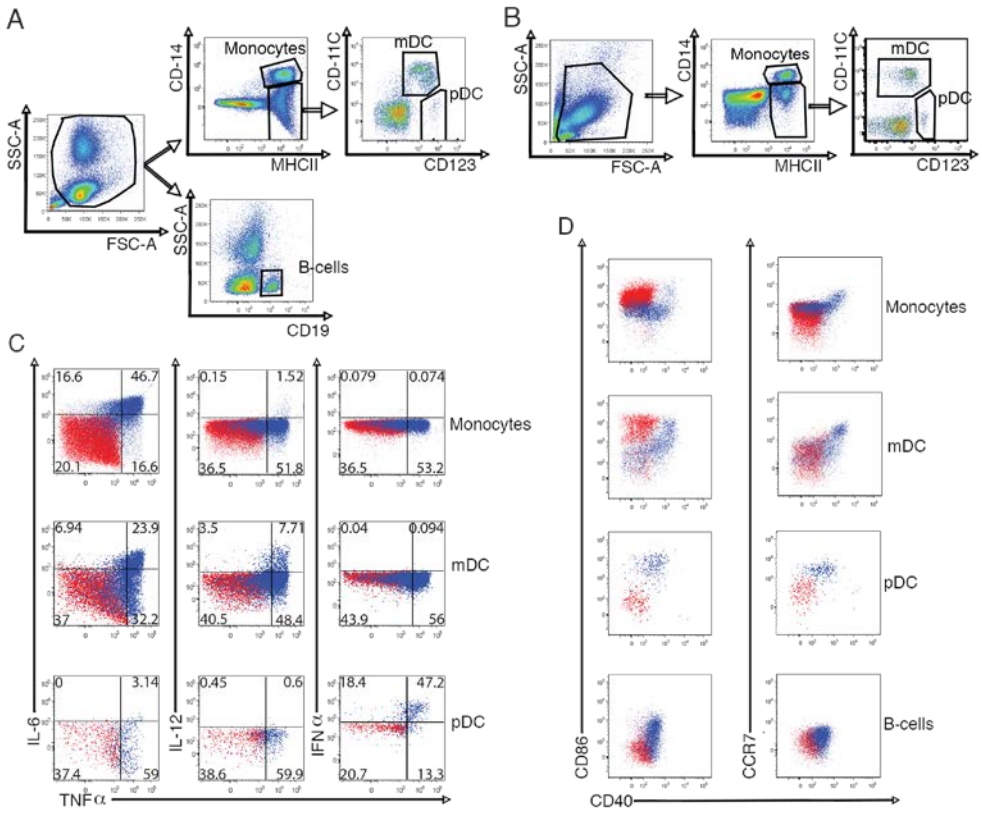
A) Panel used for intracellular cytokine staining
B) Panel used for cell surface staining

A

	Characteristic Being Measured	Antibody Name Clone Name	Vendor cat# dilution used
VIOLET			
AmCyan	Cell Surface Protein	CD123 AmCyan (9F5)	BD#custom 1:50
Pacific Blue	Intracellular Protein	IL12p40/70 (eBio: C8.6)	eBio#577129 1:100
RED			
APC	Cell Surface Protein	CD11c (5HCL3)	BD#340714 1:50
APC-Cy7	Intracellular Protein	IL6 (MQ2-13A5)	BD#custom 1:100
Alexa 700	Intracellular Protein	TNFa (Mab11)	BD#557996 1:100
BLUE			
FITC/OG	Intracellular Protein	IFNa (A11)	Antigenix#MC100133 1:100
PerCPCy5.5	Cell Surface Protein	MHCII TU36	BD#custom 1:100
PE-Cy7	Cell Surface Protein	CD14 (M5E2)	eBio #25-0149 1:50

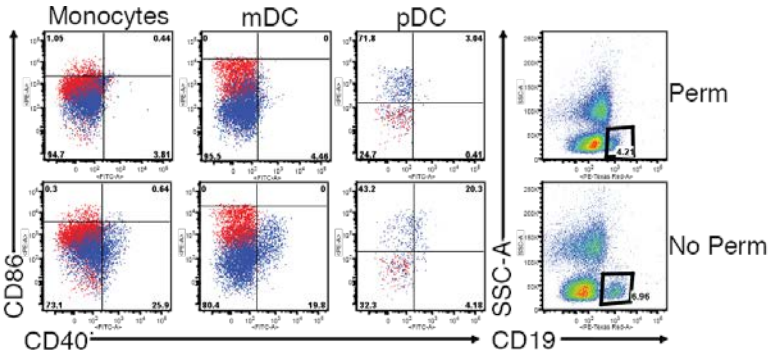
B

	Characteristic Being Measured	Antibody Name Clone Name	Vendor cat# dilution used
VIOLET			
Pacific Blue	Cell Surface Protein	CD123 (9F5)	BD#custom 1:100
RED			
APC	Cell Surface Protein	CD11c (5HCL3)	BD#340714 1:50
Alexa 700	Cell Surface Protein	CD14 (M5E2)	BD#557923 1:50
BLUE			
FITC/OG	Cell Surface Protein	CD40 (5C3)	eBio #11-0409 1:100
PE	Cell Surface Protein	CD86 (IT2.2)	eBio #12-0869 1:100
PerCPCy5.5	Cell Surface Protein	MHCII TU36*	BD#custom 1:100
PE-TexRed	Cell Surface Protein	CD19-biotin (HIB19) streptavidin PE-TR	BD#555411 BD#551487 1:50
PE-Cy7	Cell Surface Protein	CCR7 (3D12)	BD#557648 1:100



