Ontogeny of the Innate Immune Response in Healthy South African Infants

by
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Summary

Infection is a major cause of morbidity and mortality in infants within the first few months of life. Susceptibility to infectious disease in this vulnerable population is more prevalent in resource-limited regions, with a higher disease burden. Due to certain deficiencies in their adaptive immune system, neonates rely predominantly on their innate immune system for protection against infection, a vital component in the early host defence against pathogens. Several studies have described differences in neonatal innate toll-like receptor-mediated responses compared to adult counterparts, though very little is known about these receptor responses within resource-limited settings.

To address this issue, we assessed the longitudinal development of cytokine-specific responses of TLR4 and TLR7/8 in monocytes, myeloid dendritic cells and plasmacytoid dendritic cells in infants from a resource-limited setting, South Africa, within the first 12 months of life and compared it to adults. Contrary to previously published literature, we observed heightened production of T_H-1 cytokines: we showed increased responsiveness to TLR4 and TLR7/8 stimulation in infants at two and six weeks of age, which may be due to vaccination administered at birth. Unexpectedly, the hyper-inflammatory response persisted at six months in response to the LPS (TLR4) stimulus. This increased response at six months may be attributed to decreased passive immunity through infant weaning as well as increased exposure to microbial pathogens in this setting. Maturation of most cytokine responses was reached at twelve months for the TLR4 receptor, and at six months for the TLR7/8 receptor.

The first year of life represents a critical period for maturation of the immune response. Data from this study point towards an elevated response within the first six months of life. This heightened response reflects both an ability to mount a sufficient T_H-1 response in infancy, but more likely, the increased exposure to microbial stimuli in the environment. Thus, we speculate that these age-specific inflammatory responses may influence the outcome of immune responses to various vaccines administered, which may result in altered responsiveness to immunisation in infancy.

Opsomming

Die hoof oorsaak vir morbiditeit en mortaliteit in babas binne die eerste paar maande van hul lewe word toegeskryf aan infeksie. In hulpbron beperkte gebiede, gekenmerk deur `n groter siektelas, is daar `n verhoogde vatbaarheid vir infeksie in hierdie kwesbare populasie. As gevolg van sekere gebreke in die verworwe immuunstelsel, maak pasgebore babas hoofsaaklik staat op hul aangebore immuunstelsel vir beskerming teen infeksie, 'n belangrike komponent vir die vroeë verdediging teen patogene. Verskeie studies het al die verskille in toll-tipe reseptor (TTR) bemiddelde reaksies tussen pasgebore babas en volwassenes vergelyk, maar nie veel is bekend oor hierdie reaksies in areas waar hulpbronne beperk is nie.

Om hierdie kwessie aan te spreek is die longitudinale ontwikkeling van sitokien-spesifieke reaksies van die TTR4 en TTR7/8 reseptore van monosiete, miëloïede en plasmasitoïede dendritiese selle van babas in die hulpborn beperkte land Suid-Afrika, oor die eerste 12 maande geëvalueer en dit vergelyk met volwassenes. In teenstelling met vorige literatuur, het hierdie studie 'n polarisasie tot T_H-1-sitokien produksie gevind: verhoogde reaktiwiteit van die TTR4 en TTR7/8 is gevind in babas van twee en ses weke oud, wat gedeeltelik as gevolg van die inenting kan wees wat toegedien was na geboorte. Hierdie hiper-inflammatoriese reaksie teen die TTR4 stimulus (Lipopolisakkaried (LPS), het teen verwagting voortgeduur tot op ses maande en kan toegeskryf word aan die vermindering van passiewe immuniteit deur spening, sowel as die toenemende blootstelling aan mikrobiese patogene in die omgewing. Maturasie vir die meerderheid van die sitokiene reaksies, is bereik op 12 maande vir TTR4, en op ses maande vir TTR7/8.

Die eerste lewensjaar is 'n kritiese periode vir die ontwikkeling van die immuunstelsel. Data van hierdie studie dui op 'n verhoogde reaksie binne die eerste ses maande van 'n baba se lewe. Hierdie verhoogde reaksie dui op die vermoë om `n voldoende TH-1 reaksie te ontlok, maar meer waarskynlik, verhoogde blootstelling aan mikrobiese stimuli in die omgewing. Dus spekuleer ons dat hierdie ouderdom-spesifieke reaksies dalk die uitkoms van die immuunreaksie teen verskeie entstof toediening kan beïnvloed in babas.

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The most exciting phrase to hear in science, the one that heralds the most discoveries, is not "Eureka!" (I found it!) but "That's funny..."

Isaac Asimov

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List of Abbreviations

ANOVA	Analysis of variance
APC	Antigen presenting cell
BCG	Bacillus Calmette-Guérin
BFA	Brefeldin A
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
DBS	Dried blood spot
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
EDTA	Ethylene diaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
EMLA	Eutectic Mixture of Local Anaesthetics
EPI	Extended programme for immunisation
FSC	Forward scatter
g/dL	Grams per decilitre
GA	Gestational age
GPI	Glycosylphosphatidylinositol
HBAgs	Hepatitis B antigens
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HEU	HIV-exposed but uninfected
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen D receptor
HSV-1	Herpes simplex virus 1
ICC	Intracellular cytokine
ICH	Institute of Child Health
ICS	Intracellular cytokine staining
IFN	Interferon
IFN-α	Interferon alpha

IFN-δ	Interferon delta
IFN-τ	Interferon tau
IFN-ω	Interferon omega
IKK-β	Inhibitor of nuclear factor kappa-B kinase subunit beta
IKK-γ	Inhibitor of nuclear factor kappa-B kinase subunit gamma
IL	Interleukin
IL-1R	IL-1 receptor
IL-1β	Interleukin one beta
IRAK	IL-1R kinase
IRF	Interferon regulatory factor
kg	Kilogram
KIDCRU	Children's Infectious Disease and Clinical Research Unit
LDA	Limiting dilution analysis
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
Mal	MyD88 adaptor-like
MAP kinase	Mitogen activated protein kinase
MBAA	Multiplexed bead array assay
M-CSF	Macrophage colony stimulating factor
MD-2	Myeloid differentiation protein 2
mDC	Myeloid dendritic cell
mg/dL	Milligrams per decilitre
MHC	Major histocompatibility complex
ml	Millilitre
MPL-A	Monophosphoryl lipid A
MRC	Medical Research Council
MyD88	Myeloid differentiation primary response gene 88
ND	Not determined
NF-κB	Nuclear factor kappa B
NK cells	Natural killer cells
nm	Nanometre
OPV	Oral polio vaccine
PAMP	Pathogen-associated molecular patterns

PBSAN	Phosphate buffered saline containing bovine serum albumin and sodium azide
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PMT	Photomultiplier tube
PRR	Pattern recognition receptor
R-848	Resiquimod
RIP-1	Receptor-interacting protein-1
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SSC	Side scatter
ssRNA	Single-stranded ribonucleic acid
TAB	TGF-β activated kinase 1/MAP3K7 binding protein
TANK	TRAF family member-associated NF-κB activator
TB	Tuberculosis
TBK	TANK-binding kinase
TGF-β	Transforming growth factor beta
T_H-1	T-helper lymphocyte type 1
T_H-2	T-helper lymphocyte type 2
T_H-17	T-helper lymphocyte type 17
TIR	Toll/IL-1 receptor-like domain
TIRAP	TIR adaptor-like protein
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TRAF	Tumour necrosis associated factor
TRIF	TIR-domain-containing adapter-inducing interferon beta
UBC	University of British Columbia
UE	Unexposed
USP	United States Pharmacopeia
VZV	Varicella-Zoster virus
µl	Microlitre

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Chapter One
Introduction

1.1. Susceptibility to infection in early life

Neonates and young infants are at an increased risk for infectious disease compared to older children and adults (Bryce *et al*., 2005; Thaver & Zaida, 2009; WHO Health Statistics, 2010). Globally, the burden of infectious morbidity and mortality in newborns and young infants is overwhelming, with 40% of morbidity in young children (under five years) occurring within the first month of life (WHO Health Statistics, 2010). The highest incidence of infant morbidity in early life is seen in resource-limited countries, in particular African and Asian countries, with infection being the leading cause of morbidity and mortality (Bryce *et al.,* 2005; Thaver & Zaidi, 2009; WHO Health Statistics, 2011). In South Africa, the infant mortality rate stands at 49 per 1000 births, with HIV/AIDS, pneumonia, diarrhoea, and sepsis being the leading causes of infectious disease and mortality in children under the age of five years (WHO Health Statistics, 2011).

While childhood vaccination remains the primary prevention and intervention method to reduce infection, neonates and young infants tend to display suboptimal immune responses to all vaccines, with the exception of *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) where protective T_H-1 responses were observed even when given as early as birth (Adkins *et al*., 2004; Siegrist *et al*., 2001; Siegrist *et al*., 2007). The vulnerability to infection and lack of efficient vaccine responses is due to several factors, including the limited antigenic exposure *in utero* and lack of immunological memory, as well as an apparent T_H-2 bias observed in neonates (Siegrist *et al*., 2001; Siegrist *et al*., 2007; Levy 2007).

The predominant T_H-2 skewing in foetal and neonatal immunity is largely derived from the production of T_H-2-derived cytokines (e.g. IL-4) at the maternal–foetal interface (Wegmann *et al.,* 1993; Morein *et al.,* 2007). The induction of anti-inflammatory cytokine (e.g. IL-10) responses serves as a protective mechanism, as inflammatory responses are detrimental to placental integrity, resulting in preterm delivery and foetal loss (Wegmann *et al.,* 1993; Levy 2007; Morein *et al.,* 2007). This T_H-2 bias appears to affect the neonatal adaptive immunity, as T_H-2 effector functions are suboptimal for several of the diseases that strike early in life

(Adkins *et al.,* 2004), such as viral infections, *Listeria monocytogenes* and group B *Streptococcus* infections (Marodi 2006a).

Considering the limited antigenic exposure and the well-established adaptive immune defects in early infancy, neonates and infants are largely dependent on passively acquired maternal antibodies (Morein *et al.,* 2007; Belderbos *et al.,* 2009a) and their own innate immune system for protection against infection (Belderbos *et al.,* 2009a).

1.2. Pathogen recognition in innate immunity

Innate immunity represents the first line of host defence against microbial invasion. This branch of the immune system is critical for early host response as it functions efficiently without prior exposure to antigens. The innate immune system is antigen non-specific, but is able to differentiate between self and non-self antigens (Medzhitov & Janeway 1997; Akira *et al.,* 2001; Akira *et al.,* 2006; Mogensen 2009). It achieves this through the use of nonclonal, germline-encoded pattern recognition receptors (PRRs).

PRRs are expressed constitutively on antigen presenting cells, such as monocytes and dendritic cells, and recognise microbial components known as pathogen associated molecular patterns (PAMPs). PAMPs are essential for survival, invariant among classes of microbes and not produced by mammalian cells. Different PRRs are able to detect specific PAMPs regardless of microbial life-cycle stage (Medzhitov & Janeway 1997; Akira *et al.,* 2006; Medzhitov 2007; Medzhitov 2007; Mogensen 2009). Several classes of PRRs have been identified, of which the toll-like receptors (TLRs) are the most extensively studied family (Akira *et al.,* 2006; Mogensen 2009). Currently, 11 TLRs are known in mammalian species, of which ten are present in humans. TLRs are capable of recognising distinct microbial stimuli and their activation by TLR-PAMP interaction activates intracellular signalling cascades, resulting in the production of proinflammatory mediators that modulate the innate immune response (Akira *et al.,* 2001; Akira & Takeda 2004; Akira *et al.,* 2006; Mogensen 2009). Thus, these receptors are essential in initiating and orchestrating the early host response to microbes.

Given the importance of the TLR system in host defence, several studies have documented newborn responses in comparison to adults. The inability of neonates to produce T_H-1 type

proinflammatory cytokines severely hampers the efficacy of their cell-mediated immunity (De Wit *et al.,* 2004; Levy *et al.,* 2004; Angelone *et al.,* 2006; Danis *et al.,* 2008; Belderbos *et al.,* 2009b; Kollmann *et al.,* 2009). This results in an increased susceptibility of newborns to intracellular pathogens, such as viruses and *Listeria monocytogenes*. In contrast, studies have found a skewing of neonatal TLR-mediated responses towards T_H-17 and T_H-2 type immunity (with an increase in IL-6, IL-10 and IL-23) (Levy *et al.,* 2004; Kollmann *et al.,* 2009). This T_H-17 bias offers protection against certain bacterial and fungal infections (Kollmann *et al.,* 2009). Therefore, rather than simply being "immature", neonatal innate immune capabilities prime neonatal adaptive immunity differently to that of adults.

1.3. Aims and objectives

1.3.1. Aim

Although several studies have described differences in neonatal TLR-mediated cytokine responses compared to adults, very few of these studies have evaluated the development of these responses over time (Corbett *et al.,* 2010; Nguyen *et al.,* 2010). Moreover, even less data is available on the ontogeny of TLR-mediated responses in resource-limited settings, with maturation of these responses only reported in Gambian (Burl *et al*., 2011) and Latin-American infant cohorts (Teran *et al*., 2011). The investigation of these innate responses in a resource-limited setting is of considerable importance, as these infants' responses may differ significantly due to the increased microbial exposure and alternative vaccine schedules (Thaver & Zaida 2009; WHO Health Statistics, 2011). With gram-negative organisms, such as *Klebsiella pneumoniae* and *Escherichia coli* (Zaidi *et al*., 2009), and viral pathogens, including respiratory syncytial virus (RSV), (Lehmann *et al*., 1999) being the major cause of neonatal sepsis and pneumonia, especially in developing countries, it is of interest to assess the maturation of these TLR responses to the recognition of these specific pathogens.

As part of a comparative HIV Exposed but Uninfected (HEU) infant cohort study in collaboration with the University of British Columbia (UBC), we aimed to document the ontogeny of the HIV-unexposed (UE) infants' TLR-mediated proinflammatory cytokine response. We set out to assess the cytokine-specific responses to TLR4 and TLR7/8 in antigen-presenting cells (monocytes, myeloid dendritic cells and plasmacytoid dendritic cells) at 2 weeks, 6 weeks, 6 months and 12 months of age as compared to adults. This was to permit us to determine at which stage the infant response reaches a state of maturation,

thereby documenting the TLR response for normal infants with reference to the TLR4 and TLR7/8 responses.

1.3.2. Objectives

To achieve our aim, the objectives for the study were to:

1. Assess the TLR-mediated production of TNF-α, IFN-α, IL-6 and IL-12p40 in monocytes, as well as myeloid and plasmacytoid dendritic cell subsets in infants at 2 weeks, 6 weeks, 6 months and 12 months of life.
2. Assess the TLR-mediated production of TNF-α, IFN-α, IL-6 and IL-12p40 in monocytes, as well as myeloid and plasmacytoid dendritic cell subsets in clinically healthy adults at one time point.
3. To determine the time point/age at which the TLR-mediated cell-specific cytokine response in South African infants become comparable to the response measured in adults.

1.4. Hypothesis

For the purpose of this study, we hypothesise that the TLR-mediated cell-mediated cytokine responses of healthy South African infants are comparable to that of the adult responses at twelve months of age.

Chapter Two

Literature Review

2.1. Toll-like receptors

The TLR family is the most extensively studied class of PRRs. TLRs were initially identified through their homology to the *Drosophila melanogaster* Toll protein, a receptor found to be critical for dorsoventral development in embryogenesis in the fruit fly (Lemaitre *et al.,* 1996). Subsequent studies have proven this protein essential in the innate immune response against fungal infection in insects (Lemaitre *et al.,* 1996; Medzhitov *et al.,* 1997; Akira *et al.,* 2006). These findings led to the discovery of TLRs in mammals.

The mammalian TLR family currently consists of 11 members, of which ten receptors are present in humans. These receptors recognise specific PAMPs derived from various microbial pathogens, such as bacteria, viruses, fungi and protozoa (Akira *et al.,* 2001; Akira & Takeda 2004; Akira *et al.,* 2006).

2.1.1. Structure

TLRs are described as type I transmembrane glycoproteins and form part of a larger superfamily that includes the interleukin (IL)-1 receptor (IL-1R) family. These receptors are characterised as homo- or heterodimeric in nature, with an extracellular domain composed of leucine-rich repeat (LRR) motifs and a cytoplasmic signalling domain, similar to IL-1R situated in the Toll-IL-1 receptor-like (TIR) domain (Means *et al.,* 2000; Akira & Takeda 2004; Akira *et al.,* 2006; Mogensen 2009).

The LRR domains are composed of conserved regions of 16-28 tandem motifs. Each motif is 24-29 amino acids in length and is composed of a conserved LxxLxLxxN motif and a variable region. This LRR domain forms a unique horseshoe structure. The conserved leucine residues form the hydrophobic convex inner structure, creating parallel β-strands. LRRs, specifically the concave structure, are thought to be directly involved in the binding of specific PAMPs to the TLR structure (Akira & Takeda 2004; Akira *et al.,* 2006; Jin & Lee 2008).

Both TLRs and IL-1R have a conserved region of approximately 200 amino acids in their cytoplasmic tails, known as the TIR domain. The domain consists of three conserved boxes, and is essential in intracellular signalling cascade. The TIR domains of different TLRs vary in size, with approximately 20-30% of their amino acid sequence conserved among these receptors (Akira & Takeda 2004).

Crystallography of TLR1, TLR2 and TLR10 revealed the structure of the TIR domain. The TIR domain consists of five parallel β-sheets surrounded by five α-helices connected by BB-loops. The conserved boxes 1, 2, and BB-loop display most of their side chains and play a central role in TIR dimerisation and interaction with adaptor molecules (Akira & Takeda 2004; Akira *et al.,* 2006; Jin & Lee 2008).

2.1.2. TLR subfamilies

The mammalian TLR family can be separated into two distinct subfamilies based on their relative expression by cells of interest and recognition of specific PAMPs and/or ligands. The main ligands recognised by each of the 11 TLRs have been summarised in Table 1.1. The first subfamily consists of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10, and is expressed on the cell surface. These TLRs detect lipids and lipoproteins predominantly derived from bacterial products. The second subfamily consists of TLR3, TLR7, TLR8 and TLR9, and is located intracellularly on endosomes. TLR3, TLR7, TLR8 and TLR9 recognise nucleic acids from several different pathogens in endosomes and lysosomes (Akira *et al.,* 2006; Parker *et al.,* 2007; Mogensen 2009). The compartmentalisation of intracellular TLRs allows for discrimination of self versus non-self by the localisation of foreign nucleic acid, as well as differentiation of the molecular structure of invaders from that of the host (Mogensen 2009).

For the purpose of this dissertation, the next section will focus on TLR recognition and responses for TLR4 and TLR7/8 only, as these TLR responses will be the focal point of the thesis.

Table 1.1 Summary of the different PAMPs recognised by TLRs

Receptor	Ligand	Origin
TLR1	Triacyl lipopeptides	Bacteria and mycobacteria
	Soluble factors	*Neisseria meningitidis*
TLR2	Lipoproteins	Various pathogens
	Lipoteichoic acids	Gram-positive bacteria
	Zymosan	Fungi
	Porins	Neisseria
	β-glycan	Fungi
	Phospholipomannan	Candida
	Glycosylphosphatidylinositol-mucin	Protozoa
	Envelope glycoproteins	Viruses
	Heat shock protein 70	Host
TLR3	Double stranded RNA	Viruses
TLR4	Lipopolysaccharide	Gram-negative bacteria
	Fusion protein	Respiratory syncytial virus
	Envelope glycoproteins	Viruses
	Glycoinositolphospholipids	Protozoa
	Mannan	Fungi
	Fibrinogen	Host
	Heat shock protein 70	Host
TLR5	Flagellin	Bacteria
TLR6	Peptidoglycan	Gram-positive bacteria
	Diacyl lipopeptides	Mycoplasma
	Zymosan	Fungi
TLR7/8	Single stranded RNA	Viruses
	Imidazoquinoline	Synthetic compound
TLR9	CpG-containing DNA	Bacteria and viruses
TLR10	Not determined	Not determined
TLR11	Not determined	Uropathogenic bacteria

2.1.3. TLR4 recognition of different PAMPs

2.1.3.1. Recognition of lipopolysaccharide (LPS) and host proteins by TLR4

LPS is a major component of Gram-negative bacterial cell walls and recognised specifically by TLR4. LPS, also known as an endotoxin, is the most potent immunostimulant among the cell wall components. The lipid portion, lipid A, is responsible for most of the pathogenicity associated with Gram-negative bacteria, such as the inflammatory response in endotoxic shock (Akira & Takeda 2001; Ezekowitz & Hoffmann 2003; Akira *et al.,* 2006; Mogensen 2009).

TLR4 is also able to sense host-derived products secreted during inflammation and tissue injury. Examples of these products are heat-shock protein 60, fibronectin and plasma fibrinogen. The recognition of these products results in the activation of macrophages in a TLR4-dependent manner (Ezekowitz & Hoffmann 2003; Mogensen 2009).

Although TLR4 is established as the primary receptor for the recognition of LPS, several accessory molecules are involved in this recognition and binding process. Free LPS associates with the LPS binding protein, an acute-phase protein present in the bloodstream, which then binds to the CD14 receptor, and is then transferred to myeloid differentiation protein 2 (MD-2) and associates with extracellular domain of TLR4 by oligomerisation (Takeda & Akira 2001; Mogensen 2009).

Polymorphisms in the *TLR4* gene are associated with impaired LPS responses. The missense mutation in humans, Asp299Gly, impairs signalling and results in decreased inflammatory responsiveness and increased susceptibility to gram-negative bacterial infections and sepsis (Cook *et al.,* 2004; Misch & Hawn 2008). In contrast, D299G and T399I polymorphisms have been associated with resistance to *Legionella pneumophila*. Deficiencies in TLR4 have been associated with high susceptibility to *Neisseria meningitidis*, *Salmonella enterica* and *Mycobacterium tuberculosis* (Kopp & Medzhitov 2003).

2.1.3.2. Recognition of viral glycoprotein by TLR4

In addition to the endosomal TLRs, TLR4 is also involved in recognition of viral components at the cell surface. Recognition of viral PAMPs by TLR4 results in the production of proinflammatory cytokines, with the exception of type I IFNs. This suggests the induction of

an inflammatory rather than antiviral response. TLR4 recognises the fusion protein in respiratory syncytial virus (RSV), suggesting a crucial role in viral infection (Ezekowitz & Hoffmann, 2003; Mogensen, 2009). The importance of TLR4 activation in RSV was demonstrated by TLR4 knockout mice, where deficiencies resulted in lower levels of infiltrating mononuclear cells and proinflammatory cytokine production and leads to reduced viral clearance (Kopp & Medzhitov 2003; Koyama *et al.,* 2008).