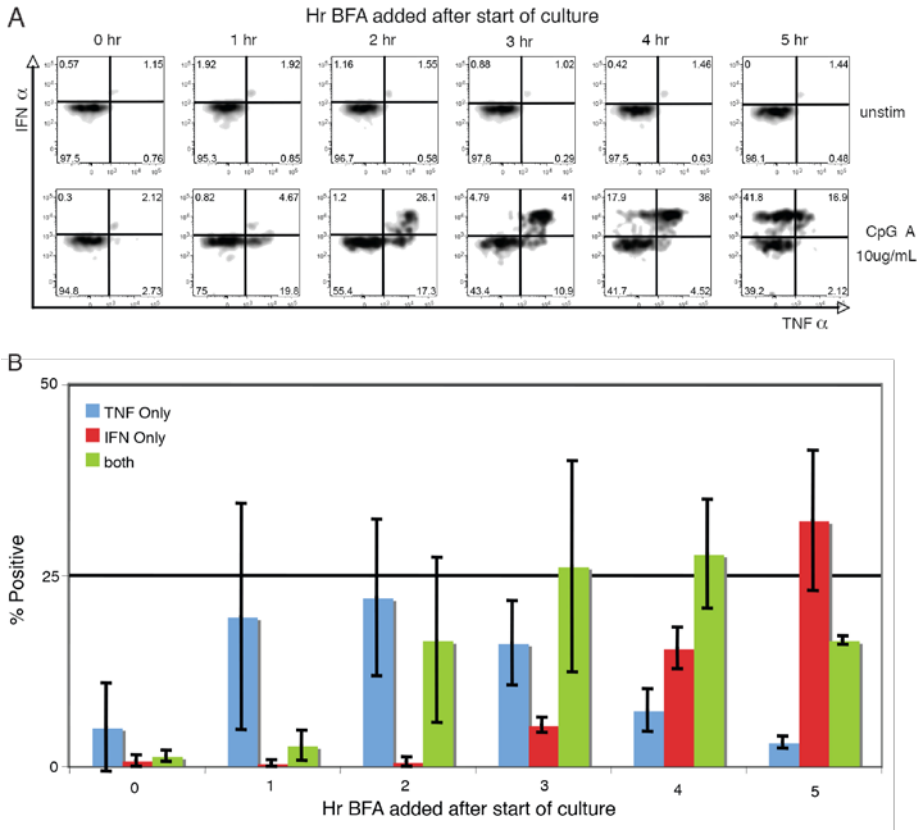
Appendix A Figure 1: Gating Strategy & Staining Profile for 8-color Flow Cytometry

Shown is a PBMC sample in A, C, and D, and a whole blood sample in B. Shown in A and B on an unstimulated sample is the gating strategy to identify various innate immune cell subsets: monocytes (MHC-II+, CD14+), B cells (MHC-II+, CD19+, myeloid DCs (MHCII+, CD14-/low, CD123-, CD11c+) and plasmacytoid DCs (MHCII+, CD14-/low, CD11c-, CD123+). C Shown is a PBMC sample from the same adult subject, either unstimulated (red) or stimulated with 10 uM of the TLR7/8 ligand 3M-003 (blue) for 6 hours in the presence of BFA. Innate immune cell subsets were identified as indicated above, then analyzed for expression of IL-6, IL-12, and IFN-α with TNF-α. C. Shown is a PBMC sample from the same adult subject, either unstimulated (red) or stimulated with TLR7/8 ligand 3M-003 (blue) for 18 hours without any Golgi-blocker. Innate immune cell subsets were identified as indicated above, then analyzed for expression of CD86, CCR7 and CD40.



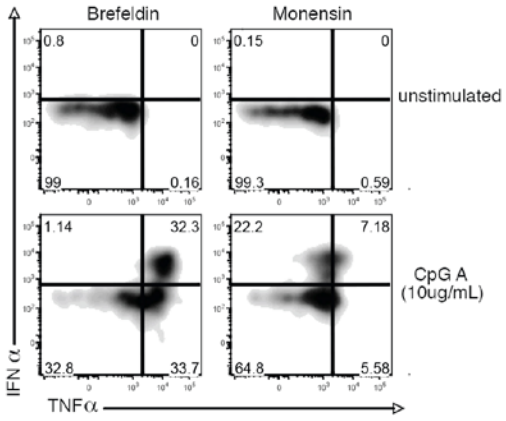
Appendix A Figure 2: Permeabilization Impacts Surface Marker Detection

Adult PBMC were either left unstimulated (red) or stimulated with 10 μM of the TLR7/8 ligand 3M-003 (blue) for 18 hours without any Golgi-blocker. Subsequently, samples were either processed as described for intracellular cytokine cytometry (i.e., permeabilized) or left untreated. Both samples were then stained to identify innate immune cell subsets as indicated in Figure 1A. The impact of permeabilization on expression of CD86 and CD40 by monocytes, mDCs and pDCs is shown in the left panels and the impact of permeabilization on CD19 expression is shown on the far-right panel.



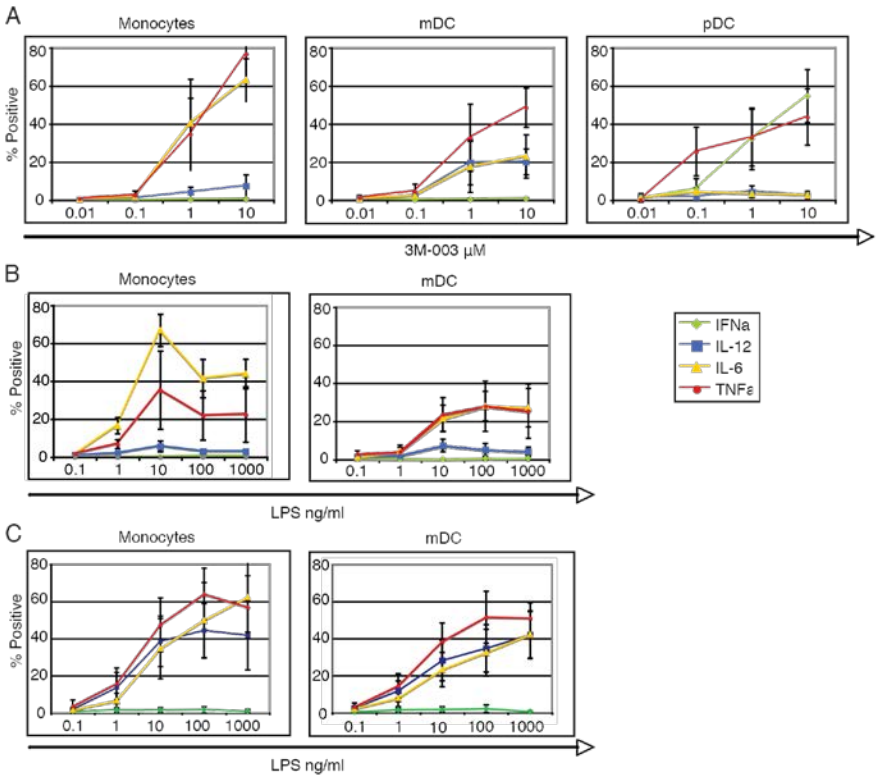
Appendix A Figure 3: Timing of Brefeldin A Addition has Significant Impact on Responses to CpG

A) Adult PBMC were either left unstimulated (top row), or stimulated with 10 μg/ml of the TLR9 ligand CpGA (lower row) for 6 hours. The Golgi-blocker Brefeldin A (BFA) was added hourly right from the start (0 hours) or up to 5 hours later. All cells were incubated for the same total time of 6 hours, and processed further for intracellular expression of cytokines in the various innate immune cell subsets. Shown is the co-expression of TNF-α and IFN-α in plasmacytoid DCs. B) Graph summarizing co-expression of TNF-α and IFN-α in plasmacytoid DCs in three different adult subjects with the bars indicating the standard deviation.



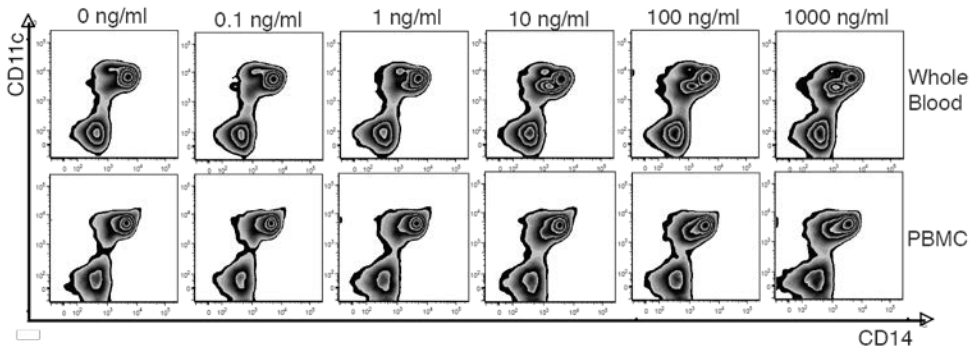
Appendix A Figure 4: Brefeldin A is the Better Golgi Blocking Reagent for Intracellular Cytokine Analysis in Innate Immune Cells

Adult PBMC were either left unstimulated (top row) or were stimulated with 10 μg/ml of the TLR9 ligand CpGA (lower row) for 6 hours. The Golgi-blockers Brefeldin A (BFA) or Monensin were added 3 hours into the incubation period. Cells were incubated for another 3 hours, then processed further for intracellular expression of cytokines. Shown is the co-expression of TNF-α and IFN-α in plasmacytoid DCs.