2.1.3.3. Recognition of fungal PAMPs by TLR4

TLR4 recognise several fungal PAMPs located in the cell wall or cell surface of fungi. TLR4 serves as a receptor for *Candida albicans*-derived mannan, as well as glucuronoxylomannan, a component of *Cryptococcus neoformans* (Roeder *et al*., 2004; Mogensen 2009). TLR4, along with TLR2, is able to both recognise components of the *Aspergillus* species. The conidia of *Aspergillus* trigger innate immune responses through recognition by TLR4, whereas TLR2 is able to recognise both hyphae and conidia of the *Aspergillus* species (Roeder *et al.,* 2004). *In vivo* experiments also suggest significant roles of TLR4 in fungal infection. TLR4-deficient mice showed increased susceptibility to disseminated *Candida* infection (Kopp & Medzhitov 2003; Netea *et al.,* 2004; Roeder *et al.,* 2004), whereas TLR2-deficient mice showed increased resistance to *Candida* (Netea *et al.,* 2004).

2.1.3.4. Recognition of protozoan PAMPs by TLR4

In comparison to viruses and bacteria, the TLR recognition of protozoa has been less well defined in literature. The primary PAMP recognised in protozoa is the glycosylphosphatidylinositol (GPI) anchors. GPI anchors or their fragments from protozoan pathogens, *Leishmania major, Trypanosoma brucei, Trypanosoma cruzi, Plasmodium falciparum* and *Toxoplasma gondii*, are able to activate both lymphoid and myeloid cells. GPI anchors are composed of a glycan core and a lipid component, and functions to secure proteins to the surface of eukaryotic cells. GPI anchors are able to activate TLR2 and result in the activation of proinflammatory signalling pathways. In addition, GPI anchors also require CD14 and TLR4 for the recognition of these protozoan pathogens, in example *Trypanosoma cruzi* (Gazzinelli & Denkers 2006).

2.1.4. TLR7 and TLR8 recognition of single stranded RNA (ssRNA)

TLR7 and TLR8 are both structurally and phylogenetically related due to the high homology of *TLR7* and *TLR8* genes (Akira *et al.*, 2006; Bowie 2007). TLR7/8 is essential in identifying ssRNA from viral genomes, such as influenza virus, vesicular stomatitis virus and human immunodeficiency virus (HIV). Both TLR7 and TLR8 are able to recognise uridine-rich or uridine/guanosine-rich ssRNA of host and viral origins. TLR7 and TLR8 are also able to detect synthetic antiviral imidazoquinolone derivatives, such as resiquimod (R-848) and guanine analogs (e.g. loxoribine) (Akira *et al.*, 2006; Kawai & Akira 2010). TLR7 and TLR8 induce the production of large amounts of type I IFNs after viral infection. Cytokine induction in response to RNA viruses is fully dependent on TLR7, which suggests that this receptor serves as the sensor of infection with ssRNA viruses (Kawai & Akira 2010).

TLR7 and TLR8 are both located on the endosomal membrane. Many enveloped viruses move through the cytosol via the endosomal compartment. The degradation of viral particles in the cytosol results in the release of ssRNA and its recognition by TLR7/TLR8. Viral RNA is also released from the phagolysosome when phagocytes take up virus-infected apoptotic cells. The RNA from the host is degraded by extracellular RNases when released from cells and rarely reach the endocytic compartment, thus preventing recognition of self-antigens (Akira *et al.*, 2006).

Certain TLR7/8 polymorphisms are implicated in the progression of HIV-1 disease. The presence of the TLR7Gln11Leu polymorphism has been associated with lower baseline CD4+ T-lymphocyte counts and possibly increased HIV-1 susceptibility in women. This is suggestive of a strong effect on the initial stages of HIV infection (Oh *et al.*, 2009). A previous study documented the TLR8 A1G mutation in a German cohort of HIV-positive patients. This polymorphism alters the start ATG of TLR8 isoform B into a GTG triplet, resulting in a truncated TLR8 peptide. The TLR8 A1G mutation results in a decrease in NF-κB activation and a lower level of immune activation (Oh *et al.*, 2008).

2.1.5. TLR signalling pathway

TLR-PAMP interactions initiate a number of signal transduction pathways. Signal transduction in TLR-mediated immune responses is mediated by a family of adaptor molecules, which include myeloid differentiation gene factor 88 (MyD88), TIR-associated

protein (TIRAP)/MyD88 adaptor-like (Mal) protein, TIR domain-containing adaptor protein inducing interferon (TRIF) and TRIF-associated adaptor molecule (Akira & Takeda 2004). The differential responses of specific TLRs are determined by the selective recruitment of adaptor molecules. MyD88 and TRIF are responsible for distinct pathways resulting in the production of proinflammatory cytokines and type I interferons (IFNs) respectively (Akira & Takeda 2004; Mogensen 2009). Figure 1.1 illustrates the TLR signalling pathway.

2.1.5.1. Proinflammatory cytokine production

The MyD88-dependent signalling pathway is activated downstream for all TLRs with the exception of TLR3. In TLR2, TLR4 and TLR5 stimulation, MyD88 associates with the cytoplasmic TIR domain and recruits IL-1 receptor kinase (IRAK) 2/1 and IRAK4 through homophilic interaction, that is shown to be essential in the NF-κB activation and in IL-R1/TLR signalling in response to stimulation of these TLRs. In TLR4 activation, the TIRAP/Mal adaptor is also required for recruitment of MyD88 (Akira & Takeda 2004; Mogensen 2009; Kawai *et al*., 2010).

The MyD88 adaptor binds to IRAK4 and IRAK2/1, resulting in the activation of IRAK1. The activated IRAK1 autophosphorylates its residues on the N-terminus, thereby allowing the binding of TRAF6. The IRAK1–TRAF6 complex then disengages from the receptor and interacts with the TAK1/TAB1/2/3 complex at the plasma membrane. TRAF6 complex activates and phosphorylates TAK1/TAB1/2/3, which then translocates together to the cytoplasm and phosphorylates the IKK-γ/NF-κB essential modulator (NEMO). The phosphorylation of IKK-β and MAP-kinases results in the modulation of NF-κB and MAP kinases, which translocates to the nucleus and initiates expression of proinflammatory cytokine genes. In addition to NF-κB, the transcription factor, interferon regulatory factor (IRF) 5 regulates the expression of IL-6, IL-12 and cytokine genes associated with NF-κB p50 (Akira *et al*., 2001; Akira & Takeda 2004; Mogensen 2009; Kawai *et al*., 2010).

2.1.5.2. Type I IFN production

Viral TLRs, such as TLR7 and TLR8, induce the production of type I IFN in addition to other proinflammatory cytokines. However, TLR4 is able to induce IFN-α and IFN-β production in a MyD88 independent manner, with the recruitment of TRIF as well as TRAM, with acts as a bridging adaptor in TLR4 signalling.

Upon stimulation, TRIF associates with receptor-interacting protein-1 (RIP-1), which is responsible for activation of NF-κB, or TRIF-family member-associated NF-κB activator (TANK) binding kinase-1 (TBK1) via TRAF3. TBK1 is comprised of a family of inducible IκB kinases that directly phosphorylates IRF-3 and IRF-7. IRF-3 and IRF-7 translocates to the nucleus and binds to the IFN-stimulated response elements, resulting in the expression IFN-inducible genes. IRF-3 and IRF-7 are essential in the production of type I IFNs in the viral mediated responses (Akira *et al.,* 2001; Akira & Takeda 2004; Parker *et al.,* 2007; Kawai *et al*., 2010).

TLR7 and TLR9 both induce IFN-α production in a MyD88-dependent manner. The IRF-7 adaptor plays a vital role in the expression of type I IFN by this TLR. Upon stimulation, MyD88, IRAK4, IRAK1, TRAF6 and IRF-7 form a complex and are recruited to the receptor. IRAK1 could potentially serve as an IRF-7 kinase, as IFN responses are abolished in deficient cells, but all other inflammatory cytokines are produced normally (Akira *et al.,* 2001; Akira & Takeda 2004; Parker *et al.,* 2007; Kawai *et al*., 2010).

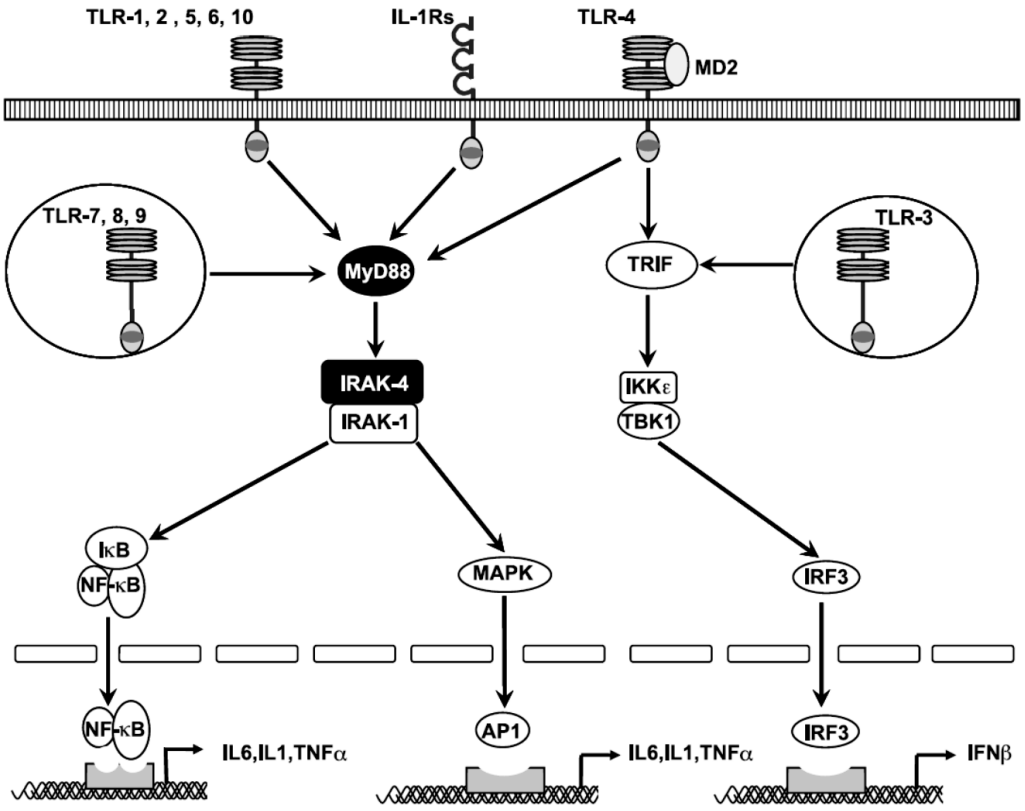

Figure 1.1 Schematic representation of TLRs signalling pathway (Source: Picard *et al.*, 2010).

2.2. Antigen presenting cells involved in TLR-mediated responses

The activation of innate immune cells relies on the recognition of PAMPs. The TLR family has emerged as primary sensors for conserved structures of bacteria, fungi and viruses. A number of innate immune cells differentially express TLRs, indicating specific roles for each cell population with regard to innate immune responses. In peripheral blood, monocytes and dendritic cells act as the primary antigen presenting cells (APCs), each specialising in an important role in pathogen recognition and specific cytokine production in response. The stimulation of most TLRs leads to T_H-1 rather than T_H-2 mediated response and cellular mediated adaptive immune differentiation. Thus, innate immunity is a key player in the inflammatory and cellular immune response against pathogens.

2.2.1. Monocytes

Monocytes are characteristically defined as non-dividing, circulating leukocytes that constitute approximately 10% of peripheral leukocytes in humans (Serbina *et al.,* 2008). Circulating monocytes are separated into two subsets based on the expression of CD14, a component of the LPS receptor complex, and CD16, the FcγRIII immunoglobulin receptor. CD14+ monocytes contribute up to 90% of circulating monocyte population (Serbina *et al.,* 2008; Yona & Jung 2010). Monocytes represent immune effector cells, and express chemokine receptors and adhesion receptors that allow the migration from peripheral blood to tissues during infection. Monocytes produce inflammatory cytokines and ingest cells, debris and toxic molecules. They can also differentiate into inflammatory monocyte-derived dendritic cells or macrophages during inflammation (Geissmann *et al.,* 2010).

Monocytes are highly responsive to TLR stimulation. Several studies have shown that monocytes express high levels of TLR4 and TLR8 but almost undetectable levels of TLR7 (Kadowaki *et al.,* 2001; Zarember & Godowski 2002; Kamgang *et al.,* 2008; Geissmann *et al.,* 2010). Monocytes are able to efficiently identify and respond to bacterial, fungal and viral PAMPs.

2.2.2. Dendritic cells

Dendritic cells (DCs) are central to the innate immune response. DCs are responsible for the capture and processing of antigens, as well as for activation and differentiation of T-helper cells into T_H-1, T_H-2 and T_H-17 lymphocytes and cytotoxic effector T-lymphocyte subsets in

lymphoid organs for the initiation of the appropriate adaptive immune response (Clark *et al.,* 2000; Iwasaki & Medzhitov 2004; Kadowaki 2007). DCs circulate in peripheral blood and tissues in an immature state and act as surveillance cells that capture and process foreign antigens and migrate to draining lymph nodes and present the processed antigens to the T-lymphocytes (Clark *et al.,* 2000; Kadowaki, 2007).

DCs are identified by their surface expression of CD45 and major histocompatibility complex class II (MHCII) molecules and absence of other haematopoietic lineage markers, such as CD3, CD14, CD15 and CD16 (Clark *et al.,* 2000). Two distinct subsets of DCs circulate through peripheral blood, specifically myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) (Clark *et al.,* 2000; Colonna *et al.,* 2006; Kadowaki, 2007).

2.2.2.1. Myeloid dendritic cells

The CD11c+ mDC subset has a similar marker expression to a myeloid derived cell (Lin123-, $CD33^{bright}$, $CD14^{dim}$, CD16-, CD11c+) (Clark *et al.,* 2000; Kadowaki 2007), but has also been identified as ILT3+/ILT1+ (Clark *et al.,* 2000). MDCs in peripheral blood have been reported to develop macrophage morphology as well as the expression of butyrate esterase and CD14 in response to macrophage-colony stimulating factor (M-CSF), suggesting the ability to differentiate into macrophages and therefore related to monocyte-derived DCs (Kadowaki 2007).

CD11c+ mDCs are the more dominant DC subset and express low levels of TLR4 and TLR8 (Jarrossay *et al.,* 2001; Kadowaki *et al.,* 2001; Zarember & Godowski 2002). Thus, they can recognise bacterial, fungal and viral pathogens. MDCs can therefore respond to bacterial and viral lipoproteins, as well as dsDNA and ssRNA from viruses.

2.2.2.2. Plasmacytoid dendritic cells

The Lin123-, CD11c-, ILT3+/ILT1- pDC subset share similar morphological characteristics with the lymphoid lineage and also express the CD123 marker on their cell surface (Clark *et al.,* 2000; Barchet *et al.,* 2005). PDCs constitute <1% of mononuclear cells in peripheral blood (Iwasaki & Medzhitov 2004; Barchet *et al.,* 2005) and express high levels of TLR7 and TLR8 (Jarrossay *et al.,* 2001; Kadowaki *et al.,* 2001; Barchet *et al.,* 2005). This subset does not express TLR4 and thus do not respond to LPS stimulation. Plasmacytoid DCs are

restricted to the recognition of DNA and RNA viruses (Kadowaki *et al.,* 2001; Iwasaki & Medzhitov 2004; Barchet *et al.,* 2005).

2.3. TLR-mediated proinflammatory cytokine responses

2.3.1. Function of proinflammatory cytokines

Cytokines produced by macrophages/monocytes and dendritic cells drive T-cell immunity, which are responsible for immunity against intracellular pathogens, elimination of cancerous cells, stimulation of hypersensitivity reactions and autoimmune responses, as well as B cell stimulation. Some of the major inflammatory cytokines produced by monocytes and DCs include tumour necrosis factor alpha (TNF-α), and several IL cytokines, such as IL-6, IL-12 as well as type I IFNs.

2.3.1.1. TNF-α

TNF-α forms part of the TNF family and is primarily secreted by activated mononuclear phagocytes, natural killer (NK) cells, mast cells and activated T-lymphocytes. TNF is a potent inflammatory mediator. TNFs induce anti-tumour immunity through cytotoxic function and by stimulating immune responses to cancerous cells. TNF is a potent chemoattractant and mediates adherence, chemotaxis and degranulation of granulocytes, such as neutrophils. TNF induces vascular leakage, has negative inotropic effects and is the primary endogenous mediator of toxic shock and sepsis. TNF also triggers apoptosis (Borish & Steinke 2003).

2.3.1.2. IL-6

IL-6 is a pleotropic cytokine secreted by various cell types, including B- and T-lymphocytes, mononuclear phagocytes, fibroblasts, keratinocytes, endothelial cells, mesenchymal cells and certain types of tumour cells. IL-6 induces the differentiation of B-lymphocytes into plasma cells; mediates activation, growth and differentiation of T-lymphocytes and IL-2 production by this cell type. It also stimulates the differentiation of macrophages and megakaryocytes. IL-6 is the most important inducer of hepatocyte synthesis of acute phase proteins and pyrexia (Borish & Steinke 2003; Blanco *et al.,* 2008). Along with transforming growth factor beta (TGF-β), IL-6 also participates in the differentiation of T_H-17 subset (Blanco *et al.,* 2008).

2.3.1.3. IL-12

IL-12 is a heterodimeric cytokine derived from monocytes, macrophages, B-lymphocytes, DCs, neutrophils and mast cells, but it is primarily produced by activated mDCs (Borish & Steinke 2003; Blanco *et al.,* 2008). The biologically active form, IL-12p70, is a heterodimer composed of p40 and p35 units. The IL-12p40 subunit is able to bind to the IL-23α chain, which is homologous to the IL-12p35 subunit (Borish & Steinke 2003). IL-12 plays an essential role in the differentiation and expansion of T_H-1 cells. This cytokine has multiple effects on T-lymphocytes and NK cells. It activates and stimulates the proliferation, cytotoxicity and cytokine production in NK cells and stimulates the proliferation of T-helper and cytotoxic lymphocytes. IL-12 also induces the production of TNF-α and IFN-γ by NK cells, as well as IFN-γ and TNF-β by T-lymphocytes (Brunda 1994). IL-23 is secreted by activated dendritic cells and, along with IL-6, is responsible for the differentiation of T_H-17-mediated lymphocyte differentiation (Hunter *et al*., 2005).

2.3.1.4. Type I IFNs

The type I IFN family is composed of IFN-α, IFN-β, IFN-δ, IFN-ω and IFN-τ, subtypes of which IFN-α and IFN-β have been described in humans. IFN-α and -β are produced by various cells types, such as fibroblasts, NK cells, T-lymphocytes, DCs in response to viruses and intracellular pathogens, but it has been found that pDCs are the largest producers of type I IFNs (Bogdan 2000).

The secretion of type I IFNs enhances cytotoxicity of NK cells and CD8+ T cells, and protects DCs and uninfected cells from the cytopathic effect of viruses, thus assisting their antigen presentation function (Bogdan 2000; Barchet *et al.,* 2005). Also, type I IFNs are secreted synergistically with other cytokines, regulating immune responses. Through secretion of IL-6 and type I IFNs, pDCs promote differentiation of memory B cells into antibody secreting plasma cells, facilitating the production of anti-viral antibodies (Barchet *et al.,* 2005). Type I IFN induces the production of IL-12 and IL-18 production in macrophages and works synergistically with IL-12 to activate NK cells to produce IFN-γ (Bogdan 2000). IFN-α has other important biological actions, including upregulation of MHC class I molecules and mediation of antitumor activity (Borish & Steinke 2003).

2.3.2. Cytokine response in monocytes

Monocytes are the most potent inducers of TNF-α in comparison to other APCs. In response to TLR2 and TLR4 stimulation, monocytes induce the production of large amounts of TNF-α, IL-6 (Kadowaki *et al.,* 2001) and IL-12 (Zarember & Godowski 2002). Also, stimulation of TLR2 and TLR4 induces the release of IL-1β, a potent pyrogen. Monocytes secrete IL-12 and TNF-α in response to TLR8 stimulation (Gorden *et al.,* 2005).

2.3.3. Cytokine response in myeloid DCs

The mDC subset is the major IL-12 producer. Myeloid DCs produce large amounts of TNF-α and lesser amounts of IL-6 and IL-12 in response to PG (TLR2/6) (Kadowaki *et al.,* 2001; Zarember & Godowski 2002). This subset of DCs also produces TNF-α, IL-12 and IL-6 in response to LPS (TLR4) (Jarrossay *et al.,* 2001). Myeloid DC also respond to TLR8, by production of IL-12 and TNF-α (Gorden *et al.,* 2005).

2.3.4. Cytokine response in plasmacytoid DCs

Plasmacytoid DCs are the primary type I IFN-producing cells during viral infection in response to TLR7 stimulation. Plasmacytoid DCs are able to prime viral-specific primary and secondary CD4+ and CD8+ T-lymphocyte immune responses *in vitro* and *in vivo* (Kadowaki *et al.,* 2001; Barchet *et al.,* 2005) . In addition to IFNs, TLR-activated pDCs produce high levels of IL-6 (Kadowaki *et al.,* 2001; Barchet *et al.,* 2005; Cao & Liu 2007; Koyama *et al.,* 2008) and stimulation with TLR7 and TLR9 agonists induced TNF-α production in pDCs (Gorden *et al*., 2005).

2.4. Neonatal TLR-mediated innate immunity

2.4.1. Infectious morbidity in neonates and young infants

Infections are the major cause of neonatal and infants morbidity and mortality. Presently, 99% of the 4 million neonatal and young infant morbidity and mortality cases occur in developing countries. Although the exact cause of neonatal deaths in developing countries is rarely known, estimates suggest that pneumonia and diarrhoea are the most common causes of neonatal and infant morbidity (Bryce 2005; WHO Health Statistics 2010). In developing countries, neonates and infants are particularly susceptible to infectious agents, such as *Staphylococcus aureus, Klebsiella pneumoniae*, *Streptococcus pneumoniae* and *Streptococcus pyogenes* (WHO Young infant group 1999), as well as group B streptococci,

Escherichia coli, HSV, CMV, RSV, varicella-zoster virus (VZV), *Candida* species (Marodi, 2006), as well as *Mycobacterium tuberculosis* and *Plasmodium* species (Gold *et al*., 2007).

2.4.2. T_H-2 bias in neonates

The foetal immune system is heavily biased against T_H-1 cytokine production and toward immunosuppression by the production of anti-inflammatory cytokines. This skewing plays a key role in the dampening of maternal proinflammatory T_H-1-type alloimmune responses. Excessive proinflammatory cytokine production, including IFN-γ and TNF, at the maternal–foetal interface is detrimental to the placental integrity and is the major cause of preterm delivery and foetal loss (Wegmann *et al*., 1993; Marodi 2006b; Levy 2007; Philbin & Levy 2010). Thus, the maternal-foetal interface secretes T_H-2 cytokines, such as IL-4, IL-5, IL-6 and IL-10, to divert the maternal immune response away from damaging T_H-l-mediated cellular immune responses (Wegmann *et al.,* 1993; Marodi 2006b; Levy 2007).

A strong bias towards T_H-2 polarisation in newborns is largely derived from the placental tissue by the production of TGF-β, prostaglandin E_2 and progesterone, as well as the endogenous T_H-2 derived cytokines (Morein *et al.,* 2007). This skewing has been associated with the increased susceptibility to infections in neonates. Cytotoxic T-lymphocyte responses are not primed in the neonate; furthermore, the T-lymphocyte responses to intracellular pathogens (with the exception of BCG) and T-lymphocyte-based vaccines responses are impaired in neonates (Siegrist *et al.,* 2001). However, antibody production in infancy as well as passive immunity acquired from the mother provides a measure of protective immunity for the neonate in the first few months of life (M'Rabet *et al*., 2008).

2.4.3. TLR-mediated cytokine responses in early life.

Innate immunity plays an important role in the induction of inflammatory response, clearance of infection and priming of adaptive immune responses. Innate immune cells are critical for early host defence and for the establishment of adaptive immunity. TLRs play a crucial role in innate immunity as they are essential for the recognition of microbial pathogens and trigger responses important to both inflammation and the instruction of adaptive immunity (Marodi 2006; Levy 2007b). Given the importance of the TLR system in host defence, several studies have documented newborn responses in comparison to adults.

2.4.3.1. TLR responses to bacterial ligand stimulation

In response to TLR2/1 and TLR2/6 stimulation, several studies observed a significantly lower production of TNF-α and IL-12p70 (Levy *et al.,* 2004; Levy *et al.,* 2006a) in neonatal monocytes and mDCs in comparison to adults. In response to TLR4 stimulation, neonates show a similar trend of cytokine impairment. LPS-mediated (TLR4) TNF-α and IL-12p70 production (Levy *et al.,* 2004; Belderbos *et al.,* 2009b) in neonates remain impaired at least up to one month of age (Belderbos *et al.,* 2009b). Soluble factors have been associated with the impairment of the TLR-mediated cytokine responses seen in neonates. Adenosine, a plasma factor present in high amounts in neonatal plasma, has been shown to increase intracellular levels of cyclic adenosine monophosphate (cAMP), thereby inhibiting TLR-mediated TNF-α production, and thereby inhibiting inflammatory response (Levy *et al.,* 2006a).

In contrast, neonates produce large amounts of IL-6 and IL-10 in response to TLR2 and TLR4. This high IL-6/TNF-α ratio and increased IL-10 production reflects a distinct polarisation of the TLR-mediated response, illustrating the T_H-2-skewing in neonates (Levy *et al.,* 2004; Angelone *et al.,* 2006). Along with increased IL-6 production, neonates produce higher levels of IL-23 than adults. The robust production of IL-6 and IL-23 induces the production of IL-17, which initiates the differentiation of naïve T_H-0 T-lymphocytes to the T_H-17 lineage. The T_H-17 subset is responsible for elimination of bacterial and fungal infections such as *Candida albicans*, *Mycobacterium tuberculosis, Klebsiella pneumoniae* and *Salmonella typhimurium* (Gold *et al.,* 2007; Levy 2007). The induction of this pathway suggests the innate immune response to TLR stimulation in neonates favours the T_H17 rather than T_H1 CD4 T-lymphocyte development.

2.4.3.2. TLR responses to viral ligand stimulation

Neonatal cord blood produces equal amounts of TNF-α in response to TLR7/8 (Levy *et al.,* 2004) and decreased amounts of IL-12p40 and IL-12p70 in response to TLR3 stimulation when compared to adults (De Wit *et al*., 2003; Belderbos *et al*., 2009b). This deficiency in IL-12p70 has been attributed to the defective production of the IL-12p35and IL-12p40 subunits, induced by the increased IL-10 and IL-6 production (Belderbos *et al.,* 2009b; Renneson *et al.,* 2009). In addition, as mDCs are the most effective IL-12 producers, the defective IL-12p70 production might be due to the immaturity of this cell type (Renneson *et*

al., 2009). However, neonatal mononuclear cells show comparable production of TNF-α (Levy *et al.,* 2004 and Levy *et al.,* 2006b) and IL-12 in response to TLR7/8 stimulation to adults (Levy *et al.,* 2006b). The mechanisms and implications for the robust response to TLR7/8 in the perinatal period are currently not understood.

The production of type I IFNs is a key feature of the host defence against invading intracellular and viral pathogens. Defective type I IFN production by neonatal pDCs is a significant feature seen in response to TLR stimulation. In response to human CMV and HSV infection, neonatal mDCs and pDCs produce lower amounts of type I IFNs in comparison to adults (Renneson *et al.,* 2009). Of note, cord blood pDCs have a decreased production of type I IFNs in response to stimulation with TLR7/8 (Danis *et al.,* 2008) and TLR9 (De Wit *et al*., 2004). These type I IFN responses in neonatal have been shown to mature rapidly in the first month of life to adult levels (Belderbos *et al.,* 2009b).

The inability of neonates to produce T_H-1 type cytokines IL-12p70 or IFN-α to TLR stimulation severely hampers the efficacy of cell-mediated immunity to HCMV, HSV and *Mycobacterium tuberculosis*. The decreased type I IFN production in pDCs is most likely due to deficient IRF-7 translocation to the nucleus, diminishing their ability to elicit antiviral responses (Danis *et al.,* 2008). Notably, the induced production of IL-6 and IL-10 in response to both agonists and infection favours the T_H-17 polarisation and immunosuppression and is essential in the induction of B-lymphocyte and anti-inflammatory responses in neonates (Angelone *et al.,* 2006; Kollmann *et al*., 2009).

Thus, neonates' innate immune capabilities are functionally different when compared to adult responses. Neonatal TLR-mediated inflammatory responses are specifically skewed towards T_H-17 and T_H-2 type immunity, thus resulting in an increased of risk of severe viral and intracellular pathogen infections, while offering protection against certain bacterial infections.

2.5. Methods of cytokine detection

Several methods have been developed for the detection of cytokine expression in response to stimulation of the innate immune cells. The enzyme-linked immunosorbent assay (ELISA) is employed for the analysis of secreted cytokine in fluids and cell supernatants, whereas

reverse transcriptase polymerase chain reaction (RT-PCR) is utilised for the semi-quantitative measurement of inducible mRNA production (Pala *et al*., 2000). While both these methods are suited for high-throughput analysis, it does not allow analysis of cell-specific cytokine production.

Enzyme-linked immunospot (ELISPOT) assays, intracellular cytokine staining (ICS) methods and limiting dilution analysis (LDA) are frequently utilised for the analysis of cell-specific cytokine production (Pala *et al*., 2000). However, these methods only allow the detection of one analyte at a time and are therefore not suitable for high- throughput analysis. The recent introduction of multiparameter analysis in flow cytometry allows for the measurement of multiple parameters in a single sample, thus providing a more comprehensive description of cellular responses.

2.5.1. Multiparameter flow cytometry

Fluorescence-activated flow cytometry is one of the leading technologies routinely used in immunology. Flow cytometry was first introduced for the phenotypic measurement of cells. This has been expanded to measure the functionality of cells through cytokine production and cytotoxicity or apoptosis on a cellular level (De Rosa *et al.,* 2003). In fact, recent technical advances allows for the simultaneous measurement of a multitude of parameters on individual cells (Chattopadhyay *et al*., 2008). This technique is known as multiparameter flow cytometry.

Multiparameter flow cytometry is defined as the use of five or more fluorescent markers to identify multiple characteristics of cells in a single sample (Perfetto *et al.,* 2006; Nomura *et al.,* 2008). This allows for a more comprehensive description of responsive cell subsets. This method has been applied to several applications, such as the identification of cellular subsets, characterisation of rare subsets and the functional characterisation of cell types (De Rosa *et al.,* 2003; Nomura *et al.,* 2008).

Multiparameter flow cytometry has several distinct advantages. The use of more colours can improve the accuracy of measurements in rare cell types and exclude debris and unwanted cell populations from analysis by the use of a "dump channel". In addition, this technique allows for improved detection of low-frequency cell populations and shared markers between

the cells of interest (De Rosa *et al.,* 2003; Perfetto *et al.,* 2004). More importantly, the technology can identify cells with complex phenotypes, such as in haematological malignancies, or cells responding to vaccines or cells from diseased states (Chattopadhyay *et al.,* 2008).

The most noteworthy disadvantage of multiparameter flow cytometry is that it requires extensive preparation and setup. The selection of markers for reagent panels for immunophenotyping needs to remain unaltered under three conditions: *in vitro* stimulation, viral infection and cryopreservation. The most suitable fluorochrome-labelled antibodies for detection need to be selected based on the background staining of and spectral overlap between the chosen fluorescent dyes, which if minimal, can be eliminated by compensation (Perfetto *et al.,* 2006; Nomura *et al.,* 2008; Chattopadhyay *et al.,* 2008).

Furthermore, accurate cytometer setup and data collection, as well as accurate data analysis of multiple combinations of positive and negative markers are essential components in multiparameter flow cytometry to obtain reliable data (Nomura *et al.,* 2008; Perfetto *et al.,* 2006; Chattopadhyay *et al.,* 2008). Once optimised, this technology allows high throughput analysis of specific cell markers and responses. One of the most important applications for multiparameter flow cytometry is the detection of multiple parameters using intracellular cytokine staining (Chattopadhyay *et al.,* 2008).

2.5.2. Intracellular cytokine staining (ICS)

ICS is a technique initially introduced for immuno-staining of tissue sections, but was adapted for detection of cytokines and markers in permeabilised and fixed cells in suspension (Arora *et al.,* 2002). Flow cytometric analysis of the intracellular cytokine (ICC) stained cells allows characterisation of a large number of cells, thus displaying the heterogeneity of the various cell populations. The advantage of ICS over existing techniques, such as ELISA and ELISPOT, is that multicolour staining in ICC analysis allows for characterisation of various cell subsets and sub-populations based on their cytokine profile, as well as their expression of cell surface markers (Maino *et al.,* 1998; Arora *et al.,* 2002; Nomura *et al.,* 2008).

Detection of cytokine positive cells is achieved by the inhibition of cytokine secretion by Golgi blockers, fixation and permeabilisation of cells of interest. These cells are then stained

for direct detection of intracellular cytokine expression with fluorochrome-conjugated antibodies and analysed using multiparameter flow cytometry (Arora *et al.,* 2002; Lamoreaux *et al*., 2006).

2.5.2.1. Application of intracellular cytokine staining

The use of ICS in multiparameter flow cytometry was first described in HIV-1 infection, where progressive disease was correlated with decreased IL-2 or IL-2 + IFN-γ CD4+ expressing T-lymphocytes. These studies employed a nine-plus colour panel, which showed that increased cytokine polyfunctionality in cells strongly correlated with reduced disease burden. These studies also revealed that polyfunctional cells express higher levels of IFN-γ per cell than cells that produce only one cytokine (Chattopadhyay *et al.,* 2008). Several other studies have also investigated the polyfunctional cytokine response to CMV in HIV infected patients (Maino *et al.,* 1998).

2.5.2.2. Applications for ICS in TLR stimulation

In innate TLR-mediated immune analysis, previously assays were limited in either the analysing the percentage of innate cells by flow cytometry (Jarrossay *et al*., 2001) or cytokine production in response to TLR stimulation subsets (Deering and Orange, 2006). Ida *et al* (2006) demonstrated a method of intracellular detection of TNF-α and IFN-α production in response to TLR-mediated DC stimulation. Although this assay was able to detect TLR-mediated response in using 4-colour flow cytometry, its disadvantage was that only a limited response can be detected at one time.

Recently, Jansen et al (2008) described a new method for detection of intracellular cytokine production in innate immune cells in response to TLR stimulation. This method of cytokine stimulation and detection was employed in this study. This intracellular cytokine stimulation method has numerous advantages: it has been optimised for use of peripheral blood mononuclear cells as well as whole blood allowing the comparison of responses in the presence and absence of soluble factors. This assay is suitable for cryopreservation and shipment on dry ice after freezing, eliminating the need for immediate analysis.

This multiparameter flow cytometric approach allows identification of the pattern of the cytokine response to TLR stimulation at the single cell level (monocytes, myeloid dendritic

cells, plasmacytoid dendritic cells). More importantly, this assay was employed in this study, as it requires minimal blood volumes, such as those obtainable in a young infant cohort. It is also easily adaptable to include coupling to other multiplexed assays, such as cytokine bead arrays, without increasing the necessary sample volume. Thus, this multiparameter flow cytometric approach is ideal for robust screening of various TLR responses and perfectly suited for use in larger multicentre infant cohorts.

Chapter Three

Materials and Methods

3.1. Ethical approval

Ethical approval was obtained from the Stellenbosch Committee for Human Research for longitudinal follow-up of an infant cohort and analysis of an adult control group (Addendum A). Project number N08/10/289 was assigned to the study. The study was conducted according to the Declarations of Helsinki, as well as the guidelines stated by the Medical Research Council (MRC) and Institute of Child Health (ICH).

3.2. Study design

This study is described as a prospective comparative longitudinal study. It forms part of a multidisciplinary, international, collaborative pilot study aimed at investigating the innate immune responses in HIV-Exposed Uninfected (HEU) and HIV unexposed (UE) neonates. The working hypothesis of the larger pilot study is that transient innate immune abnormalties are present in the HEU infants. This research project is a sub-study of the larger pilot study aimed at describing the ontogeny of these innate immune responses in the UE infant population only in order to establish a baseline for normal responses in South African infants.

3.3. Participant recruitment

Infants born to HIV-negative mothers were recruited postnatally at the Tygerberg Academic Hospital, Western Cape from March 2009 to June 2009. A total of 29 UE infants were enrolled into the study after signed informed consent from the mother, of which one infant was subsequently excluded from the study and the infant-mother pair was withdrawn from the study. A list of maternal exclusion criteria and infant inclusion criteria was employed for the recruitment of mother-infant pairs that were eligible for enrolment and participation in the study.

***Maternal exclusion criteria*:**

- Active tuberculosis (TB) infection or currently on TB treatment
- Pre-eclampsia
- Hypertension with protein urinary loss of more than 300mg/dL
- Perinatal pyrexia of more than 38°C or prolonged rupture of membranes

- Maternal haemoglobin of less than 8g/dL at time of delivery
- Syphilis with treatment completion of less than 4 weeks

Infant inclusion criteria

- Birth weight greater than 2kgs
- Gestational age (GA) greater than 37 weeks
- No congenital abnormalities

The study objectives, procedures and information on the consent forms were carefully explained to each mother in her preferred language by the study coordinator or trained counsellors at the Children's Clinical Research Unit (KIDCRU), Tygerberg Hospital (copy presented in Addendum B). The consent forms were signed by the mothers before enrolment of their infants into the study. Mothers were informed of their right to withdraw their consent and exclude their infants at any point in the study. All information regarding the participants was handled in a confidential manner. To ensure anonymity of participants, each patient was assigned a subject study number during recruitment and all data were linked to the assigned subject number.

In addition, ten clinically healthy adult controls were recruited, with informed consent, as a separate group for comparative analysis at the KIDCRU facilities and Division of Medical Virology, Tygerberg Hospital, from October to November 2009. A subject number was assigned to each adult control to ensure anonymity and all the data was linked to the assigned subject number. The cross-sectional group of healthy adults served as a standard of comparison for the maturation of the TLR-mediated innate cytokine response in the infants at the different time points. This allowed for the determination of the age at which the infant's response is similar to the adult group. The decision not to include the infant mothers as a comparative group was based on their hormonal changes post-partum, which would not be at a stable baseline level. As no published data exists on the TLR-mediated innate immune responses of mothers around birth, we speculated that the hormonal responses could be a confounding factor in the analysis of the *in vitro* TLR-mediated cytokine responses, thus making the mothers an unsuitable group of controls.

3.4. Clinical evaluation and follow-up of infant cohort

Mothers of enrolled babies were referred to KIDCRU when the infants were 2 weeks, 6 weeks, 12 weeks, 6 months and 12 months of age for clinical assessment. Medical practitioners experienced in Paediatric Medicine examined the infants at each visit. During each visit, all infections, socio-economic factors and nutritional information was recorded by the medical practitioner or recruitment nurse. In addition, demographic information, vaccination history, medical history, growth parameters, feeding methods, episodes of illness and medication used was recorded for the all the infant participants (Addendum C, D and E).

3.5. Sample collection

3.5.1. Preparation of sodium heparin syringes

The sodium heparin syringes were prepared under sterile conditions using a Class II Biosafety Cabinet in a Biosafety Level 3 (BSL3) facility at the Division of Medical Virology, NHLS, Tygerberg Hospital. A sterile 23 gage needle (BD Biosciences, USA) was utilised to prime sterile 5ml syringes (Neomedic, UK) with 10 000 USP units/ml sodium heparin (Pharmaceutical Partners of Canada, Canada). Approximately 250µl sodium heparin was transferred from the sodium heparin vial to the 5ml syringe. The syringes were then sealed with a syringe cap (BD Biosciences, USA), taken to the KIDCRU facilities, and kept at room temperature until blood collection. Prepared syringes older than 24 hours were not utilised for blood collection.

3.5.2. Blood collection

A total of 5ml blood was obtained from the infants at each visit. After the application of EMLA anaesthetic cream, a study nurse performed venepuncture via peripheral vein in the cubital fossa or the external jugular vein in the neck. For routine analysis of full blood count and differential blood count, CD4/CD8 analysis and HIV testing, 500µl of whole blood was collected into an EDTA paediatric tube (BD Biosciences, USA). For the immune analysis, 4.5ml of whole blood was collected into the sodium heparin-primed syringe. The blood was stored at room temperature until analysis or *in vitro* stimulation. If the blood specimens were clotted, the patient was recalled for a follow-up visit to obtain another blood sample.

3.6. Methodology for routine analysis

3.6.1. Full blood count and differential blood count analysis

At each visit, full and differential blood count analysis was performed to avoid blood draw from severely anaemic infants. For analysis, the remaining 350µl EDTA blood was analysed on the ADVIA 2120 Haematology System® (Siemens Healthcare Diagnostics, USA) by a qualified technologist at the Division of Haematology, NHLS, Tygerberg Hospital.

Medical practitioners employed a grade toxicity table (Division of AIDS Toxicity Grading Table for Severity of Paediatric Adverse Events, September 1993) to assess whether a subsequent blood draw could be performed on the infant. If the infant's haemoglobin level was Grade 1 category on the toxicity scale, a maximum of 2.5ml blood was collected from the infant. If the patient's haemoglobin level was in the Grade 2 category, no blood draw was performed at the visit and iron supplementation was administered to the infant. The patient was then rescheduled for a later date.

3.6.2. HIV-1 dried blood spot (DBS) PCR

A DBS HIV-1 PCR was performed on the EDTA blood sample at 2, 6 and 12 weeks of age to ensure the HIV-negative status of the recruited infants. The AMPLICOR HIV-1 DNA Test kit version 1.5 (Roche Diagnostics, USA) was employed for HIV testing at the 2 and six week follow-up visits and the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 was employed at the 12 week follow-up visit. The assays have four major processes: (1) sample preparation, (2) PCR amplification of target DNA using HIV-1 specific complimentary primers, (3) hybridisation of the amplified product to oligonucleotide probes specific to the target DNA and (4) the detection of probe-bound amplified products by colorimetric determination.

The DBS was prepared in a Class II biosafety cabinet in the BSL3 facility at the Division of Medical Virology, NHLS, Tygerberg Hospital within 6 hours after blood collection. DBS were prepared by placing two blood spots of 75µl EDTA blood onto a specimen collection card (Munktell Filter AB, Sweden), and dried overnight in the biosafety cabinet. The qualitative DBS HIV-1 PCR was performed by a qualified molecular technologist. The amplification process and temperature is depicted in Table 3.1. Results were read on the Anthos Plate Reader (Biochrom Ltd, UK) at 450nm, and reported as positive or negative. For

inconclusive or positive results, the sample was repeated to avoid reporting of false positive results.

Table 3.1 Amplification process for HIV-1 detection

Process	Temperature	Time
5 cycles		
Denaturation of DNA	95°C	10 seconds
Annealing of target primers	52°C	10 seconds
Extension of PCR product	72°C	10 seconds
35 cycles		
Denaturation of DNA	90°C	10 seconds
Annealing of target primers	52°C	10 seconds
Extension of PCR product	72°C	10 seconds
Hold	72°C	15 minutes

3.6.3. HIV-1 mini-pool ELISA for adult controls

To confirm the HIV-negative status of the 10 adult controls, an HIV-1 mini-pool ELISA was performed on stored plasma samples of blood collected for the innate immune analysis. The AxSYM system and AxSYM HIV Ag/Ab Combo reagent kit (Abbot, Germany) was employed for the mini-pool ELISA assay. This assay is a microparticle enzyme immunoassay for the simultaneous qualitative detection of antibodies to HIV-1and HIV-2 transmembrane protein and HIV p24 antigen in human serum or plasma. The presence or absence of antibodies or p24 antigen in the sample is determined by comparing the rate of formation of fluorescent product to the cut-off rate, previously calculated by the AxSYM HIV Ag/Ab Combo Index Calibrator. If the rate of formation is greater than or equal to the cut-off rate, the sample is considered reactive.

The AxSYM HIV Ag/Ab ELISA was performed by a qualified medical technologist. For the adult control samples, a 1:10 dilution was prepared by mixing 50µl plasma of each adult participant sample into a 2ml tube. The samples were then placed into the AxSYM system for HIV testing. Results were reported as positive or negative.

3.7. Methodology for innate immune stimulation

3.7.1. TLR ligand stimulation

For this study, the TLR stimulation was performed by the investigator at the Division of Medical Virology, NHLS, Tygerberg Hospital. Endotoxin-free reagents and materials were utilised for blood sampling, processing and stimulation. Endotoxin (LPS) is a potent immune modulator and contamination can result in overstimulation of the cells of interest. All the preparation and *in vitro* stimulation was performed under sterile conditions using a Class II Biosafety Cabinet in a BSL3 laboratory.

3.7.1.1. Preparation of TLR ligands

The TLR ligands employed were specific for the appropriate TLR stimulated and lacked activity for all the other TLRs. LPS was used for the stimulation of TLR4 (Invivogen, USA) and R-848 was employed for the stimulation of TLR7/8 (Invivogen, USA). The concentrations and volumes for each TLR ligand used in the preparation of the TLR stimulation plates are described in Table 3.2.

Table 3.2 TLR ligand panel for in vitro stimulation of innate immune cells.

TLR	Ligand	Stock Concentration	Concentration per well	Volume per well
TLR4	LPS	200ng/ml	10ng/ml	10µl
TLR7/8	R848	200µM	10µM	10µl

Before dilution, the LPS ligand was sonicated for 10 minutes in a Branson B200 sonicator (Branson Untrasonic Corporation, USA) in order to disrupt lipids aggregated on the wall of tubes and distribute them evenly within the solution. Both ligands were vortexed at high speed for 30 seconds and diluted from the stock concentration with sterile endotoxin-free RPMI 1640 media (Lonza, Biowhittaker, USA) into sterile endotoxin-free 1.8ml cryovials (Corning, USA). Endotoxin-free filter tips (Axygen, USA) were employed for this procedure.