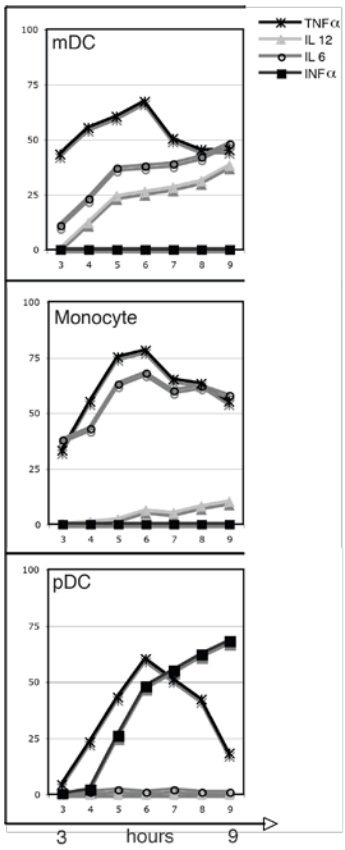
Appendix A Figure 5: Dose Response Profiles for 3M-003 and LPS

Adult PBMC were either left unstimulated or stimulated with different concentrations of the TLR7/8 ligand 3M-003 (A) or the TLR4 ligand LPS (B). C) Adult whole blood samples were either left unstimulated or stimulated with different concentrations of the TLR4 ligand LPS. Shown in all graphs is the result of intracellular cytokine detection in samples stimulated for 6 hours in the presence of BFA. These graphs depict the % cytokine positive cells for each of the cytokines analyzed from 5 different adult subjects (n=5).



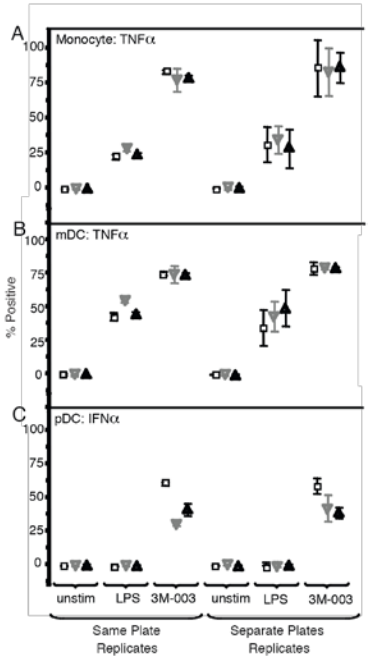
Appendix A Figure 6: Lineage Marker Expression is Altered with TLR Stimulation

Adult whole blood (top row) or PBMC (bottom row) were either left unstimulated or stimulated for 6 hours with the indicated concentrations of the TLR4 ligand LPS in the presence of the Golgi-blocker BFA. Samples were processed as described for intracellular cytokine cytometry and analyzed for the surface expression of CD11c and CD14.



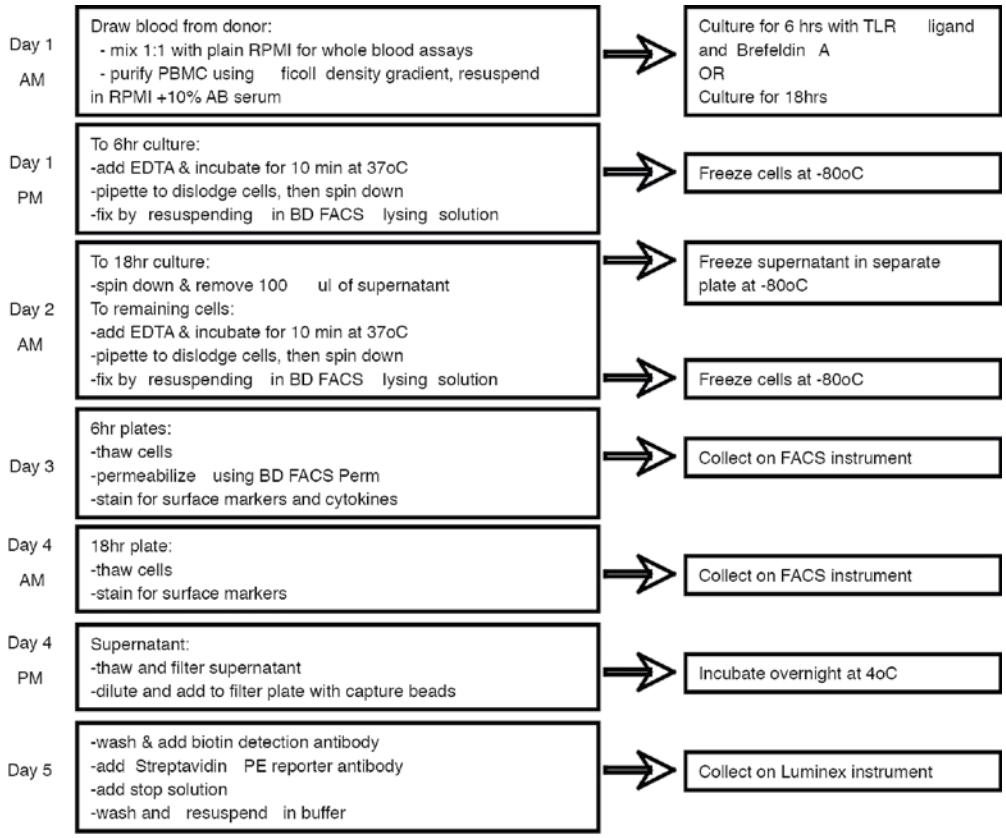
Appendix A Figure 7: Optimal Time Point of Total Incubation Length