3.7.1.2. Preparation of 6-hour ICC TLR stimulation plates

Sterile endotoxin-free 96-well round bottom polystyrene plates (Corning, USA) were used for the preparation of TLR stimulation plates. A final concentration of 2mg/ml Brefeldin A (BFA) (Sigma-Aldrich, Germany) was prepared from stock concentration of 10mg/ml using

endotoxin-free, RNase- and DNase-free ethanol. BFA acts as a Golgi-secretion inhibitor. For whole blood stimulation, 20mM EDTA (Fisher-Scientific, USA) was prepared with deionised water (Lonza, Biowhittaker, USA) in order to stop cell stimulation. A 1:10 dilution of BD FACSLysing Solution (BD Biosciences, USA) was also prepared with deionised water (Lonza, Biowhittaker, USA) in order to lyse red blood cells. The FACSLysing solution also acts as a fixative to aid in cryopreservation of cells.

The TLR stimulation plates were prepared as previously described by Jansen *et al* (2008) and Kollmann *et al* (2009). The LPS ligand was sonicated for 10 minutes and both LPS and R-848 ligands were vortexed at high speed for 30 seconds before aliquoting into the 96-well stimulation plate. Ten microlitres of RPMI 1640 media was aliquoted into the first well as a control and a measure of basal stimulation. Ten microlitres of LPS and R-848 ligands was then aliquoted into the relevant demarcated wells. One microlitre of BFA was also added into each of the wells. The stimulation plates were sealed with aluminium plate sealers (Nunc, Denmark) and enclosed in Parafilm (Pechiney Plastic Packaging Company, USA). The plates were stored at -80^{o}C until whole blood *in vitro* stimulation.

3.7.2. *In vitro* stimulation

Innate immune analysis was performed at the 2-week, 6-week, 6-month and 12-month time points. Blood processing and *in vitro* stimulation were performed under sterile conditions in a BSL3 facility at the Division of Medical Virology, NHLS, Tygerberg Hospital. As described earlier, whole blood was collected directly into a sodium heparin-primed syringe and stored at room temperature. The blood was processed within 2 hours of venepuncture. The pre-prepared TLR stimulation plates were thawed for 20-30 minutes in an incubator at 37˚C in 5% CO_2 to warm up the plates to prior to use. A sterile 15ml conical tube (BD Biosciences, USA) was labelled with the patient study number and 1ml RPMI 1640 media was pipetted into the tube. The tube was incubated at 37˚C in 5% CO_2 for 20-30 minutes to warm up the media prior to use.

A second sterile 15ml conical tube was labelled with the patient study number. The 4.5ml heparinised blood was transferred from the syringe to the 15ml conical tube and the blood was examined for clots by carefully pipetting the blood up and down using a 1000µl pipette. For *in vitro* stimulation, 400µl heated RPMI media was transferred to a third 15ml conical

tube labelled with a patient study number and 400µl heparinised whole blood was added to the media in a 1:1 ratio and mixed thoroughly. Two hundred microlitres of the blood-RPMI mixture was aliquoted into the stimulation wells in the 96-well plates and mixed thoroughly. The stimulation plate was then incubated at 37°C in 5% CO_2.

After 6 hours of incubation, 25ul of 20mM EDTA was added to each well at a final concentration of 2mM per well and the plate was incubated for a further 10 minutes at 37°C in 5% CO_2. Four sterile 2ml screw-cap microtubes (Quality Scientific Plastics, USA) were labelled with the corresponding stimulation well numbers and 1400µl of 1:10 BD FACSLysing solution was added to each tube. After incubation, the stimulated whole blood mixture in each well was transferred into each corresponding labelled tube and resuspended in the FACSLysing solution. The tubes were incubated for 10 minutes at room temperature and stored at -80^{o}C until the samples were shipped to the Child & Family Research Institute, University of British Columbia, Vancouver, Canada for sample processing and analysis.

3.8. Methodology for flow cytometric analysis

The flow cytometric training and subsequent analysis of the TLR-stimulated samples was performed by the investigatior at the Child and Family Research Institute, UBC, as part of the international collaborative study. All samples were permeabilised, stained with antibodies from the established 8-colour flow cytometric panel (Jansen *et al*., 2008; Blimkie *et al*., 2011) and samples were run on the BD FACSAria II flow cytometer (BD Biosciences).

3.8.1. Description of instrumentation

The BD FACSAria II flow cytometer (BD Biosciences, USA) was employed for the analysis of samples. This instrument is a high-speed fixed-alignment benchtop cell sorter that can be operated at varied pressures to acquire up to 70,000 events per second. It enables multicolour analysis of up to 13 fluorescent markers and two scatter parameters, forward scatter (FSC) and side scatter (SSC) at a time. It is equipped with blue (488-nm), red (633-nm) and violet (405-nm) solid state lasers for excitation of fluorochrome-conjugated antibodies. It is also equipped with two types of signal detectors; photomultiplier tubes (PMTs) and a photodiode detector. PMTs detect signals generated by SSC and all fluorescence channels whereas the photodiode signal generated by FSC.

The BD FACSAria II possesses both octagon and trigon detector arrays. The octagon detector array contains six PMTs that detect SSC and up to seven fluorescence signals excited by the blue laser. The trigon arrays detect fluorescence signals excited by the red and violet lasers, respectively. Each trigon contains two PMTs that detect up to three fluorescence channels. The PMTs within each array convert light into electrical pulses that can be processed by the electronics system and converted into data. Acquisition and data analysis, as well as most BD FACSAria II cytometer functions, are performed and controlled by BD FACSDiva software (BD FACSAria II User Guide).

For the purpose of our study, Table 3.3 illustrates the light path, filters, detectors and voltage settings for the BD FACSAria II employed in our study. The detectors and filters are listed in the order that the emitted light hits them, with the exception of FSC, as it is measured from light that passes through the cell. All other detectors detect light scattered 90º.

Table 3.3 BD FACSAria II set up for the flow cytometric analysis

Laser	Detector	Filter 1 (LP)	Filter 2 (BP)	Parameter detected	Detector voltage	Amplification Type
Blue Laser (488 nm)	SSC (PD)	na	488/10 BP	SSC-A	440	LINEAR
	488 A (PMT)	735 LP	780/60 BP	PE-Cy7-A	605	LOG
	488 B (PMT)	655 LP	695/40 BP	PerCP-Cy5.5-A	585	LOG
	488 C (PMT)	595 LP	610/20 BP	PE-TexRed	na	
	488 D (PMT)	556 LP	575/26 BP	PE	498	LOG
	488 E (PMT)	502 LP	530/30 BP	FITC	480	LOG
Violet Laser (407 nm)	407 A (PMT)	502 LP	530/30 BP	Alexa-430-A	na	
	407 B (PMT)	blank	450/40 BP	Pacific Blue-A	530	LOG
Red Laser (633 nm)	633 A (PMT)	755 LP	780/60 BP	APC-Cy7-A	605	LOG
	633 B (PMT)	685 LP	720/40 BP	Alexa700-A	515	LOG
	633 C (PMT)	blank	660/20 BP	APC-A	492	LOG

3.8.2. Optimisation of eight-colour flow cytometric panel

The development of 8-colour panel was previously described in Jansen *et al* (2008). The antibody conjugates, their clones as well as their respective titrations used in this study are illustrated in Table 3.4.

Table 3.4 Antibody staining panel for ICC

Fluorochrome	Marker measured	Clone	Supplier	Dilution	Volume per well
PerCP Cy5.5	HLA-DR	TU36	BD #custom	1:100	1µl
PE-Cy7	CD14	M5E2	eBioscience #25-0149	1:100	1µl
APC	CD11c	5HCL3	BD #340714	1:50	2µl
PE	CD123	6H6	eBioscience #12-1239	1:200	0.5µl
FITC	IFN-α	A11	Antigenix #MC100133	1:100	1µl
APC-Cy7	IL-6	AS12	BD #custom	1:100	1µl
eFluor450	IL-12p40/70	C8.6	eBioscience # 48-7139	1:400	0.25µl
AlexaFluor700	TNF-α	Mab11	BD #557996	1:100	1µl

Fluorescence spillover was analysed by use of fluorescence minus one (FMO) controls for each monoclonal antibody conjugate included in the 8–colour staining panel (Table 3.4). FMO controls were performed in order to assess the delineation of the true positive and populations for the regent of interest. This distinguishes the fluorescent cells expressing the marker of interest from cells that do not express the marker but detected as positive within that channel.

The FMO control was performed using R-848 stimulated and RPMI-stimulated (unstimulated) cell controls. The frozen control tubes were thawed in a water bath at 37˚C for 5-10 minutes and spun in a microcentrifuge at 600xg for 5 minutes at room temperature. After centrifugation, the supernatant was aspirated from each tube and the remaining pellets were resuspended in 200µl of 1:10 FACSPermeabilising Solution (BD Biosciences). The samples were incubated in the dark for 10 minutes at room temperature. After incubation, the cells were resuspended in 100µl PBSAN and centrifuged at 600xg for 5 minutes at room temperature to stop the permeabilisation process. The supernatant was discarded and the pellets were washed in 200µl PBSAN and centrifuged at 600xg for 5minutes. This process was repeated and the pellets were resuspended in 75µl PBSAN after centrifugation.

For the FMO staining of the cell control for each tested antibody, each tested antibody FMO was prepared by aliquoting the volumes of the antibodies as described in Table 3.4, with the

exception of the tested antibody and a total of 17.25µl PBSAN per well was added to the tube. The mastermix was then vortexed for 10-15 seconds and 25µl of the mixture was pipetted into each cell control well to make up a final well volume of 100µl per well.

The cell controls were then acquired on the BD FACSAria II and analysed for the percentage of spillover for each tested antibody. The compensation matrix was then adjusted to account for the spillover, when detected, and these adjusted settings were then employed for the analysis of the samples.

Table 3.5 Representative compensation matrix for the analysis of samples

	FITC	PE	PerCP-Cy5-5	PE-Cy7	APC	APC-Cy7	Pacific Blue	Alex 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-A	0.1415	0.1788	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	0.05744	-0.172		-0.2704
Alex 700	0.1859	0.208	1.44	0.9827	0.2883	29.7	0.662	

3.8.3. Permeabilisation and cell staining

All the reagents were prepared prior to use. For permeabilisation of samples, a 1:10 dilution of BD FACSPermeabilisation Solution (BD Biosciences, USA) was prepared with deionised water. The PBSAN washing and staining solution was prepared with 50ml of 5% bovine serum albumin (Invitrogen, USA) (5g/100ml in deionised water), 5ml of 10% sodium azide (10g/100ml in deionised water), and 50ml of 10X Dulbecco's phosphate-buffered saline (DPBS) (Gibco, USA) in 395ml deionised water. The solution was stored at 4ºC until use. The paraformaldehyde solution (BD Biosciences, USA) used as a fixative, was prepared as a 1:10 dilution with DPBS. The following antibodies were employed for the staining process:

HLA-DR PerCp-Cy5.5 (BD Biosciences, USA);

CD14 PE-Cy7 (BD Biosciences, USA);

CD11c APC (BD Biosciences, USA);

CD123 PE (eBiosciences, USA);

IFN-α FITC (Antigenix, USA);

IL-6 APC-Cy7 (BD Biosciences, USA);

IL-12p40/70 eFluor450 (eBiosciences, USA)
TNF-α AlexaFluor700 (BD Biosciences, USA)

For the compensation, Anti-Mouse Ig CompBeads were employed (BD Biosciences, USA). The dilutions were for each antibody is illustrated in Table 3.3. For the innate immune analysis, no live/dead discriminator was employed as the stimulated cells were fixed and frozen prior to flow cytometric analysis. At the time of the study no product for live/dead cell discrimination was available that would endure the fix-freeze-thaw process and if this marker was included during the staining process, all the cells would have been identified as dead cells.

For each set of experiments, an RPMI-stimulated negative control and an R848- stimulated positive control were prepared with the patient samples. For the preparation of the controls, whole blood was obtained from a clinically healthy adult donor. The *in vitro* stimulation for controls was similar to that of the patient samples. Ten patient samples along with one R-848 positive cell control and one unstimulated negative control were prepared for each run.

The frozen sample tubes were placed in a water bath at 37˚C for 5-10 minutes until thawed. When thawed, the samples were spun in a microcentrifuge at 600xg for 5 minutes at room temperature. A 96-well round bottom plate was labelled with the patient study numbers (rows) and the different TLR ligands (columns). After centrifugation, the supernatant was aspirated from each tube. The remaining pellets in each stimulation tube were resuspended in 200µl of the 1:10 FACSPermeabilising Solution and the patient samples was transferred into their respective rows in the 96-well round bottom plate. The plate was incubated in the dark for 10 minutes at room temperature. After incubation, the pellets in the wells were resuspended in 100µl PBSAN and centrifuged at 600xg for 5 minutes at room temperature to stop the permeabilisation process. The supernatant was discarded and the pellets were washed in 200µl PBSAN and centrifuged at 600xg for 5minutes. This process was repeated and the pellets were resuspended in 75µl PBSAN after centrifugation.
For the antibody staining process, a mastermix of the antibodies was prepared in the dark as the fluorochromes are light-sensitive. The volumes of each antibody were added to the mastermix as described in Table 3.3. A total of 17.25µl PBSAN per well was added to the mastermix. The mastermix was then vortexed for 10-15 seconds and 25µl of the antibody

mastermix was pipetted into each well to make up a final well volume of 100µl per well. The wells were mixed thoroughly by pipetting and the plate was incubated in the dark at room temperature for 45 minutes. After incubation, 100µl PBSAN was added to the wells and the plate was centrifuged at 600xg for 5 minutes at room temperature. The supernatant was discarded and the pellets were washed in 200µl PBSAN and centrifuged at 600xg for 5 minutes. This process was repeated and the pellets were resuspended in 100µl of 1:10 dilution paraformaldehyde. The samples were stored overnight at 4˚C. The samples were protected from light.

For compensation controls, single-stained bead controls were prepared for each antibody in the staining panel. A 96-well round bottom plate was labelled with the antibodies included in the staining panel and 92µl PBSAN was added to each well. The positive and negative anti-Mouse Ig CompBeads were vortexed at high speed for a minimum of 15 seconds. Three microlitres of the positive and negative anti-Mouse Ig CompBeads was added to each well. Two microlitres of each antibody was added to the demarcated wells and mixed thoroughly. The bead mix was incubated in the dark for a minimum of 20 minutes at room temperature. After incubation, 100µl PBSAN was added to the wells and the plate was centrifuged at 600xg for 5 minutes at room temperature and the supernatant was discarded. After centrifugation, the beads were washed in 200µl PBSAN and centrifuged at 600xg for 5 minutes. This process was repeated and the beads were resuspended in 100µl of 1:10 dilution paraformaldehyde. The single-stained bead controls were stored overnight at 4˚C and protected from light. The stained beads were protected from light.

3.8.4. Acquisition

Before acquisition, the sample tubes and beads were vortexed for 5 seconds to ensure that the cells and beads were well suspended. All samples were analysed on the BD FACSAria II flow cytometer, which employed BD FACSDiva™ software for data acquisition and analysis. The FSC and SSC gain was set up in dot plot using the unstained control. The positive R-848 control was employed to set the gain for each fluorescent channel. This enabled positioning of the high positive cells between the 3rd and 4th decade on the plot. The unstimulated negative cell control was used to position the negative cells within the 1st decade. After setup, the experimental samples, negative unstimulated and positive R-848 stimulated cell controls were acquired on the BD FACSAria II. For sample acquision, a

maximum of 500 000 and a minimum of 250 0000 gated events were collected for each sample tube. Thereafter, the single-stained bead control tubes were acquired. A maximum of 5000 bead events were acquired for each bead control.

3.8.5. FlowJo analysis

The FCS files for the samples were analysed using FlowJo (Tree Star, OR, USA). Before analysis, the compensation for each experiment was set using the single stained CompBeads sample FCS files. In each single-stained bead control histogram, the positive and negative gate was defined for that specific fluorescent channel and these were used to define the compensation matrix. The sample FCS files were compensated with the matrix. Further subset gating was applied to enable identification of the specific cellular subsets of interest and the production of intracellular cytokines for each specific cell population.

3.8.5.1. Identification of cell subsets

Figure 3.1 demonstrates the dot plots employed for the identification of monocytes, mDCs and pDCs. Back gating was performed on each population of interest to verify their position in relation to the FSC versus SSC axis (Figure 3.2 to 3.4). The unstimulated samples for each participant was used to apply the gating strategy, therefore each gating strategy was specifically applicable to each participant. The primary gating strategy (FSC versus SSC) was used to yield a live gate to include all cells and exclude debris and dead cells. Secondary gating was applied to identify monocytes and dendritic cells. Monocytes were identified based on their expression of MHCII+ and CD14+ surface markers. Dendritic cells were identified based on MHCII+ CD14- expression. Further gating was applied to dendritic cell gate to identify the mDC (CD11c+) and surface pDC (CD123+) subpopulations. Bulk gating was used to apply these gate coordinates to the rest of the samples for that specific participant. After bulk gating, the gates were checked and adjusted manually for each participant sample.

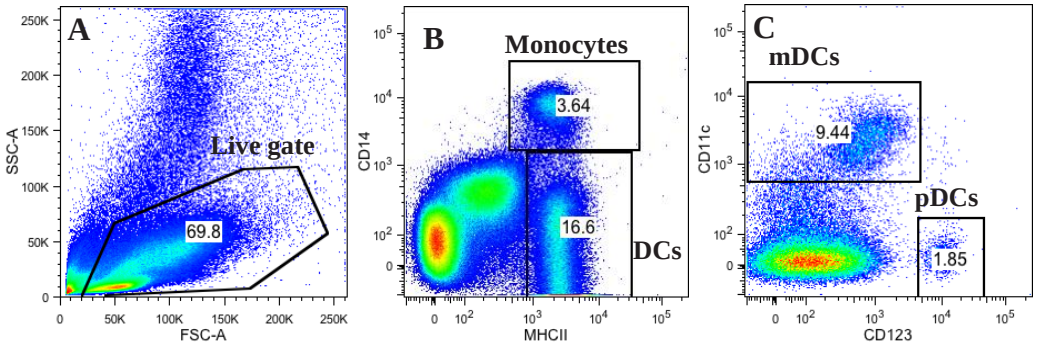

Figure 3.1 Example of identification of monocytes, mDCs and pDCs by flow cytometry. Dot plot A indicates the live gate, which included all cells. Dot plot B indicates the differentiation of monocytes (MHCII+CD14+) and DCs (MHCII+CD14-). Dot plot C indicates the CD11c and CD123 expression by DCs. Myeloid DCs were differentiated by expression of CD11c marker and pDCs were differentiated by expression of CD123 marker

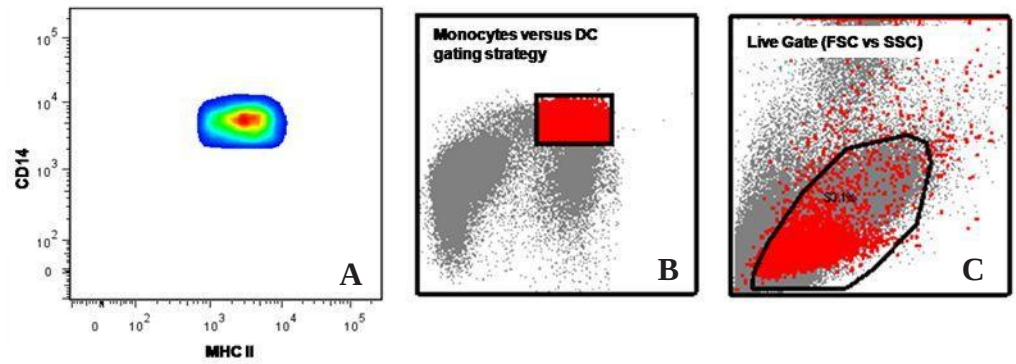

Figure 3.2 Example of backgating strategy for the monocyte population using FlowJo. Dot plot A represents the monocyte population in the secondary gate used to identify monocytes and DCs (CD14 vs MHCII). Dot plot B indicates the gated monocyte population relative to the monocyte versus DC gate. Dot plot C indicates the gated monocyte population in the live gate (FSC vs SSC).

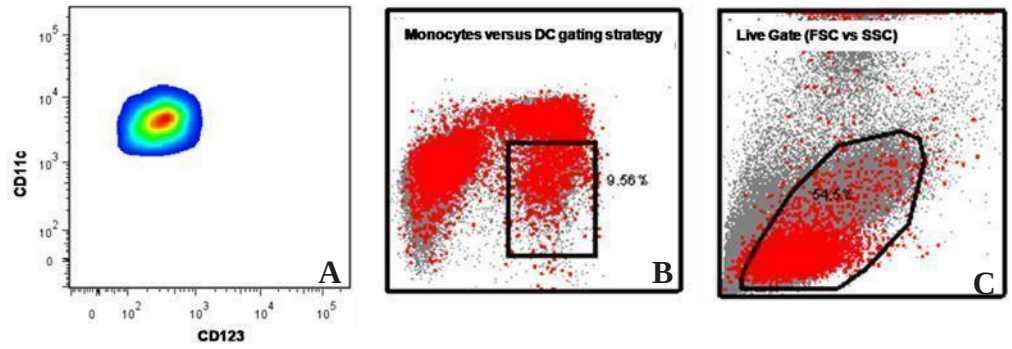

Figure 3.3 Example of backgating strategy for the mDC population using FlowJo. Dot plot A represents the mDC population in the tertiary gate used to identify mDCs and pDCs (CD11c vs CD123). Dot plot B indicates the gated mDC population relative to the monocyte versus DC gate. Dot plot C indicates the gated mDC population in the live gate (FSC vs SSC).

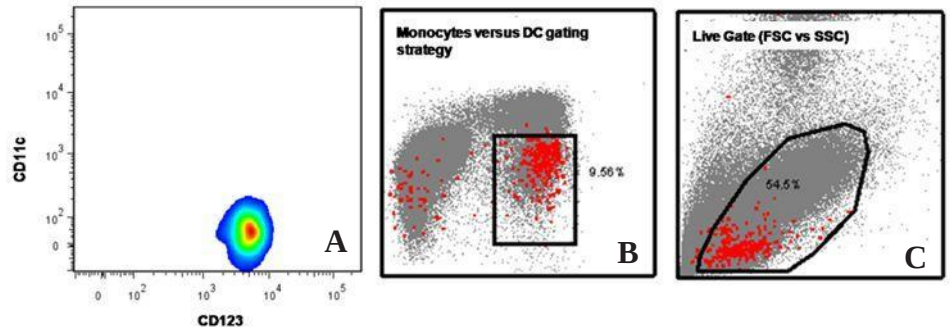

Figure 3.4 Example of backgating strategy for the pDC population using FlowJo. Dot plot A represents the pDC population in the tertiary gate used to identify mDCs and pDCs (CD11c vs CD123). Dot plot B indicates the gated pDC population relative to the monocyte versus DC gate. Dot plot C indicates the gated pDC population in the live gate (FSC vs SSC).

3.8.5.2. Evaluation of intracellular cytokine production

Monocytes, mDCs and pDCs were analysed for the intracellular production of TNF-α, IL-6, IL-12p40, and IFN-α in response to each TLR ligand. Figure 3 demonstrates the gating strategy for the identification of intracellular cytokine response profile in an unstimulated and R848 (TLR7/8) stimulated sample in monocytes, mDCs and pDCs.