Adult PBMC were either left unstimulated or stimulated with 10μM of the TLR7/8 ligand 3M-003. Shown is the result of intracellular cytokine detection in myeloid DC, monocytes, or plasmacytoid DC stimulated for 3 to 9 hours in the presence of BFA. The graphs depict the % cytokine positive cells for each of the cytokines analyzed. This graph is representative of results obtained with 3 different adult subjects.



Appendix A Figure 8: Precision of the Assay

PBMC from 3 different adults were either left unstimulated or stimulated with 10μM of the TLR7/8 ligand 3M-003 or 10ng/ml of the TLR4 ligand LPS for 6 hours in the presence of BFA. Triplicate wells were assayed either within the same plate (Same Plate) or across three different plates (Separate Plates). Furthermore, samples from these 3 adult subjects were taken three months apart between the assay using the Same Plate (time = 0 months) and those taken to assay triplicates across 3 different plates (time = 3 months), to estimate changes in a given subject over time. Shown is the result of intracellular TNF-α detection in monocytes (A) and mDCs (B), and for INF-α in pDCs (C); error bars = standard deviation.



Appendix A Figure. 9: Outline of Standard Operating Procedure

APPENDIX B: MIFlowCyt [MINIMUM INFORMATION ABOUT A FLOW CYTOMETRY EXPERIMENT] DATA

MIFlowCyt standard compliant information for submitted flow cytometric data.

Experiment overview

Purpose: The purpose of this experiment was to track the changing responses of monocytes, B cells (by negative gating), plasmacytoid dendritic cells, and myeloid dendritic cells to TLR stimulation during the first two years of life. Comparison to adult peripheral blood was performed to give an idea as to the relationship between adult and changing early life TLR responses. As previous work had accomplished comparison between whole blood and PBMC fractionated cell populations, only PBMCs were the focus of this study. We hypothesized that–given the changes in environment and ontogeny–that PBMC TLR responses would vary over time in the very young, and that this knowledge could form the basis of future work in rational vaccine design for the pediatric field.

Keywords: HEU, HIV Exposed Uninfected, UE, Innate Immune Development, Immune ontogeny

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Primary Contact: *Principal Investigator*: Dr. Tobias Kollmann tkollmann@cw.bc.ca

Graduate Student: Dr. Brian Reikie reikieb@myumanitoba.ca

Research Assistant: Bing Cai caibing@interchange.ubc.ca

Experiment Date: Experiments were set up from 5/24/07 to 5/25/10 and stained from 6/6/07 to 6/25/10.

Conclusions: Responses to TLR ligands change significantly from birth to 2 years of age. For some indicators of TLR response and certain TLR stimuli (e.g. IFN-α2 in TLR8/9 stimulation, TNF-α in TLR4 stimulation), levels approached those observed in similarly treated adult cells by 1 year of age. Conversely, others made an apparent decline from birth to 2 years of age (e.g IL-6), indicating an alternate TLR response by the very young. It is not yet known how or when these responses reach adult-like status. In sum, the neonatal immune system is distinct from that of the adult, and develops through age-specific phases instead of linear trend towards an adult-like pattern.

Quality Control Measures: Unstimulated controls were set up for each condition tested. Single stain controls were set up by staining 3 ul of Anti-Mouse Ig CompBeads (BD #552843) and 3 ul of anti-FBS negative control beads (included with BD #552843) with 3ul of each antibody used.

Flow Sample/Specimen Description

Biological Samples: *Biological Sample Name*: Cord and adult mononuclear cells *Biological Sample Source*: Healthy human adult peripheral blood; cord blood obtained and processed within < 4h from a healthy full-term baby born via C-section (no labor); healthy infant peripheral blood obtained and processed within < 4h. *Biological Sample Source Organism*:

Taxonomy: Kingdom Animalia Subkingdom Metazoa Phylum Chordata Subphylum Vertebrata Superclass Tetrapoda Class Mammalia Subclass Theria Infraclass Eutheria Order Primates Suborder Anthropoidea Family Hominidae Subfamily Homininae Tribe Hominini Genus Homo Subspecies sapiens

Age: Birth (for cord blood) to 2 years of age (venous blood); 21-55 years old (adult venous blood)

Gender: Male and Female

Phenotype: Healthy (none)

Genotype: Not Applicable

Treatment: PBMCs were isolated from peripheral blood using Ficoll gradient centrifugation. After purification, cells were washed twice with DPBS then resuspended at 2.5E6 cells/mL in RPMI supplemented with 10% human AB serum and 1% Penicillin/Streptomycin. Whole blood was diluted 1:1 in RPMI.

Environmental Samples: Not Applicable

Control Sample Description: Single stain controls were set up by staining 3 ul of Anti-Mouse Ig CompBeads (BD #552843) and 3 ul of anti-FBS negative control beads (included with BD #552843) with 3ul of each antibody used. Also, for cytokine analysis, fluorescence minus one (FMO) staining was applied, in which the antibody for the cytokine of interest is omitted from the normal antibody staining panel. Lastly, unstimulated control were used as controls to set analysis gates.

Sample Treatment Description: Cells were plated in a 96 well plate and cultured for 6 hrs. Cells were stimulated with PAM3CSK4 (TLR2/1, EMC microcollections); Peptidoglycan (TLR2, NOD1/2, Invivogen); poly I:C (TLR3, Amersham); 0111:B4

LPS (TLR4, InVivogen); R848 (TLR7/8, Invivogen); CpGA (TLR9, Coley); or plain RPMI. After culture, cells were treated with 2mM EDTA for 15 min at 37°C, then centrifuged @400g for 5min @22°C and resuspended in 100ul of BD FACS Lysing solution (BD 349202) for 10 minutes at room temperature before before being frozen at -80°C.

Instrument and Reagent Details

Fluorescence Reagent Description: See Appendix B Table 1

Manufacturer: BD Biosciences

Model: BD LSR II 4 Laser, Blue/Red/Violet/UV cat # 347545

Instrument Configuration and Settings: All lasers, filters and mirrors were manufactured by BD Biosciences. All filters and mirrors came with the machine and were installed March 2005. See Appendix B Table 2.

Light Sources: The light path, filters and detectors are described below in Table 2. The lasers are listed in the order the cells pass through them. The detectors and filters are listed in the order the light hits them, with the exception of FSC which is measured from light that passes through the cell/bead while all the other 488nm detectors detect light that has been scattered 90°, in the order listed. For example, for blue laser detector A light passes through or is reflected off of filter 1, 735 LP, then the light passes through filter 2, 780/60 BP, then it hits the PMT detector. Light that is reflected off the long pass goes to detector B and so on. For parameters used in this experiment, it is indicated whether Area (-A), Height (-H) or Width (-W) was used.

Abbreviations:

PMT = photomultiplier tube

PD = photodiode,

BP = band pass filter, first number is center of interval, second number is the width of the interval.

LP = long pass filter, lets light waves through that have a longer wavelength than the number specified. All LP filters are dichroic and reflect at an angle of incidence at 11.25°.

Data Analysis

FCS Data File

To request raw data please contact Dr. Kollmann tkollmann@cw.bc.ca or Dr. Reikie reikieb@myumanitoba.ca

Total Count of Events: Recorded within individual FCS files, 200 000 events per tube.

Compensation Description: Compensation was done in FlowJo using BDCompBeads as single stain controls. Representative compensation matrix shown in Appendix B Table 3.

Gating (Data Filtering) Description

Gate Summary Information: Gate Descriptions, subpopulations and gate statistics are shown in Appendix B Table 4. Example of gating summary can be seen in Appendix B Figure 1.

Data Transformation Description: Data was transformed using FlowJo's "Define BiExponential Transformation" function using the above mentioned compensation matrix, with an additional negative display size set at 0.5 and Positive Decades of "log" Display set at 5.

Appendix B Tables and Figures

Appendix B Table 1: Fluorescence reagent descriptions

	Characteristic Being Measured	Antibody Name *Clone Name*	Vendor cat# *dilution used*
VIOLET			
AmCyan/ PO	Cell Surface Protein	CD123 AmCyan (9F5)	BD#custom *1:50*
Pacific Blue	Intracellular Protein	IL12p40/70 *(eBio: C8.6)*	eBio#577129 *1:100*
RED			
APC	Cell Surface Protein	CD11c (5HCL3)	BD#340714 *1:50*
APC-Cy7	Intracellular Protein	IL6 *(AS12)*	BD #custom *1:100*
Alexa 700	Intracellular Protein	TNFa *(Mab11)*	BD#557996 *1:100*
BLUE			
FITC/OG	Intracellular Protein	IFNa *(A11)*	Antigenix#MC100133 *1:100*
PerCPCy 5.5	Cell Surface Protein	MHCII *TU36*	BD#custom *1:100*
PE-Cy7	Cell Surface Protein	CD14 (M5E2)	BD #557742 *1:50*