Dot plots were set up for each of the cell types (monocytes, mDCs and pDCs), as described in section 3.8.5.1. The unstimulated sample for each participant was used to determine the negative break points for each cytokine combination for each cell population (e.g. TNF-α versus IL-6). The negative response was defined as where the unstimulated cell populations lie within the unstimulated sample gate. The coordinates of these break points for each of the cytokine combinations in each cell break points were applied to each cell type. A histogram plot was then chosen and the break points determined for the positive and negative dot plot populations were applied to the histogram for each of the cytokines for each cell type.

Boolean gating was employed in order to identify the single-, double-, triple-, and quadruple cytokine-expressing populations. Using the histograms created to depict the positive and negative cytokine populations, the positive cytokine-expressing cells of interest were grouped together (e.g. TNF-α and IL-6) and the AND function selected. This allows for the determination of the double positive TNF-α+IL-6+ population within that cell type. Bulk gating was then used to apply these gates to the stimulated samples for that cell population for that specific participant. This process was followed for each participant at each time point.

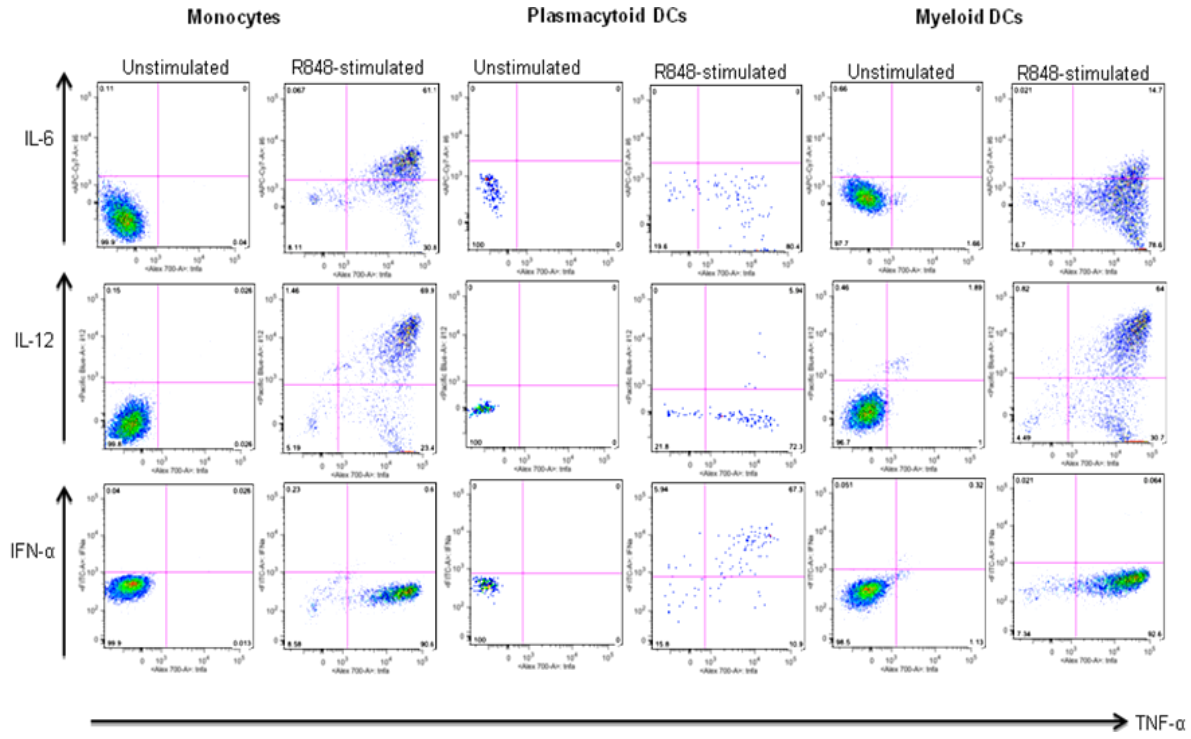

Figure 3.5 Example of intracellular cytokine production in monocytes, mDCs and pDCs of infants and adults. Monocytes, mDCs and pDCs were analysed for the intracellular production of IL-6, IL-12, TNF-α and IFN-α.

3.9. Data collection and statistical analysis

The whole blood samples were evaluated for cytokine production in response to TLR stimulation at the 2 weeks, 6 weeks, 6 months and 12 months of age. The analysed data from each participant at each time point was transferred to a Microsoft Excel spreadsheet (Microsoft, USA). For the determination of total cytokine responses in monocytes, mDCs and pDCs, the following calculations were performed in Microsoft Excel; for the total TNF-α production, the sum of TNF-α+, TNF-α+IL-6+, TNF-α+IL-12+ and TNF-α+IL-6+IL-12+ populations were determined. For the total IL-6 production, the sum of IL-6+, TNF-α+IL-6+, IL-6+IL-12+ and TNF-α+IL-6+IL-12+ populations were determined. For the total IL-12+ production, the sum of IL-12+, IL-6+IL-12+, TNF-α+IL-12+ and TNF-α+IL-6+IL-12+ populations were determined. For the total IFN-α production, the sum of IFN-α+, IFN-α+IL-6+, TNF-α+IFN-α+ and IFN-α+TNF-α+IL-6+ populations were determined. These values were analysed using statistical software.

For the determination of polyfunctional responses in monocytes, mDCs and pDCs, the following populations were analysed: the TNF-α+, IL-6+, IL-12+ and IFN-α+ single positive

populations were analysed for single-positive cytokine responses. The TNF-α+IL-6+, TNF-α+IL-12+, IL-6+IL-12+, IFN-α+IL-6+ and TNF-α+IFN-α+ double-positive populations were analysed for double-positive cytokine responses. The TNF-α+IL-6+IL-12+ and IFN-α+TNF-α+IL-6+ triple-positive populations were analysed for triple-positive cytokine responses.

For statistical analysis of the participant flow cytometric data, GraphPad Prism 5 (GraphPad Software Incorporated, USA) and Statistica 10 (StatSoft, USA) statistical software was employed. To analyse the statistical differences between the pairwise comparison of means between infant responses (at 2 weeks, 6 weeks, 6 months and 12 months) and adult responses, an analysis of variance (ANOVA) estimation method was employed, with type III sum of squares to account and compensate for unequal *n*. The same method, ANOVA with type III sum of squares, was employed to determine the statistical significance of the pairwise comparison of means in infant responses between each time point. This was performed with the Variance Estimation and Precision method in Statistica 10. Confidence intervals were set at 95%. The mean values with standard deviations as well as p-values were reported. Results were displayed graphically as follows; for the total cytokine responses, bar graphs were used, depicting the mean and standard deviation and for the poyfunctional responses, line graphs were used, depicting the mean and 95% confidence intervals. P-values of <0.05 were regarded as significant and p-values of <0.01 were regarded as highly significant.

Chapter Four

Results

4.1. Participant characteristics

4.1.1. Demographics

From the period of March 2009 to June 2009, 28 UE infants were included for innate immune analysis. Most of the participants lived in the residential areas surrounding Tygerberg Hospital, Cape Town. Ten clinically healthy adult controls were recruited as a separate group for comparative analysis during October and November 2009. The infants recruited into the study were predominantly of coloured or mixed ethnicity with a mean gestational age of 36±7.5 weeks (Table 4.1). The majority of the adults recruited into the control group were of coloured or mixed ethnicity and between the age of 23 and 54 years with a mean age of 37±10 years. All infants received their vaccinations at the scheduled timelines outlined in the extended programme of immunisation (EPI) implemented by the Department of Health, South Africa.

4.1.2. Feeding practices

In our study, all the infants were exclusively breastfed from birth up to a minimum of 12 weeks of life (Table 4.1). At the six months visit, we recorded that the infants were on a mixed diet consisting of either breast milk or formula and solids. The reason for this was that many of the mothers recruited on the study had already returned to employment around this time point. At 12 months, all the infants were on mixed feeding that consisted of formula and solids (data not shown).

4.1.3. Rate of attrition

The rate of attrition was documented throughout the duration of the study (Table 4.1). A substantial loss to follow-up of infants was noted during the course of study. At the two-week time point, one infant did not attend the visit, but re-entered the study at the 6-week visit. At the 6-week time point, two infants were lost to follow-up. The highest attrition rate was observed at the six-month follow up visits, as four infants were lost to follow-up. At the 12-month time point, a further two infants did not arrive for the follow-up visit.

Data was collected to ascertain the causes for the loss to follow-up in the infant group. The key reasons for the high attrition rate were: (1) refusal of further participation in the study for

unknown reasons (n=4) and (2) mothers resuming work responsibilities (n=3). One infant died during the course of the study due to adenovirus infection. No loss to follow-up was observed in the adult group, as no follow-up visits were required.

Table 4.1 General characteristics of participants. This table describes the distribution and number of participants recruited in each group.

Number of infant participants (%)	28 (100)
Gestational age in weeks (mean ± SD)	36 ± 7.5
Gender	
Male	15 (54)
Female	13 (46)
Ethnicity	
Black	8 (29)
Coloured/Mixed race	19 (68)
Caucasian	1 (3)
Feeding practices	
Breastfed at birth	28 (100)
Duration of exclusive breastfeeding	± 12 weeks
Introduction of solids/mixed feeding	± 6 months
Follow-up visits	
Two week time point	28 (100)
Six week time point	28 (100)
Six month time point	23 (82)
Twelve month time point	20 (71)
Number of adult participants (%)	10 (100)
Age in years (mean ± SD)	37.4 ± 10
Gender	
Male	2 (20)
Female	8 (80)
Ethnicity	
Black	2 (20)
Coloured/Mixed race	6 (60)
Caucasian	2 (20)

4.2. Comparison of cytokine responses in infants versus adults

Using the multiparameter flow cytometric approach, we assessed the total production of TNF-α, IL-6, IL-12/23p40, and IFN-α2 at the single-cell level at basal level (unstimulated control), as well as in response to LPS (TLR4) and R-848 (TLR7/8) stimulation in monocytes, mDCs and R-848 (TLR7/8) stimulation in pDCs. At each time point (2 weeks, 6 weeks, 6 months and 12 months); the infant responses were compared to the adult group for each cell type.

The monocytes and mDCs responded to TLR4 and TLR7/8 stimulation, whereas the pDC population responded to TLR7/8 stimulation. No response was observed to TLR4 stimulation in pDCs. We observed TNF-α, IL-6, and IL-12/23p40 production by monocytes and mDCs, but not IFN-α2 (data not shown). In pDCs, we observed the production of TNF-α and IFN-α2, but not IL-6 or IL-12/23p40 (data not shown). Thus, only the analysis for each of these cytokines will be discussed for each respective cell type. Data comparisons were defined as significant if $p<0.05$ and highly significant if $p<0.01$.

4.2.1. Monocytes

4.2.1.1. Total TNF-α response

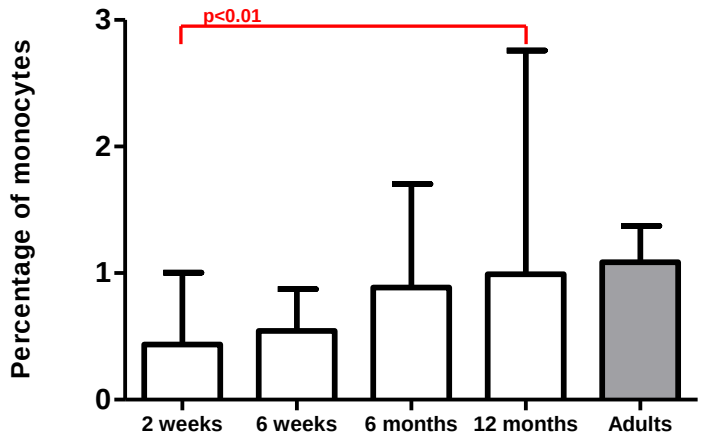

Figure 4.1 Percentage of TNF-α-producing monocytes at basal level. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

At basal level, no significant differences were observed in the percentage of TNF-α-producing monocytes between the adult group and infants groups when measured at two weeks, six weeks, six months and 12 months of age. The highest percentage of TNF-α-producing monocytes was observed at the 12 month time point and was comparable to the adult group.

In the comparison of responses over time, no significant differences in the percentage of TNF-α-producing monocytes were observed between any of the time points, with the exception of two weeks and 12 months. A significantly higher percentage of TNF-α-producing monocytes was observed at 12 months compared to the two-week time point ($p<0.05$).

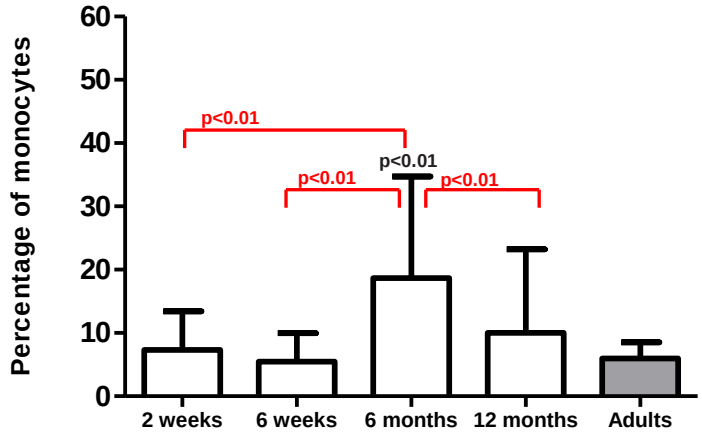

Figure 4.2 Percentage of TNF-α-producing monocytes in response to LPS stimulation. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

In response to TLR4 stimulation, no significant difference was observed in the percentage of TNF-α-producing monocytes between the adults and infants at 2 and 6 weeks of age, though a slightly higher percentage of TNF-α-producing cells at 2 weeks and slightly lower percentage of TNF-α-producing cells at 6 weeks was seen compared to adults. At 6 months, however, the percentage of TNF-α-producing monocytes was significantly higher in infants compared to the adult group ($p<0.01$). At the 12 month time point, no significant differences were observed, though the percentage of TNF-α-producing monocytes remained higher in infants.

When comparing each time point in the infant population, several changes over time were also observed. Although no significant difference was observed in the percentage of TNF-α-producing monocytes between the two and six weeks, the six month time point revealed significantly higher responses to TLR4 stimulation compared to the 2 weeks, 6 weeks and 12 month time points ($p<0.01$). No significant differences were observed 12 months and the two and six week time points.

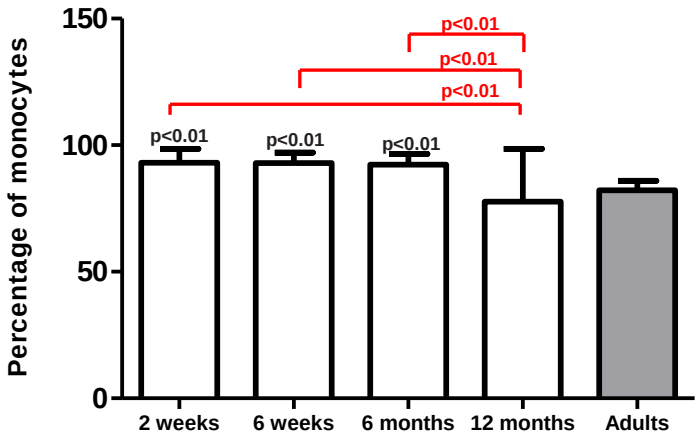

Figure 4.3 Percentage of TNF-α-producing monocytes in response to R848 stimulation. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

In response to TLR7/8 stimulation, the percentage of TNF-α-producing monocytes in infants were significantly higher compared to the adult monocyte responses at 2 weeks ($p<0.01$), six weeks ($p<0.01$) and six months ($p<0.01$) of age. At 12 months, though infants showed a lower percentage of TNF-α-producing monocytes, no significant difference was observed when compared to adults.

The comparison in infant responses over time showed no significant difference in the percentage of TNF-α-producing monocytes between two week and six weeks, as well as the between the six week and six month time points. The 12 month time point showed significantly lower percentage of TNF-α-producing monocytes when compared to 2 weeks, 6 weeks and 6 months time points ($p<0.01$).

4.2.1.2. Total IL-6 response

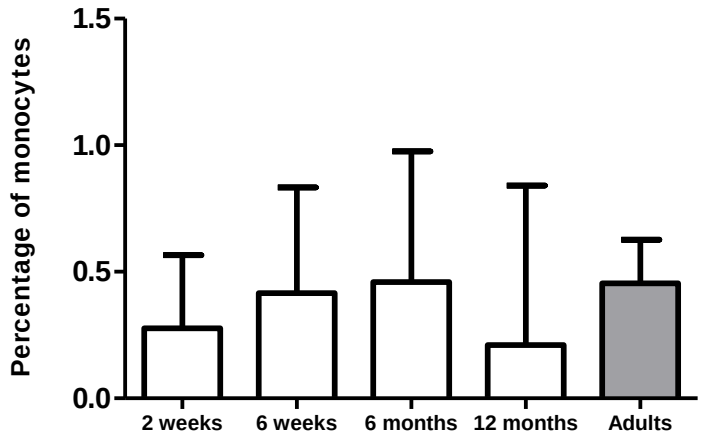

Figure 4.4 Percentage of IL-6-producing monocytes at basal level. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

At basal level, no significant differences were observed in the percentage of IL-6-producing monocytes between the adult group and infants groups when compared at two weeks, six weeks, six months and 12 months of age. The highest percentage of IL-6-producing monocytes was observed at the 6 month time point and was comparable to the adult group, with a significant decline at the 12 month time point in comparison to the adult group.

In the comparison of responses over time, no significant differences were observed in the percentage of IL-6-producing monocytes between any of the time points.

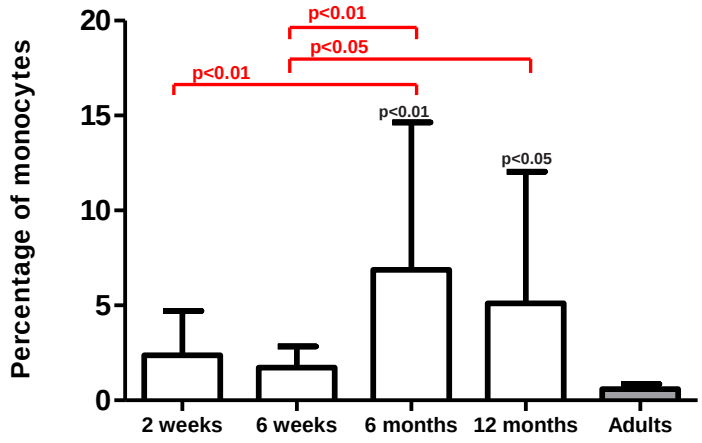

Figure 4.5 Percentage of IL-6-producing monocytes in response to LPS stimulation. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

In both the infant and adult groups, a low percentage of IL-6-producing monocytes were observed in both the infant and adult groups in response to TLR4 stimulation. At two and six weeks of age, no significant difference in the percentage of IL-6-producing monocytes was observed, though infants produced a higher percentage of IL-6-producing monocytes in comparison to the adult monocytes. At six months and 12 months, the percentage of IL-6-producing monocytes in the infant group were significantly higher at both time points compared to the adult monocytes, with p-value of less than 0.01 and 0.05 respectively.

Comparison of the infant's responses at two weeks and six weeks revealed no significant differences between the time points. At six months, the percentage of IL-6-producing monocytes was significantly higher compared to the two-week and six-week time points ($p<0.01$). At 12 months of age, the percentage of IL-6-producing monocytes was only significantly higher compared to the 6-week time point ($p<0.05$).

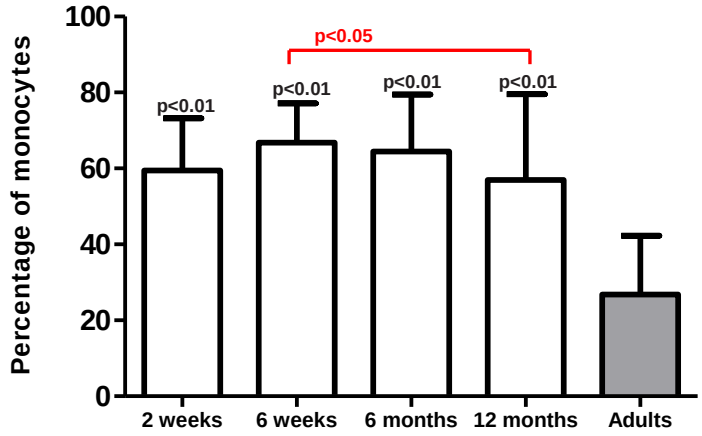

Figure 4.6 Percentage of IL-6-producing monocytes in response to R-848 stimulation. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

In response to TLR7/8 stimulation, significantly higher percentages of IL-6-producing monocytes were observed in infants at 2 weeks, 6 weeks, 6 months and 12 months of age when compared to the adult responses ($p<0.01$).

In comparison of infant responses over time, no significant difference was observed in infants between two weeks and six weeks, two weeks and six months as well as six weeks and six months of age. At 12months of age, infants showed a significantly lower percentage of IL-6-producing monocytes compared to six weeks ($p<0.05$), but no significant differences were observed between two week and twelve months, and six months and twelve months of age.

4.2.1.3. Total IL-12/23p40 response

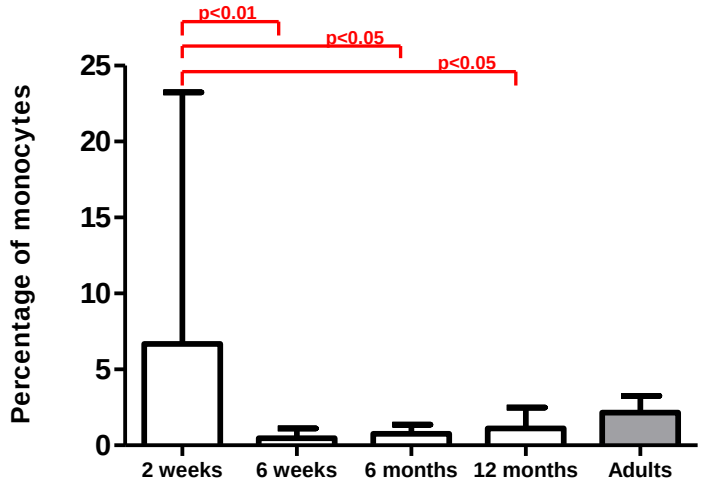

Figure 4.7 Percentage of IL-12/23p40-producing monocytes at basal level. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

At basal level, no significant differences were observed in the percentage of IL-12/23p40-producing monocytes between the adult group and infants groups when compared at two weeks, six weeks, six months and 12 months of age. The highest percentage of IL-12/23p40-producing monocytes was observed at the 2 weeks time point, with the high standard deviation indicating that certain infants showed much higher cytokine responses at this time point. At 12 months the percentage of was comparable to the adult group.

In the comparison of responses over time, infants at 2 weeks showed significantly higher percentages of IL-12/23p40-producing monocytes compared to six weeks ($p<0.01$), six months ($p<0.05$) and 12 months ($p<0.05$) of age. No significant differences were observed between the other time points.