Appendix B Table 2: Instrument Configuration Settings

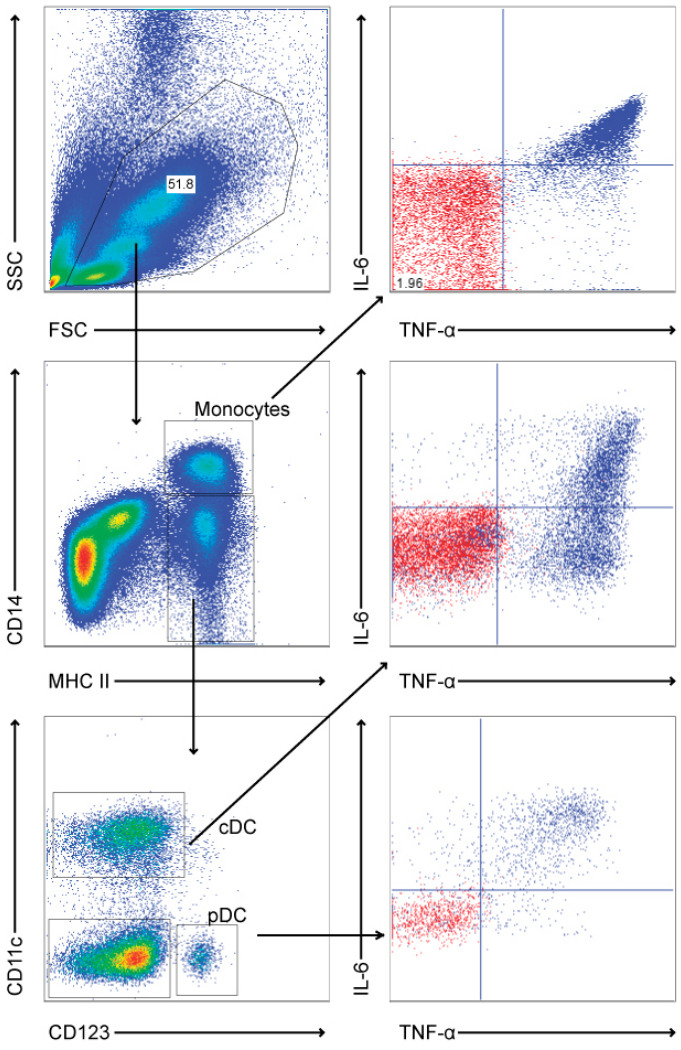
Laser	Detector Name (Type)	Filter 1 (1st filter)	Filter 2 (2nd Filter)	Parameter detected	Detector voltage	Amplification Type
Blue Laser (488 nm)	FSC (PD)	488/10 BP	na	FSC-A	390	LINEAR
Solid state Coherent Sapphire blue laser 20 mW	488 A (PMT)	735 LP	780/60 BP	PE-Cy7-A	625	LOG
	488 B (PMT)	685 LP	712/21 BP	PerCP-Cy5.5-A	750	LOG
	488 C (PMT)	655 LP	670/14 BP	PerCP	na	
	488 D (PMT)	595 LP	610/20 BP	PE-TexRed	na	
	488 E (PMT)	550 LP	575/26 BP	PE	na	
	488 F (PMT)	505 LP	530/30 BP	FITC, Ax488-A	450	LOG
	488 G (PD)	blank	488/10 BP	SSC-A	410	LINEAR
	488 H (PMT)	blank	na	blank	na	
Violet Laser (405 nm)	405 A (PMT)	505 LP	585/42 BP	AmCyan-A	650	LOG
Coherent VioFlame PLUS laser 25mw	405 B (PMT)	blank	440/40 BP	Pacific Blue-A	530	LOG
	405 C (PMT)	blank	na	blank	na	
UV Laser (355 nm)	355 A (PMT)	505 LP	530/30 BP	Ca++ Blue	na	
Solid state Coherent Lightwave Xcyte 20mW	355 B (PMT)	blank	440/40 BP	Alexa350	na	
	355 C (PMT)	blank	na	blank	na	
Red Laser (637 nm)	637 A (PMT)	755 LP	780/60 BP	APC-Cy7-A	700	LOG
Solid State Coherent laser 25 mW	637 B (PMT)	685 LP	710/50 BP	Alexa700-A	600	LOG
	637 C (PMT)	660/20 BP	na	APC-A	505	LOG

Appendix B Table 3: Representative Compensation Matrix

	FITC	PE	PERCP-Cy5.5	PE-Cy7	APC	APC-Cy7	Pacific Blue	Alexa 700
FITC		29.11	1.847	0.2719	0	0.1527	0.6106	0.4128
PE	0.6607		6.615	1.07	-0.249	0.3758	0.3544	0.6247
PERCP-Cy5.5	0.1683	0.2436		33.73	2.026	11.66	1.175	30.06
PE-Cy7	0.1653	1.982	0.3268		0	15.32	0.1029	0.5033
APC	0.1415	0.1768	1.576	0.4223		20.5	0.9705	65.71
APC-Cy7	0.08945	0.119	0.3078	2.183	12.56		0.3553	13.26
Pacific Blue	0.1281	0.1833	0	0	0.0574	-0.172		0.2704
Alexa 700	0.1859	0.208	1.44	0.9827	0.2883	29.7	0.662	