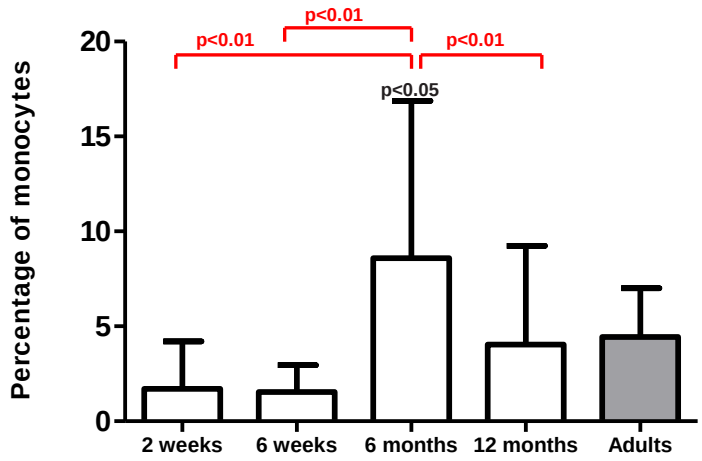

Figure 4.8 Percentage of IL-12/23p40-producing monocytes in response to LPS stimulation. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

In response to TLR4 stimulation, no significant difference in the IL-12/23p40-producing monocytes was observed between the adult group and infants at two and six weeks of age, though infants had lower percentages of IL-12/23p40-producing monocytes compared to the adults. At six months of age, the percentage of IL-12/23p40-producing monocytes was significantly higher in infants at six months compared to the adult ($p<0.05$). At twelve months of age, no significant difference was observed between infants and adults at this time point, and was comparable to adults.

The comparison of infant responses over time showed no significant difference in the percentage of IL-12/23p40-producing monocytes between the 2-week and 6-week time points. At six months, significantly higher percentages of IL-12/23p40-producing monocytes was observed compared to two weeks and six weeks ($p<0.01$). At 12 months, no significant difference was observed compared to the other time points, with the exception of six months, where a significantly lower percentage of IL-12/23p40-producing monocytes was observed at twelve months compared to 6-month time point ($p<0.01$).

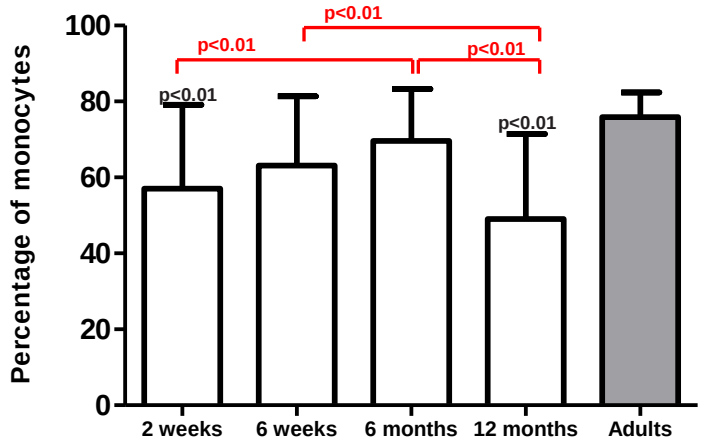

Figure 4.9 Percentage of IL-12/23p40-producing monocytes in response to R-848 stimulation. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

In response to TLR7/8 stimulation, the percentage of IL-12/23p40-producing monocytes was lower in infants at two weeks, six weeks-, six months- and twelve months of age when compared to the adults. This was only found to be significant at 2 weeks and 12 months of age ($p<0.01$)

In the comparison of infants' responses over time, no significant differences in the percentage of IL-12/23p40-producing monocytes were observed between the two-week and six-week and six week and six month time points. Significantly higher responses were observed at 6 months compared to the two week and 12 month time points. Furthermore, significantly lower responses were observed at 12 months compared to the six-week time point.

4.2.2. Myeloid DCs

4.2.2.1. Total TNF-α response

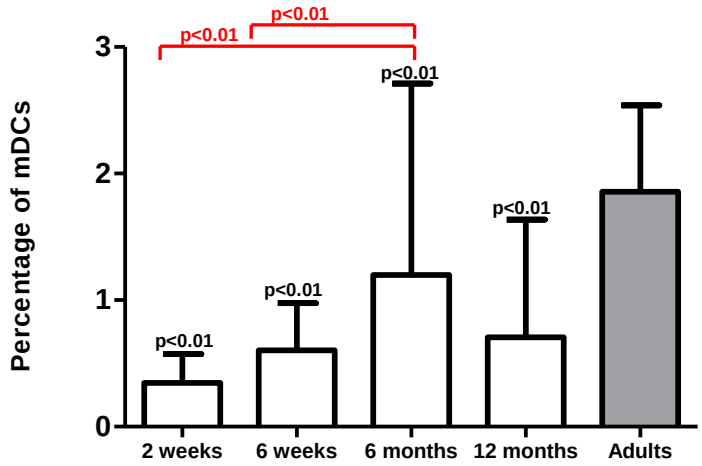

Figure 4.10 Percentage of TNF-α-producing mDCs at basal level. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

At basal level, the percentage of TNF-α-producing mDCs in infants at two weeks, six weeks, six months and 12 months were significantly lower in comparision to the adult group ($p<0.01$).

In the comparison of responses over time, no significant difference was observed in the percentage of TNF-α-producing mDCs between infant groups at 2 weeks and six weeks of age. At six months, infants had significantly higher percentages of TNF-α-producing mDCs compared to two weeks ($p<0.01$) and six weeks ($p<0.01$). At 12 months, a decline in the percentage of TNF-α-producing mDCs was observed in comparison to six months, but this was not found to be significant.

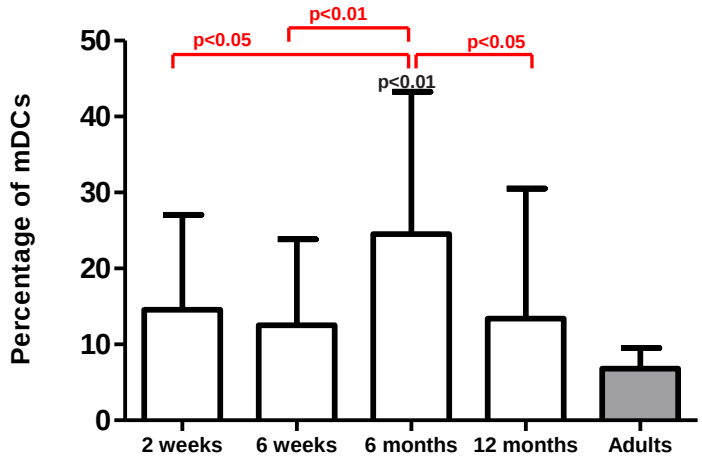

Figure 4.11 Percentage of TNF-α-producing mDCs in response to LPS stimulation. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

In response to TLR4 stimulation, no significant difference was observed in the percentages of TNF-α-producing mDCs between infants at two- and six week time points and adults, although it was higher compared to adults. At six months, a significantly higher percentage of TNF-α-producing mDCs was observed in infants compared to adults ($p<0.01$). At 12 months, higher percentages of TNF-α-producing mDCs was observed in infant mDCs compared to the adults, but was not statistically significant.

The comparison of infant responses over time revealed no significant differences in the percentage of TNF-α-producing mDCs in infants between 2 weeks and 6 weeks of age. At six months, a significantly higher percentage of TNF-α-producing mDCs was observed in infants when compared to the two weeks ($p<0.05$) and six weeks ($p<0.01$) time points. At 12 months, a significant decline in the percentage of TNF-α-producing mDCs was observed compared to the 6 month time point ($p<0.05$).

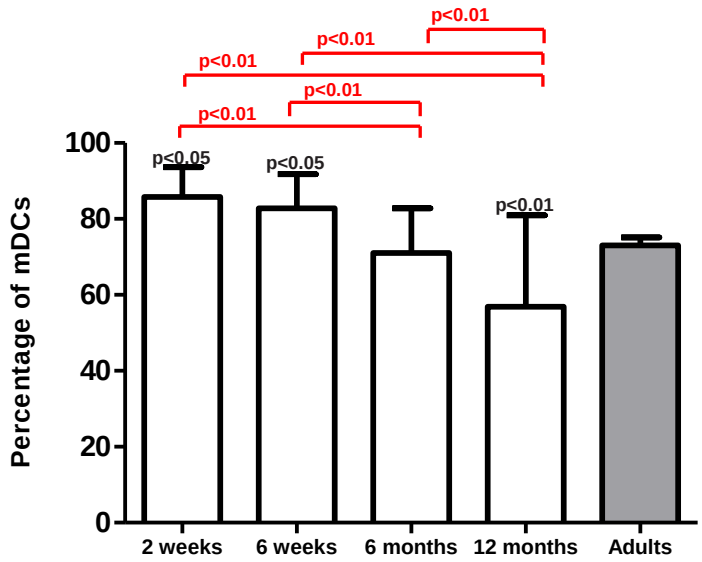

Figure 4.12 Percentage of TNF-α-producing mDCs in response to R-848 stimulation. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

In response to TLR7/8 stimulation, infants at two weeks and six weeks of age showed significantly higher percentages of TNF-α-producing mDCs when compared to the adults ($p<0.05$). At 6 months and 12 months of age, infants showed lower percentages of TNF-α-producing mDCs compared to the adult group. This was only significant at 12 months ($p<0.01$).

The comparison over time showed a trend towards decreasing percentages of TNF-α-producing mDCs in infants over time. The six-week time point showed a somewhat lower response compared to the infants at 2 weeks, though this was not significant. At six months, infants showed higher showed significantly lower percentages of TNF-α-producing mDCs compared to two weeks ($p<0.01$) and 6 weeks ($p<0.01$). The most substantial decrease was observed at 12 months, which was significantly lower compared to the two-week, six-week and six-month time points ($p<0.01$).

4.2.2.2. Total IL-6 response

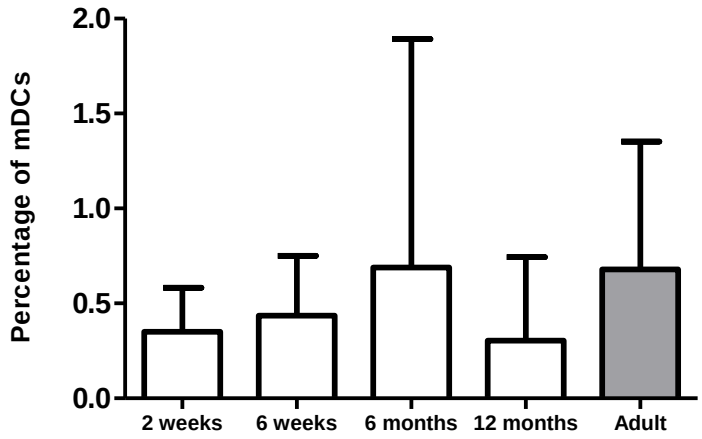

Figure 4.13 Percentage of IL-6-producing mDCs at basal level. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

At basal level, no significant differences were observed in the percentage of IL-6-producing mDCs in infants at two weeks, six weeks, six months and 12 months in comparision to the adult group. At six months of age, the percentage of IL-6-producing mDCs were comparable to the adult. A decline in this response was observed at 12 months.

In the comparison of responses over time, no significant difference was observed in the percentage of TNF-α-producing mDCs between infant groups at any of the progressive time points.

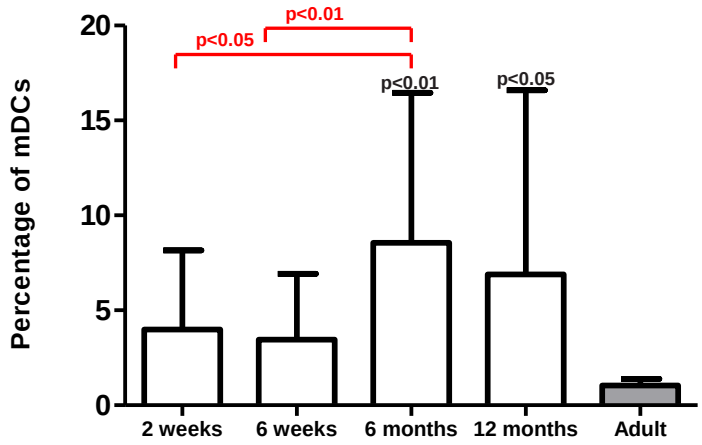

Figure 4.14 Percentage of IL-6-producing mDCs in response to LPS stimulation. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

In response to TLR4 stimulation, infants demonstrated higher percentages of IL-6-producing mDCs at all four time points in comparison to the adults. This was found to be significant at the 6 months and 12 month time points, with $p<0.01$ and $p<0.05$ respectively.

In comparison of infant responses over time, no significant difference in the percentages of IL-6-producing mDCs was observed between two weeks and six weeks of age. At 6 months, infants showed significantly higher percentages of IL-6-producing mDCs compared to the two-week ($p<0.05$) and six-week time point ($p<0.01$). At 12 months, though a decline in the percentage of IL-6-producing mDCs was observed compared to six months, this was not significant.

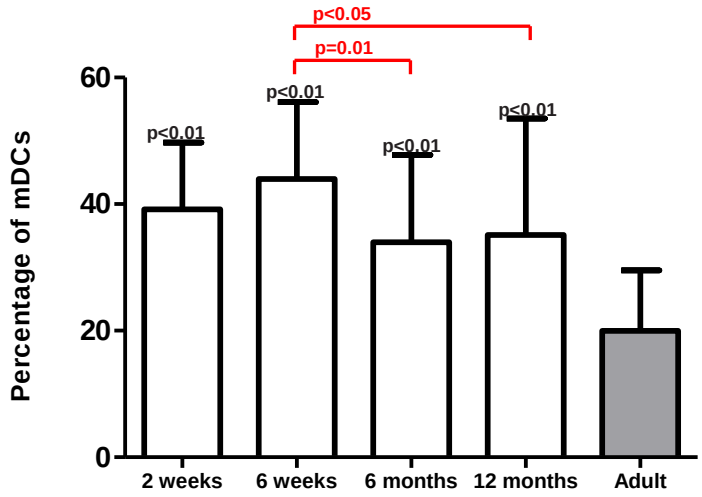

Figure 4.15 Percentage of IL-6-producing mDCs in response to R-848 stimulation. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values with brackets depicted in red denote comparison between infant time points.

In response to TLR7/8 stimulation, infants showed significantly higher percentages of IL-6-producing mDCs at 2 weeks, 6 weeks, 6 months and 12 months compared to the adult responses ($p<0.01$).

In the comparison of infants responses over time, no significant difference was observed in the percentage of IL-6-producing mDCs between two weeks showed and six weeks of age. At 6 months, infants showed significantly lower percentages of IL-6-producing mDCs compared the 6 week time point ($p=0.01$). At 12 months, no significant differences were observed in infants' responses compared to the other time points, with the exception of the six-week time point where significantly lower IL-6 responses were observed ($p<0.05$).

4.2.2.3. Total IL-12/23p40 response in mDCs

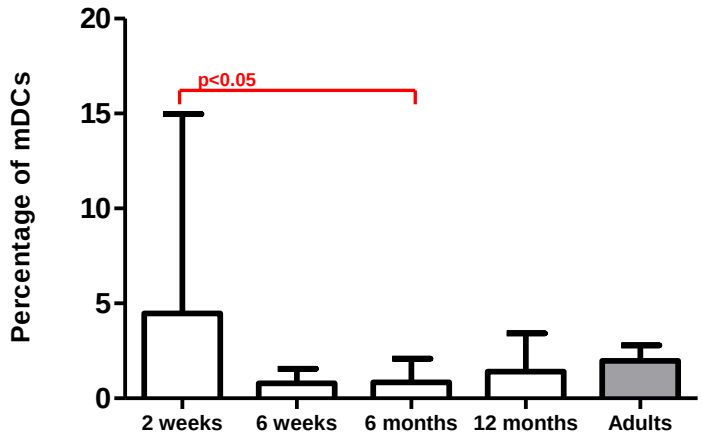

Figure 4.16 Percentage of IL-12/23p40-producing mDCs at basal level. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points

At basal level, no significant differences were observed in the percentage of IL-12/23p40-producing mDCs between the adult group and infants groups at two weeks, six weeks, six months and 12 months of age. The highest percentage of IL-12/23p40-producing monocytes was observed at the 2 weeks time point, with the high standard deviation indicating that certain infants showed much higher spontaneous cytokine responses at this time point. At 12 months the percentage of was comparable to the adult group.

In the comparison of responses over time, no significant differences were observed when comparing the infant groups with time point comparison, with the exception of the two week-six month time point comparison. Infants at 2 weeks showed significantly higher percentages of IL-12/23p40-producing mDCs compared the six month time point ($p<0.05$).

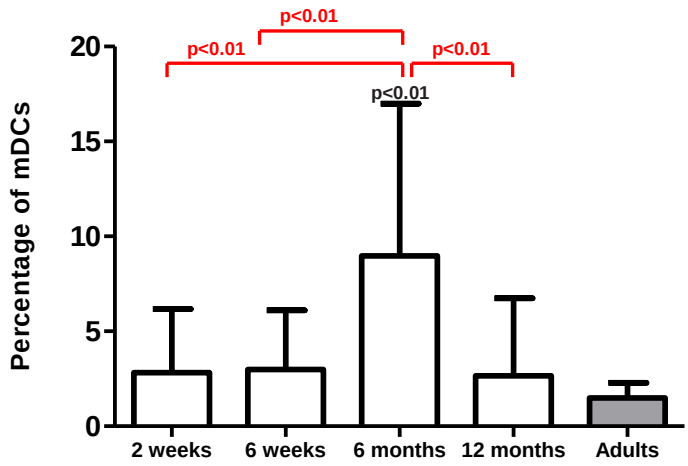

Figure 4.17 Percentage of IL-12/23p40-producing mDCs in response to LPS stimulation. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

In response to TLR4 stimulation, no significant differences were observed in the percentages of IL-12/23p40-producing mDCs in infants at two weeks and six weeks of age compared to the adults. At the 6 months, a significantly highly percentage of IL-12/23p40-producing mDCs was observed in infants compared to the adults ($p<0.01$). At 12 months of age, no significant difference was observed in the percentage of IL-12/23p40-producing mDCs in infants compared to the adults.

The comparison over time revealed no significant difference between the two week and six week time points. In addition, significantly higher percentages of IL-12/23p40-producing mDCs was observed in infants at six months when compared to the two-week and six-week time points ($p<0.01$). At 12 months, a significant decline in the percentage of IL-12/23p40+ mDCs was observed compared to the 6 month time point ($p<0.01$).

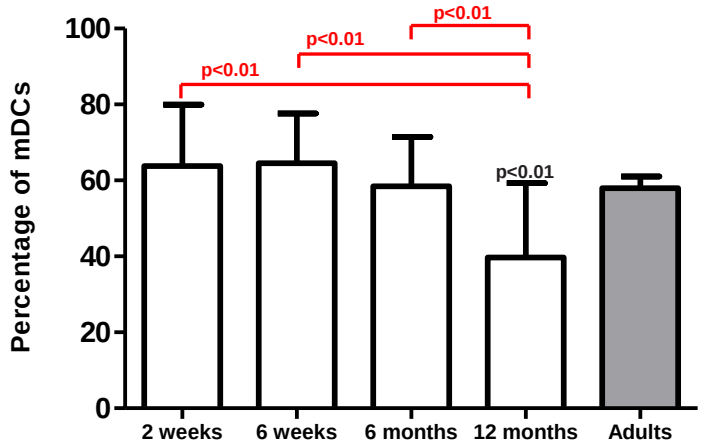

Figure 4.18 Percentage of IL-12/23p40-producing mDCs in response to R-848 stimulation. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

In response to TLR7/8 stimulation, no significant differences in the percentage of IL-12/23p40-producing mDCs were observed between infants and adult groups at two weeks, six weeks and six months of age. At 12 months, infants had significantly lower percentages of IL-12/23p40-producing mDCs compared to the adults ($p<0.01$).

In the comparison of infant responses over time, no differences were observed in the percentage of IL-12/23p40-producing mDCs between the two-week and six-week, two-week and six months as well as six-week and six-month time points. At 12 months, a significant decline in the percentage of IL-12/23p40-producing mDCs was observed compared to two weeks, six weeks and six months of age ($p<0.01$).

4.2.3. Plasmacytoid DCs

4.2.3.1. Total TNF-α response

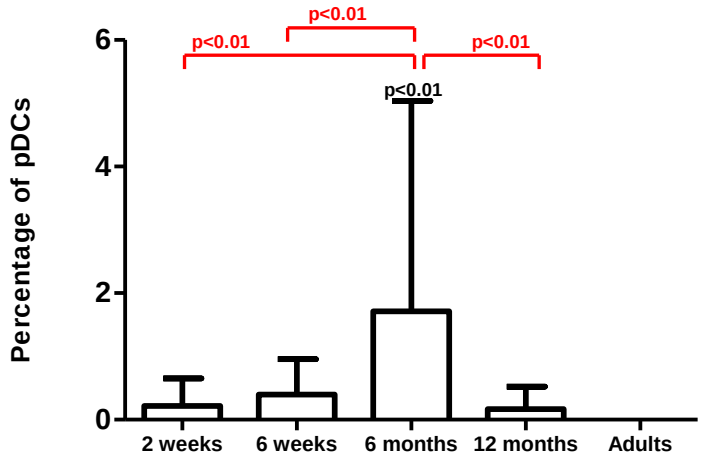

Figure 4.19 Percentage of TNF-α-producing pDCs at basal level. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

At basal level, no significant difference in the percentage of TNF-α-producing pDCs was observed between infants and adult groups at two weeks and six weeks of age. At 6 months, infants showed significantly higher percentages of TNF-α-producing pDCs compared to the adults ($p<0.01$). At the 12-month time point, no significant difference was observed in the percentage of TNF-α-producing pDCs between infants and adults.

In the comparison of infant responses over time, no differences were observed in the percentage of TNF-α-producing pDCs between the two-week and six-week time points. At 6 months, a significant higher percentage of TNF-α-producing pDCs was observed compared to six weeks and six weeks of age ($p<0.01$). Furthermore, infants at 12 months showed a significantly decline in the percentage of TNF-α-producing pDCs ($p<0.01$).

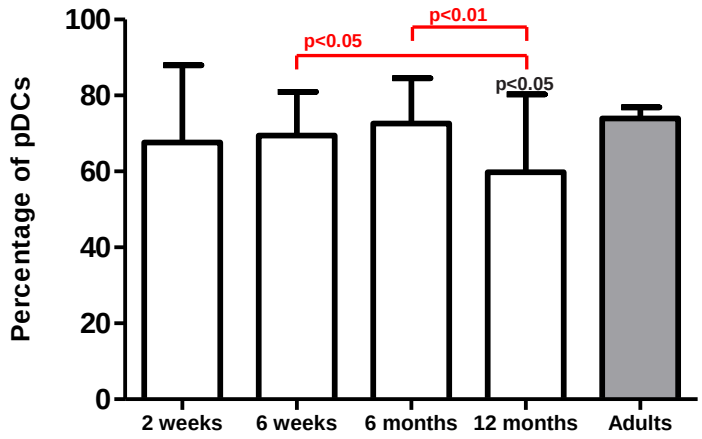

Figure 4.20 Percentage of TNF-α-producing pDCs in response to R-848 stimulation. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

In response to TLR7/8 stimulation, no significant difference was observed in the percentage of TNF-α-producing pDCs between adults and infants at 2 weeks and 6 weeks of age. The same trend was observed at 6 months, with comparable percentages of TNF-α-producing pDCs between the infant and adult group at this time point. At 12 months, however, a significant decline in TNF-α-producing pDCs was observed in infants compared to the adults ($p<0.05$).

In the evaluation of the response over time, a trend towards increased TNF-α response was observed in the first six months, although no significant differences was observed between these time points. At 12 months of age, lower percentages of TNF-α-producing pDCs were observed in infants when compared to the two-week, six-week and six-month time points. This was only found to be significant at the six-week and 12 month ($p<0.05$) and six-month and 12 months time point comparisons ($p<0.01$).

4.2.3.2. Total IFN-α response

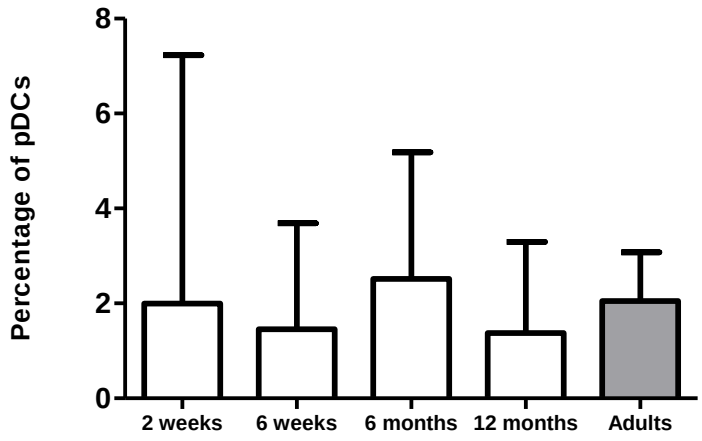

Figure 4.21 Percentage of IFN-α-producing pDCs at basal level. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

At basal level, no significant difference in the percentage of IFN-α-producing pDCs was observed between adult and infants groups at two weeks, six weeks, six months and 12 months of age. At the 2-week and 6-month time point, infant pDC responses were comparable to the adult group.

In the comparison of infant responses over time, no differences were observed in the percentage of TNF-α-producing pDCs between tehifnat groups at any of the time points.

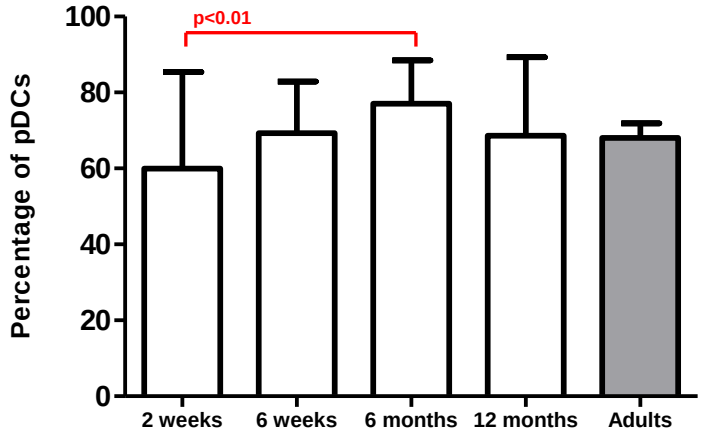

Figure 4.22 Percentage of IFN-α-producing pDCs in response to R-848 stimulation. Bar graphs represent mean and standard deviation. The grey bar depicts the adult group. P-values depicted in black denote comparisons between infant groups and the adult group. P-values depicted in red denote comparison between infant time points.

In response to TLR7/8 stimulation, no significant difference in the percentage of IFN-α-producing pDCs was observed between infant and adults at any of the time points. Infants produced slightly lower percentages of IFN-α-producing pDCs compared to the adults at 2 weeks and slightly higher percentages of IFN-α-producing pDCs at 6 months. At the 6 week and 12 month time points, infants showed percentages of IFN-α-producing pDCs that were comparable to the adults.

The comparison of infants' responses over time showed no significant difference when comparing all the progressive time points, with the exception of two-week and six-month time point where a significantly higher percentage of IFN-α-producing pDCs was observed at six months compared to two weeks of age ($p<0.01$). Furthermore, a trend towards increased percentages of IFN-α-producing pDCs was observed in the first three time points. A decline in this trend was observed at the 12-month time point.

Table 4.2 Flow cytometric comparison of percentage of cells expressing cytokines for longitudinal infant and adult responses to LPS and R-848 stimulation. Analysis of the mean percentage of monocytes, mDC, or pDC expressing a particular cytokine. Statistically significant differences between age groups are indicated in bold.

			LPS (TLR4)						R-848 (TLR7/8)					
	Cytokine		Mean ± SD		p-value				Mean ± SD		p-value			
Monocytes														
	Total TNF-α	**2 wk**	7.30 ± 6.10					**2 wk**	93.12 ± 5.42					**2 wk**
	% positive	**6 wk**	5.52 ± 4.13				**6 wk**	0.5351	92.92 ± 4.18				**6 wk**	0.9348
		6 mo	18.67 ± 16.05			**6 mo**	**0.0000**	**0.0002**	92.37 ± 4.16			**6 mo**	0.8481	0.7964
		12 mo	10.02 ±13.21		**12 mo**	**0.0068**	0.1427	0.3790	77.63 ± 20.87		**12 mo**	**0.0000**	**0.0000**	**0.0000**
		Adult	5.95 ± 2.55	**Adult**	0.3070	**0.0014**	0.9107	0.7239	83.16 ± 3.83	**Adult**	0.2453	**0.0084**	**0.0048**	**0.0043**
	Total IL-6	**2 wk**	2.38 ± 2.32					**2 wk**	59.49 ± 13.75					**2 wk**
	% positive	**6 wk**	1.73 ± 1.11				**6 wk**	0.6370	66.83 ±10.32				**6 wk**	0.0959
		6 mo	6.88 ± 7.77			**6 mo**	**0.0004**	**0.0021**	64.49 ± 14.97			**6 mo**	0.6006	0.2701
		12 mo	5.11 ± 6.93		**12 mo**	0.2434	**0.0230**	0.0677	56.91 ± 22.65		**12 mo**	0.1154	**0.0349**	0.5830
		Adult	0.59 ± 0.26	**Adult**	**0.0197**	**0.0011**	0.5361	0.3335	26.79 ± 15.49	**Adult**	**0.0000**	**0.0000**	**0.0000**	**0.0000**
	Total IL-12/23p40	**2 wk**	1.71 ± 2.50					**2 wk**	57.03 ± 22.06					**2 wk**
	% positive	**6 wk**	1.54 ± 1.40				**6 wk**	0.9051	63.14± 18.19				**6 wk**	0.2420
		6 mo	8.59 ± 8.28			**6 mo**	**0.0000**	**0.0000**	69.62 ± 13.66			**6 mo**	0.2245	**0.0206**
		12 mo	4.05 ± 5.19		**12 mo**	**0.0025**	0.0828	0.1071	49.07 ± 22.39		**12 mo**	**0.0004**	**0.0122**	0.1551
		Adult	4.45 ± 2.56	**Adult**	0.8298	**0.0264**	0.1072	0.1299	75.84 ± 6.54	**Adult**	**0.0003**	0.3779	0.0685	**0.0079**
mDCs														
	Total TNF-α	**2 wk**	14.57 ± 12.49					**2 wk**	85.81 ± 7.83					**2 wk**
	% positive	**6 wk**	12.53 ± 11.33				**6 wk**	0.6138	82.78 ± 8.96				**6 wk**	0.4191
		6 mo	24.52 ± 18.74			**6 mo**	**0.0043**	**0.0181**	71.07 ± 11.68			**6 mo**	**0.0027**	**0.0002**
		12 mo	13.39 ± 17.15		**12 mo**	**0.0126**	0.8421	0.7841	56.88 ± 24.06		**12 mo**	**0.0007**	**0.0000**	**0.0000**
		Adult	6.80 ± 2.74	**Adult**	0.2379	**0.0015**	0.2844	0.1504	73.00 ± 2.12	**Adult**	**0.0023**	0.7019	**0.050**	**0.0116**
	Total IL-6	**2 wk**	3.98 ± 4.17					**2 wk**	39.17 ± 10.55					**2 wk**
	% positive	**6 wk**	3.45 ± 3.46				**6 wk**	0.7628	43.98 ± 12.11				**6 wk**	0.2029
		6 mo	8.55 ± 7.91			**6 mo**	**0.0054**	**0.0131**	33.95 ± 13.84			**6 mo**	**0.0103**	0.1812
		12 mo	6.89 ± 9.70		**12 mo**	0.3873	0.0677	0.1246	35.12 ± 18.41		**12 mo**	0.7756	**0.0286**	0.3172
		Adult	1.04 ± 0.35	**Adult**	**0.0176**	**0.0020**	0.3033	0.2122	19.99 ± 9.53	**Adult**	**0.0044**	**0.0072**	**0.0000**	**0.0002**

	Total IL-12/23p40	**2 wk**	2.82 ± 3.35					**2 wk**	63.77 ± 16.15						**2 wk**
	% positive	**6 wk**	2.98 ± 3.12				**6 wk**	0.9061	64.53 ± 13.06					**6 wk**	0.8544
		6 mo	8.98 ± 8.01			**6 mo**	**0.0000**	**0.0000**	58.43 ± 13.05				**6 mo**	0.1527	0.2145
		12 mo	2.65 ± 4.08		**12 mo**	**0.0000**	0.8164	0.9044	39.67 ± 19.63			**12 mo**	**0.0000**	**0.0000**	**0.0000**
		Adult	1.48 ± 0.81	**Adult**	0.5276	**0.0000**	0.3997	0.4534	57.95 ± 3.06	**Adult**	**0.0019**	0.9319	0.2347	0.2957	
pDCs															
	Total TNF-α	**2 wk**	Undetectable						67.62 ± 20.35						**2 wk**
	% positive	**6 wk**	Undetectable						69.45 ± 11.53					**6 wk**	0.6788
		6 mo	Undetectable						72.63 ± 11.89				**6 mo**	0.4815	0.2728
		12 mo	Undetectable						59.76 ± 20.51			**12 mo**	**0.0086**	**0.0406**	0.0983
		Adult	Undetectable						73.94 ± 2.94	**Adult**	**0.0218**	0.8271	0.4452	0.2854	
	Total IFN-α	**2 wk**	Undetectable						59.95 ± 25.44						**2 wk**
	% positive	**6 wk**	Undetectable						69.26 ± 13.64					**6 wk**	0.0649
		6 mo	Undetectable						77.03 ± 11.46				**6 mo**	0.1305	**0.0013**
		12 mo	Undetectable						68.59 ± 20.70			**12 mo**	0.1244	0.9004	0.1087
		Adult	Undetectable						68.01 ± 3.89	**Adult**	0.9321	0.1895	0.8504	0.2294	

			Basal Stimulation					
	Cytokine		Mean ± SD			p-value		
Monocytes								
	Total TNF-α	**2 wk**	0.42 ± 0.56					**2 wk**
	% positive	**6 wk**	0.56 ± 0.35				**6 wk**	0.5661
		6 mo	0.89 ± 0.81			**6 mo**	0.2265	0.0816
		12 mo	0.99 ± 1.77		**12 mo**	0.7127	0.1235	**0.0408**
		Adult	1.09 ± 0.28	**Adult**	0.7918	0.5708	0.1329	0.0562
	Total IL-6	**2 wk**	0.27 ± 0.29					**2 wk**
	% positive	**6 wk**	0.40 ± 0.41				**6 wk**	0.2766
		6 mo	0.45 ± 0.51			**6 mo**	0.6587	0.1280
		12 mo	0.21 ± 0.63		**12 mo**	0.0709	0.1458	0.6585
		Adult	0.45 ± 0.17	**Adult**	0.1619	0.9738	0.7602	0.2687
	Total IL-12/23p40	**2 wk**	6.60 ± 16.22					**2 wk**
	% positive	**6 wk**	0.47 ± 0.64				**6 wk**	**0.0070**
		6 mo	0.75 ± 0.61			**6 mo**	0.9051	**0.0132**
		12 mo	1.12 ± 1.3		**12 mo**	0.8815	0.7879	**0.0249**
		Adult	2.15 ± 1.1	**Adult**	0.7421	0.6479	0.5766	0.1432
mDCs								
	Total TNF-α	**2 wk**	0.35 ± 0.22					**2 wk**
	% positive	**6 wk**	0.63 ± 0.38				**6 wk**	0.2582
		6 mo	1.19 ± 1.5			**6 mo**	**0.0222**	**0.0009**
		12 mo	0.71 ± 0.93		**12 mo**	0.0666	0.7549	0.1766
		Adult	1.85 ± 0.68	**Adult**	**0.0009**	**0.0484**	**0.0003**	**0.0000**
	Total IL-6	**2 wk**	0.37 ± 0.26					**2 wk**
	% positive	**6 wk**	0.49 ± 0.41				**6 wk**	0.5412
		6 mo	0.68 ± 1.20			**6 mo**	0.2963	0.1064
		12 mo	0.30 ± 0.44		**12 mo**	0.6466	0.3565	0.7252

		Adult	0.68 ± 0.67	**Adult**	0.1537	0.9697	0.4456	0.2274
	Total IL-12/23p40	**2 wk**	4.32 ± 10.30					**2 wk**
	% positive	**6 wk**	0.77 ± 0.76				**6 wk**	0.0154
		6 mo	0.85 ± 1.24			**6 mo**	0.9564	**0.0229**
		12 mo	1.40 ± 2.02		**12 mo**	0.7300	0.6871	0.0646
		Adult	1.98 ± 0.81	**Adult**	0.7790	0.5719	0.5352	0.2328
pDCs								
	Total TNF-α	**2 wk**	0.23 ± 00.43					**2 wk**
	% positive	**6 wk**	0.48 ± 0.66				**6 wk**	0.5687
		6 mo	1.71 ± 3.32			**6 mo**	**0.0077**	**0.0018**
		12 mo	0.17 ± 0.35		**12 mo**	**0.0022**	0.5083	0.9020
		Adult	0.00 ± 0.00	**Adult**	0.7881	**0.0059**	0.4194	0.7064
	Total IFN-α	**2 wk**	2.06 ± 5.32					**2 wk**
	% positive	**6 wk**	1.36 ± 2.17				**6 wk**	0.4295
		6 mo	2.51 ± 2.66			**6 mo**	0.2053	0.6279
		12 mo	1.38 ± 1.92		**12 mo**	0.2493	0.9880	0.4776
		Adult	2.04 ± 1.03	**Adult**	0.5927	0.6998	0.5658	0.9872

4.3. Comparison of polyfunctional cytokine responses

We assessed the percentage of single, double and triple cytokine-producing cells in monocyte, mDC and pDC populations at basal (unstimulated) level as well as in response to LPS and R-848 stimulation. At each time point (2 weeks, 6 weeks, 6 months and 12 months); the infant responses were compared to the adult group as well as between time points for each respective cell type.

The basal level cytokine response was assessed in both monocytes and mDC populations. These cell populations responded to TLR4 and TLR7/8 stimulation, whereas the pDC population responded only to TLR7/8 stimulation. No response was observed to TLR4 stimulation in pDCs. For the monocytes and mDCs, we assessed the polyfunctional responses of TNF-α+, IL-6+, IL-12+ single-cytokine producing cells, TNF-α+IL6+, TNF-α+IL-12+ double-cytokine producing cells and TNF-α+IL-6+IL-12+ triple-cytokine producing cells.

The basal level cytokine response was assessed in the pDC population. However, for TLR stimulation, pDCs only responsed to TLR7/8 stimulation, the production was IL-12/23p40 and IL-6 was not observed, with the exception of the TNF-α+IFN-α+IL-6+ triple-cytokine producers (Addendum F). Therefore, in the pDC population we assessed the polyfunctional production of TNF-α and IFN-α+ single-cytokine producing cells, the TNF-α+ IFN-α+ double-cytokine producing cells as well as the TNF-α+IFN-α+IL-6+ triple-cytokine producing cells as observed in both infant and adult pDCs in response to TLR7/8 stimulation.

Line graphs were employed to illustrate the variation over time, with the exception of the basal stimulation data, which is depicted in tabular format. Data comparisons were defined as significant if $p<0.05$ and highly significant if $p<0.01$.

4.3.1. Monocytes

4.3.1.1. Single cytokine expression

Table 4.3 Percentage of single cytokine producers in monocytes at basal level. Table illustrates mean and SD of TNF-α+, IL-6+ and IL-12+ single producers in infants and adults as well as the p-values for the infant-adult comparison. Significant values are denoted in bold.

Monocytes		Mean ± SD	Infant vs Adult (p-value)
TNF-α+ % positive	**2 wk**	0.32 ± 0.40	0.2254
	6 wk	0.47 ± 0.32	0.5515
	6 mo	0.59 ± 0.55	0.9317
	12 mo	0.74 ± 1.22	0.6233
	Adult	0.61 ± 0.17	
IL-6+ % positive	**2 wk**	0.21 ± 0.20	0.7588
	6 wk	0.34 ± 0.41	0.1107
	6 mo	0.25 ± 0.25	0.4784
	12 mo	0.11 ± 0.30	0.5869
	Adult	0.17 ± 0.07	
IL-12/23p40+ % positive	**2 wk**	6.53 ± 16.22	0.1166
	6 wk	0.40 ± 0.62	0.6525
	6 mo	0.52 ± 0.51	0.6885
	12 mo	0.92 ± 1.22	0.7904
	Adult	1.76 ± 1.03	

At basal level, no robust expression of TNF-α+ IL-6+ and IL-12/23p40+ was observed in both infant and adult monocytes. In TNF-α+ response, no significant differences were observed in the the percentage of TNF-α+ monocytes in infants at 2 weeks, 6 weeks, 6 months and 12 months of age in comparison to the adults.

A similar trend was observed for the IL-6+ monocytes. No significant differences were observed in the percentages of IL-6+ monocytes between the infants and the adults at each time point.

The percentage of IL-12/23p40+ monocytes in infants at 2 weeks, 6 weeks, 6 months and 12 months showed no significant differences when compared to the adult group. Though, infants at 2 weeks showed a higher percentage of IL-12/23p40+ monocytes, which may be due to spontaneous cytokine responses produced by some infants at that time point.

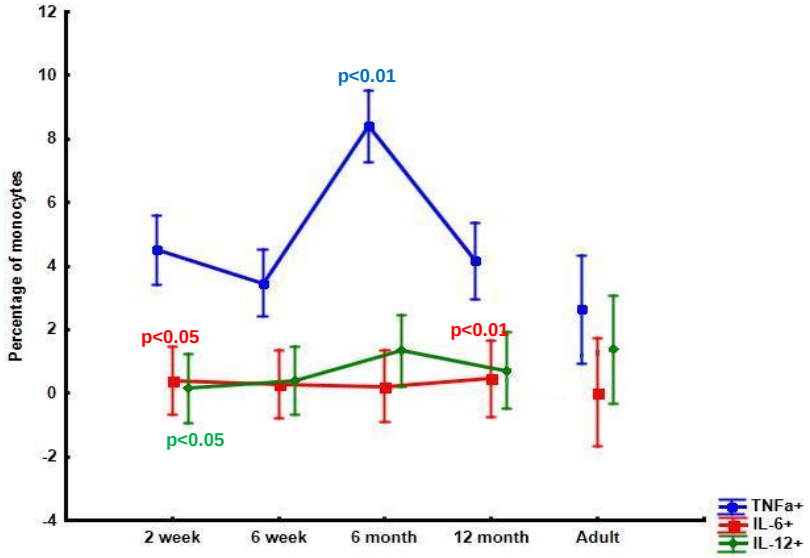

Figure 4.23 Percentage of single cytokine producers in monocytes in response to LPS. Figure illustrates TNF-α+, IL-6+ and IL-12+ single producers in infants versus adults. Vertical bars denote 95% confidence intervals.

In response to TLR4 stimulation, monocytes demonstrated the robust production of TNF-α and very low production of IL-6+ and IL-12+. In comparison to the adults, no significant difference was observed in the percentage of TNF-α+ monocytes in infants at two and six weeks of age. At six months, infants demonstrated a significantly higher percentage of TNF-α+ monocytes compared to adults ($p<0.01$). At 12 months, no significant difference was observed in the percentage of TNF-α+ monocytes between infants and adults.

The percentage of IL-6+ monocytes was significantly higher in infants at two weeks in comparison to the adults ($p<0.05$), with no significant differences observed at in infants 6 weeks and 6 months of age when compared to adults. At 12 months, a significantly higher percentage of IL-6+ monocytes was observed infants compared to adults ($p<0.01$).

The percentage of IL-12/23p40+ monocytes was significantly higher in infants at the 2 week time point when compared to the adults ($p<0.05$). No significant differences were observed at the other subsequent time points in comparison to the adult group.

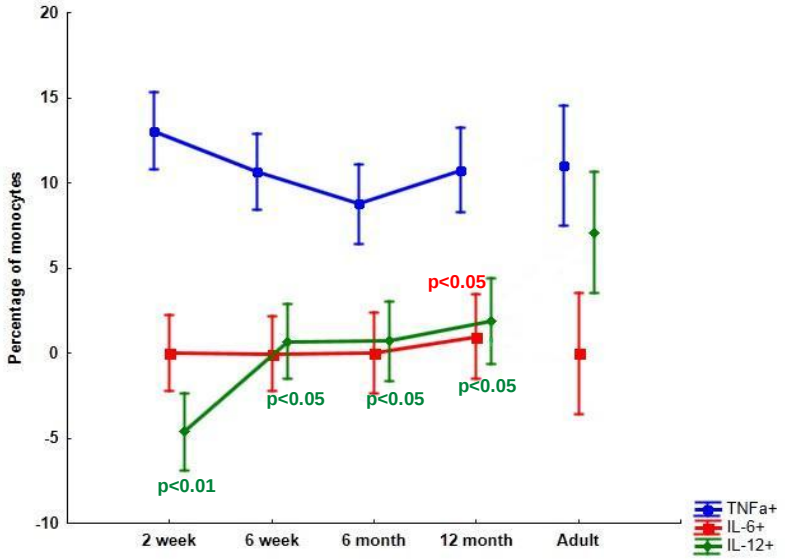

Figure 4.24 Percentage of single cytokine producers in monocytes in response to R-848. Figure illustrates TNF-α+, IL-6+ and IL-12+ single producers in infants versus adults. Vertical bars denote 95% confidence intervals.

In response to TLR7/8 stimulation, monocytes showed the highest production of TNF-α+.and lowest production of IL-6+. In comparison to the adult group, no significant differences were observed in the percentage of TNF-α+ monocytes at 2 weeks, 6 weeks, 6 months and 12 months time points.