Appendix B Table 4: Gating (data filtering) Description

		Gate Statistics (% Parent Gate)	
Gate Description:	**Qualitative Description of the Subpopulation**	**Unstim**	**R848 stim**
Live Cells	High cell density excluding lower left corner population	79.6	71.9
Monocytes	CD14 high, MCHII high	24.9	13.2
Other MHCII+ cells	MHCII high, CD14 mid to low	15.3	19.2
Myeloid Dendritic Cells (cDCs)	MHCII high, CD11c high, CD123 low	22.9	26.9
Plasmacytoid Dendritic Cells (pDCs)	MHCII high, CD11c low, CD123 high	2.39	2.42
Monocyte TNF+ IL-6	"Monocyte" TNFa high, IL-6 low	0.29	16.6
Monocyte TNF+ IL-6+	"Monocyte" TNFa high, IL-6 high	0.15	46.7
Monocyte TNF- IL-6+	"Monocyte" TNFa low, IL-6 high	0.28	16.6
Monocyte TNF- IL-6	"Monocyte" TNFa low, IL-6 low	99.3	20.1
Monocyte TNF+ IL-12	"Monocyte" TNFa high, IL-12 low	0.35	61.8
Monocyte TNF+ IL-12+	"Monocyte" TNFa high, IL-12 high	0.093	1.52
Monocyte TNF- IL-12+	"Monocyte" TNFa low, IL-12 high	0.048	0.15
Monocyte TNF- IL-12	"Monocyte" TNFa low, IL-12 low	99.5	36.5
Monocyte TNF+ IFNa	"Monocyte" TNFa high, IFNa low	0.43	63.2
Monocyte TNF+ IFNa+	"Monocyte" TNFa high, IFNa high	0.00757	0.074
Monocyte TNF- IFNa+	"Monocyte" TNFa low, IFNa high	0.033	0.079
Monocyte TNF- IFNa	"Monocyte" TNFa low, IFNa low	99.5	36.6
cDC TNF+ IL-6	"cDC" TNFa high, IL-6 low	0.98	32.2
cDC TNF+ IL-6+	"cDC" TNFa high, IL-6 high	0.018	23.9
cDC TNF- IL-6+	"cDC" TNFa low, IL-6 high	1.66	6.94
cDC TNF- IL-6	"cDC" TNFa low, IL-6 low	97.3	37
cDC TNF+ IL-12	"cDC" TNFa high, IL-12 low	0.98	48.4
cDC TNF+ IL-12+	"cDC" TNFa high, IL-12 high	0.018	7.71
cDC TNF- IL-12+	"cDC" TNFa low, IL-12 high	0.32	3.5
cDC TNF- IL-12	"cDC" TNFa low, IL-12 low	98.6	40.5
cDC TNF+ IFNa	"cDC" TNFa high, IFNa low	1	56
cDC TNF+ IFNa+	"cDC" TNFa high, IFNa high	0	0.094
cDC TNF- IFNa+	"cDC" TNFa low, IFNa high	0.14	0.04
cDC TNF- IFNa	"cDC" TNFa low, IFNa low	98.8	43.9
pDC TNF+ IL-6	"pDC" TNFa high, IL-6 low	0.69	59.2
pDC TNF+ IL-6+	"pDC" TNFa high, IL-6 high	0.17	3.16
pDC TNF- IL-6+	"pDC" TNFa low, IL-6 high	0.69	0
pDC TNF- IL-6	"pDC" TNFa low, IL-6 low	98.3	37.6
pDC TNF+ IL-12	"pDC" TNFa high, IL-12 low	0.86	60.2
pDC TNF+ IL-12+	"pDC" TNFa high, IL-12 high	0	0.6
pDC TNF- IL-12+	"pDC" TNFa low, IL-12 high	0.17	0.45
pDC TNF- IL-12	"pDC" TNFa low, IL-12 low	98.8	38.8
pDC TNF+ IFNa	"pDC" TNFa high, IFNa low	0.86	13.4
pDC TNF+ IFNa+	"pDC" TNFa high, IFNa high	0	47.4
pDC TNF- IFNa+	"pDC" TNFa low, IFNa high	0.34	18.5
pDC TNF- IFNa	"pDC" TNFa low, IFNa low	98.6	20.8



Appendix B Figure 1: Example of gating strategy

Shown is a WB unstimulated sample in the panels on the left. The gating strategy was to identify various innate immune cell subsets: monocytes (MHC-II+, CD14+), B cells (MHC-II+, CD19+ – unlabeled), myeloid DCs (MHCII+, CD14-/low, CD123-, CD11c+) and plasmacytoid DCs (MHCII+, CD14-/low, CD11c-, CD123+). The panels on the right show unstimulated (red) or stimulated with 10 μM of the TLR7/8 ligand R848 (blue) for 6 hours in the presence of BFA. Monocyte and cDC cell subsets were identified as indicated above, then analyzed for expression of IL-6, IL-12, and TNF-α (examples shown), pDCs were analyzed for expression of IL-6, TNF-α and IFN-α (example shown).