No significant difference was observed in the percentage of IL-6+ monocytes in infants at the 2 weeks, 6 weeks and 6 months time points in comparison to the adult group. At the 12 month time point, infants had significantly higher percentage of IL-6+ monocytes compared to the adult group ($p<0.05$).

The percentage of IL-12/23p40+ monocytes was significantly lower in the infant group at the 2 week ($p<0.01$), 6 week ($p<0.05$), 6 month ($p<0.05$) and 12 month ($p<0.05$) time points when compared to the adults.

4.3.1.2. Double cytokine expression

Table 4.4 Percentage of double cytokine producers in monocytes at basal level. Table illustrates mean and SD of TNF-α+IL-6+, TNF-α+IL-6+and IL-6+IL-12+ double producers in infants and adults as well as the p-values for the infant-adult comparison. Significant values are denoted in bold.

Monocytes		Mean ± SD	Infant vs Adult (p-value)
TNF-α+IL-6+ % positive	**2 wk**	0.04 ± 0.09	**0.0460**
	6 wk	0.03 ± 0.02	**0.0387**
	6 mo	0.11 ± 0.17	0.6386
	12 mo	0.07 ± 0.22	0.1732
	Adult	0.14 ± 0.07	
TNF-α+IL-12+ % positive	**2 wk**	0.05 ± 0.07	**0.0008**
	6 wk	0.05 ± 0.07	**0.0006**
	6 mo	0.13 ± 0.10	**0.0439**
	12 mo	0.17 ± 0.32	0.1458
	Adult	0.26 ± 0.07	
IL-6+IL-12+ % positive	**2 wk**	0.02 ± 0.02	**0.0003**
	6 wk	0.01 ± 0.01	**0.0003**
	6 mo	0.05 ± 0.06	0.2027
	12 mo	0.02 ± 0.05	**0.0006**
	Adult	0.07 ± 0.02	

At basal level, low expression of TNF-α+IL-6+, TNF-α+ IL-12/12p40+ and IL-6+IL-12/23p40+ was observed in both infant and adult monocytes. For the TNF-α+IL-6+ response, infants produced significantly lower percentage of TNF-α+IL-6+ monocytes at the two weeks and six week time points in comparison to the adults ($p<0.05$). At the six months and twelve months time points, no significant differences were observed in the percentage of TNF-α+IL-6+ monocytes in infants compared to the adults.

The percentage of TNF-α+IL-12/23p40+ monocytes in infants was significantly lower in infants at two weeks, six weeks and six months when compared to the adult group ($p<0.01$). At 12 months, no significant differences were observed in the percentages of TNF-α+IL-12/23p40+ monocytes between the infants and the adults at each time point.

The percentage of IL-6+IL-12/23p40+ monocytes was found to be significantly lower in infants at the 2 week and 6 week time points compared to adults ($p<0.01$). At 6 months, the percentage of IL-6+IL-12/23p40+ monocytes between infants and adults were found to be comparable. At 12 months, the percentage of IL-6+IL-12/23p40+ monocytes showed a significant decline in infants when compared to the adult group ($p<0.01$).

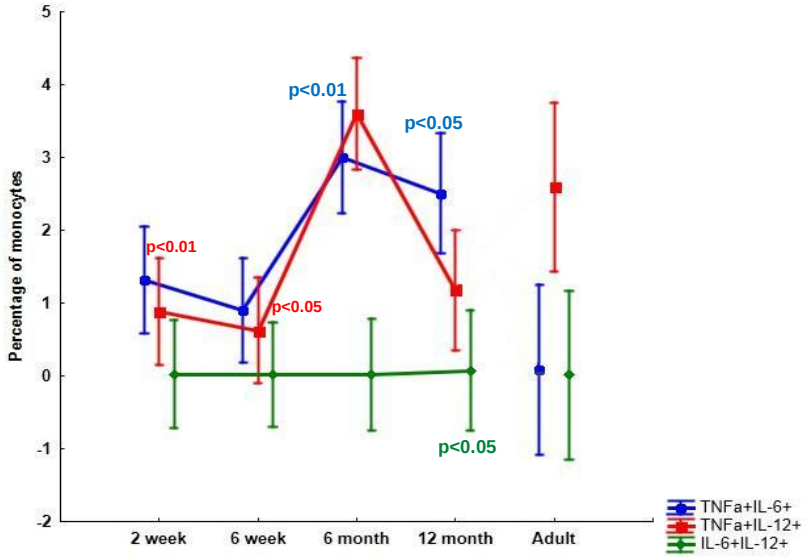

Figure 4.25 Percentage of double cytokine producers in monocytes in response to LPS. Figure illustrates TNF-α+IL-6+, TNF-α+IL-12+ and IL-6+IL-12+ double producers in infants versus adults. Vertical bars denote 95% confidence intervals.

In response to TLR4 stimulation, monocytes demonstrated high expression of TNF-α+IL-6+ and TNF-α+IL-12/23p40+. At two and six weeks of age, no significant difference in the percentage of TNF-α+IL-6+ monocytes was observed in infants when compared to adults. At 6 and 12 months of age, infants showed significantly higher percentages of TNF-α+IL-6+ monocytes compared to the adults, with p-value of less than 0.01 and 0.05 respectively.

With respect to the TNF-α+IL-12/23p40+ monocytes, infants showed significantly lower percentages at 2 weeks and six weeks of age in comparison to the adults ($p<0.01$). No significant differences in the percentage of TNF-α+IL-12/23p40+ monocytes were observed between infants and adults at the 6 month and 12 month time points.

No significant differences in the percentages of IL-6+IL-12/23p40+ monocytes were observed in infants in comparison to the adults at the 2 week, 6 week and 6 month time points. At 12 months, infants showed a significantly higher percentage of IL-6+IL-12/23p40+ monocytes when compared to the adult group ($p<0.01$).

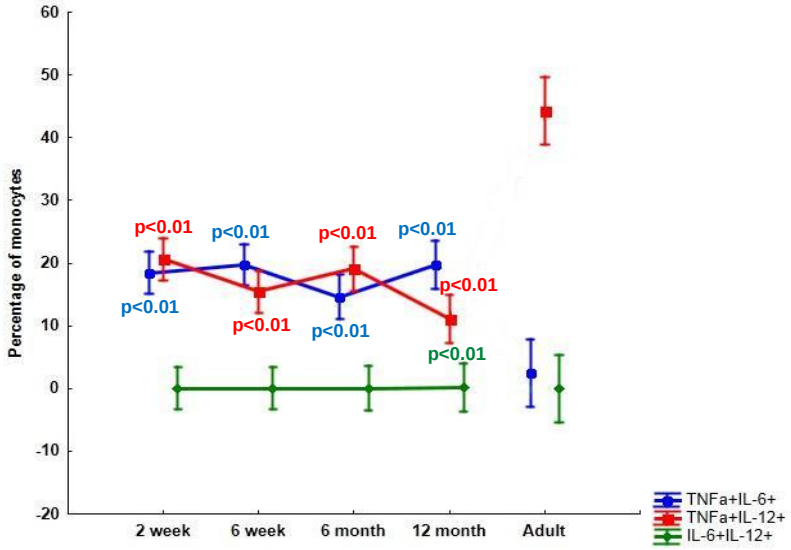

Figure 4.26 Percentage of double cytokine producers in monocytes in response to R-848. Figure illustrates TNF-α+IL-6+, TNF-α+IL-12+ and IL-6+IL-12+ double producers in infants versus adults. Vertical bars denote 95% confidence intervals.

In response to TLR7/8 stimulation, monocytes demonstrated high expression of TNF-α+ IL-6+ and TNF-α+IL-12/23p40+ and low expression of IL-6+IL-12/23p40+. In comparison to the adult group, infants showed significantly higher percentages of TNF-α+IL-6+ monocytes at the 2 week, 6 week, 6 month and 12 month time points ($p<0.01$).

The opposite trend was observed for the TNF-α+IL-12/23p40+ response. The percentages of TNF-α+IL-12/23p40+ monocytes were significantly lower in infants at the 2 week, 6 week, 6 month and 12 month in comparison to the adult group ($p<0.01$).

The percentage of IL-6+IL-12/23p40+ monocytes were comparable to the adult group at the 2 week, 6 week and 6 month time points, as no significant differences were observed. At 12 months, infants showed significantly higher percentages of IL-6+IL-12/23p40+ monocytes than the adult group ($p<0.01$).

4.3.1.3. Triple cytokine expression

Table 4.5 Percentage of triple cytokine producers in monocytes at basal level. Table illustrates mean and SD of TNF-α+IL-6+IL-12+ triple producers in infants and adults as well as the p-values for the infant-adult comparison. Significant values are denoted in bold.

Monocytes		Mean ± SD	Infant vs Adult (p-value)
TNF-α+IL-6+IL-12+ % positive	**2 wk**	0.01 ± 0.02	**0.0000**
	6 wk	0.01 ± 0.01	**0.0000**
	6 mo	0.04 ± 0.06	**0.0398**
	12 mo	0.02 ± 0.05	**0.0003**
	Adult	0.07 ± 0.02	

At basal level, low expression of TNF-α+IL-6+IL-12/12p40+ was observed in both infant and adult monocytes. At each time point, infants showed significantly lower percentages of TNF-α+IL-6+IL-12/23p40 monocytes at two weeks ($p<0.01$), six weeks ($p<0.01$), six months ($p<0.05$) and 12 months ($p<0.01$) in comparison to the adults.

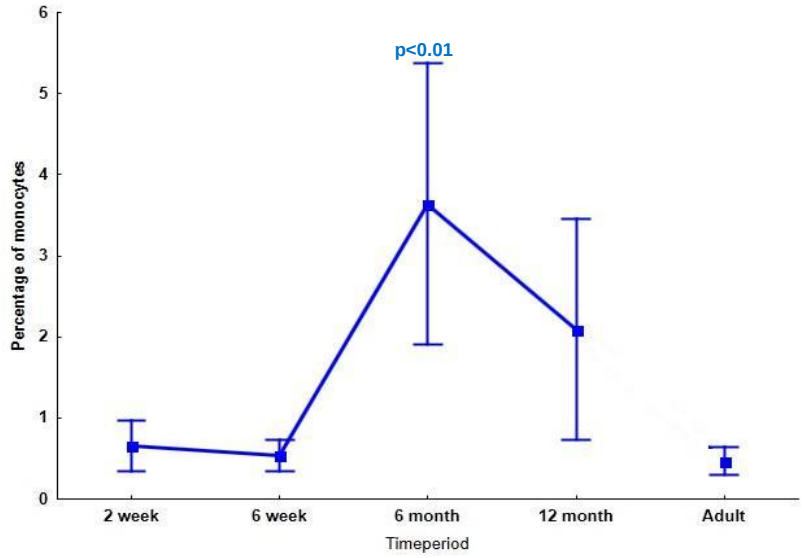

Figure 4.27 Percentage of triple cytokine producers in monocytes in response to LPS. Figure illustrates the expression of TNF-α+IL-6+IL-12+ monocytes in infants versus adults. Vertical bars denote 95% confidence intervals.

In response to TLR4 stimulation, low production of TNF-α+IL-6+IL-12/23p40+ was observed in infant and adult monocytes. No significant difference was observed in the percentages of TNF-α+IL-6+IL-12+ monocytes in infants at the 2 week and six week time points when compared to the adult group. At six months of age, infants produced significantly higher percentages of TNF-α+IL-6+IL-12/23p40+ monocytes in comparison to the adult group ($p<0.01$). At 12 months of age, no significant differences were observed in the percentage of TNF-α+IL-6+IL-12/23p40+ monocytes between infants and adults.

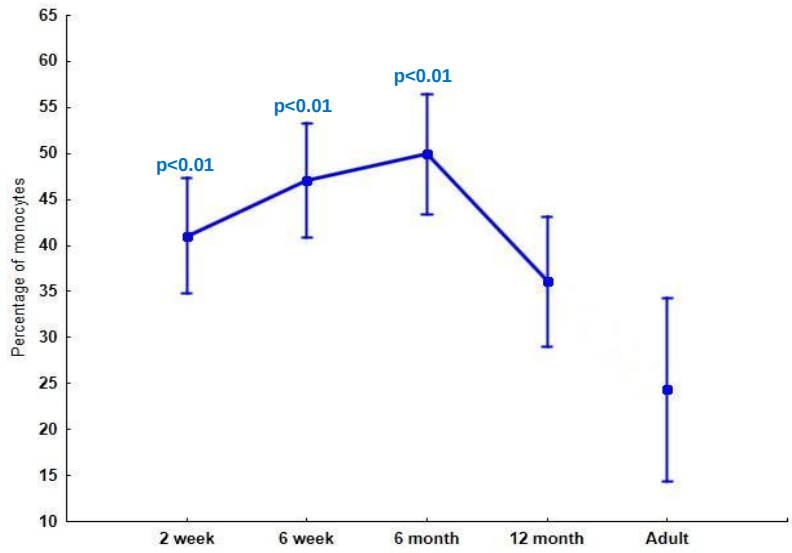

Figure 4.28 Percentage of triple cytokine producers in monocytes in response to R-848. Figure illustrates the expression of TNF-α+IL-6+IL-12+ monocytes in infants versus adults. Vertical bars denote 95% confidence intervals.

In response to TLR7/8 stimulation, robust production of TNF-α+IL-6+IL-12/23p40+ was observed in infant and adult monocytes. The percentages of TNF-α+IL-6+IL-12/23p40+ monocytes was significantly higher in infants at the two week, six week and six month time points in comparison to the adults ($p<0.01$). At 12 months, no significant difference in the percentage of TNF-α+IL-6+IL-12/23p40+ monocytes was observed between the infant and adult group.

4.3.2. Myeloid dendritic cells

4.3.2.1. Single cytokine expression

Table 4.6 Percentage of single cytokine producers in mDCs at basal level. Table illustrates mean and SD of TNF-α+, IL-6+ and IL-12+ single producers in infants and adults as well as the p-values for the infant-adult comparison. Significant values are denoted in bold.

mDCs		Mean ± SD	Infant vs Adult (p-value)
TNF-α+ % positive	**2 wk**	0.28 ± 0.15	**0.0000**
	6 wk	0.49 ± 0.33	**0.0000**
	6 mo	0.79 ± 0.77	**0.0031**
	12 mo	0.54 ± 0.67	**0.0000**
	Adult	1.38 ± 0.41	
IL-6+ % positive	**2 wk**	0.30 ± 0.22	0.2274
	6 wk	0.38 ± 0.41	0.4456
	6 mo	0.35 ± 0.45	0.9697
	12 mo	0.21 ± 0.22	0.1537
	Adult	0.38 ± 0.35	
IL-12/23p40+ % positive	**2 wk**	4.25 ± 10.29	0.1754
	6 wk	0.65 ± 0.67	0.6262
	6 mo	0.50 ± 0.55	0.5796
	12 mo	1.30 ± 1.98	0.8815
	Adult	1.60 ± 0.80	

At basal level, low expression of TNF-α+ IL-6+ and IL-12/23p40+ was observed in both infant and adult mDCs. In comparison to the adults, infants produced significantly lower percentages of TNF-α+ mDCs at the two week, six week, six month and twelve month time points in comparison to the adult group ($p<0.01$).

The percentage of IL-6+ mDCs was comparable between the infant and the adult groups at all the progressive time points, as no significant differences was observed in the percentages of IL-6+ mDCs between the infants and the adults at each subsequent time point.

A similar trend was observed in the percentage of IL-12/23p40+ mDCs. The percentages of IL-12/23p40 mDCs in infants at two weeks, six weeks, six months and twelve months showed no significant differences when compared to the adult group. Though, infants at 2 weeks showed a higher percentage of IL-12/23p40+ mDCs, which may be due to spontaneous cytokine responses produced by a few infants at that time point.

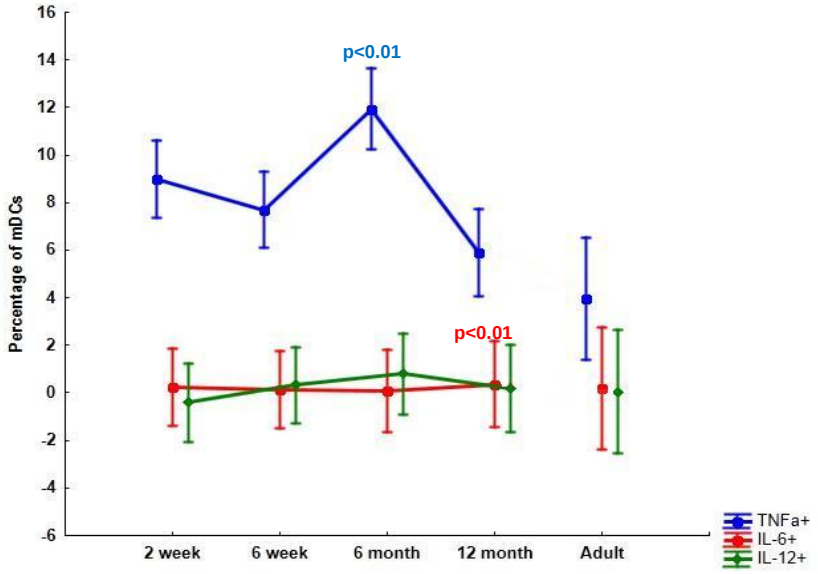

Figure 4.29 Percentage of single cytokine producers in mDCs in response to LPS. Figure illustrates TNF-α+, IL-6+ and IL-12+ single producers in infants versus adults. Vertical bars denote 95% confidence intervals.

In response to TLR4 stimulation, mDCs demonstrated high production of TNF-α+ and low production of IL-6+ and IL-12/23p40+. Although higher percentages of TNF-α+ mDCs were observed at 2 weeks and 6 weeks, no significant differences were observed at these time points when compared to the adult group. At six months, infants showed a significantly higher percentage of TNF-α+ mDCs when compared to the adult mDCs ($p<0.01$). No significant difference was observed in the percentage of TNF-α+ mDCs between infants at 12 months of age and the adult group.

The percentage of IL-6+ mDCs between the infants and adults showed no significant difference at the two week, six week and six month time points. At 12 months, infants showed a significantly higher percentage of IL-6+ mDCs when compared to the adult group ($p<0.05$).

No significant differences were observed in the percentage of IL-12/23p40+ mDCs in infants at two weeks, six weeks, six months and twelve months of age compared to the adult group.

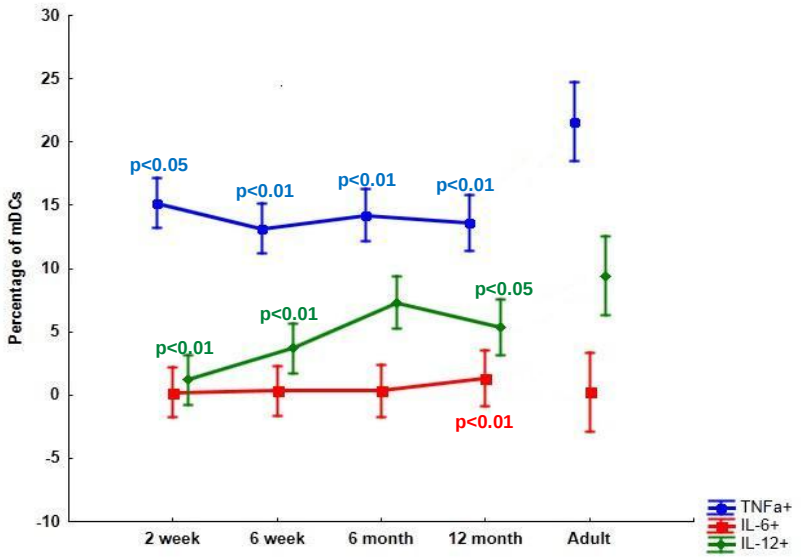

Figure 4.30 Percentage of single cytokine producers in mDCs in response to R-848. Figure illustrates TNF-α+, IL-6+ and IL-12+ single producers in infants versus adults. Vertical bars denote 95% confidence intervals.

In response to TLR7/8 stimulation, mDCs demonstrated high production of TNF-α+ and low production of IL-6+ and IL-12/23p40+. Infants showed significantly lower percentages of TNF-α+ mDCs at 2 weeks ($p<0.05$), 6 weeks ($p<0.01$), 6 months ($p<0.01$) and 12 months ($p<0.01$) of age when compared to the adult group.

The percentage of IL-6+ mDCs showed no significant difference between the adult group and the infant groups at the two week, six week and six month time points. At 12 months of age, the infants showed a significantly higher percentage of IL-6+ mDCs when compared to the adult group ($p<0.01$).

The percentages of IL-12/23p40+ mDCs were significantly lower in infants at 2 weeks and 6 weeks of age compared to the adults ($p<0.01$). At 6 months, the percentage of IL-12/23p40+ mDCs was comparable to adults as no significant difference was observed between the two groups. At 12 months, the percentage of IL-12/23p40+ mDCs showed a significant decline compared to the ($p<0.05$).

4.3.2.2. Double cytokine expression

Table 4.7 Percentage of double cytokine producers in mDCs at basal level. Table illustrates mean and SD of TNF-α+IL-6+, TNF-α+IL-12+ and IL-6+IL-12+ double producers in infants and adults as well as the p-values for the infant-adult comparison. Significant values are denoted in bold.

mDCs		Mean ± SD	Infant vs Adult (p-value)
TNF-α+IL-6+ % positive	**2 wk**	0.03 ± 0.04	0.0539
	6 wk	0.06 ± 0.05	0.1122
	6 mo	0.16 ± 0.33	0.8620
	12 mo	0.07 ± 0.18	0.1795
	Adult	0.17 ± 0.20	
TNF-α+IL-12+ % positive	**2 wk**	0.03 ± 0.04	**0.0007**
	6 wk	0.06 ± 0.05	**0.0024**
	6 mo	0.16 ± 0.29	0.2099
	12 mo	0.08 ± 0.15	**0.0127**
	Adult	0.24± 0.09	
IL-6+IL-12+ % positive	**2 wk**	0.02 ± 0.03	0.3190
	6 wk	0.03 ± 0.04	0.3514
	6 mo	0.09 ± 0.23	0.5498
	12 mo	0.01 ± 0.03	0.2019
	Adult	0.07 ± 0.07	

At basal level, low expression of TNF-α+IL-6+, TNF-α+ IL-12/12p40+ and IL-6+IL-12/23p40+ was observed in infant and adult mDCs. No significant differences were observed in the percentages of TNF-α+IL-6+ mDCs between infant and adult mDCs at any of the progressive time points.

The percentage of TNF-α+IL-12/23p40+ mDCs in infants was significantly lower in infants at the two week and six week time points compared to the adult group ($p<0.01$). At the 6 month time point, the percentage of TNF-α+IL-12/23p40+ mDCs in infants was comparable to the adult group. At 12 months, infants showed a significant decline in the percentage of TNF-α+IL-12/23p40+ mDCs when compared to the adult group ($p<0.05$).

The comparison of the percentage of IL-6+IL-12/23p40+ mDCs between the infants and the adults were not found to be significant at any of the progressive time points.

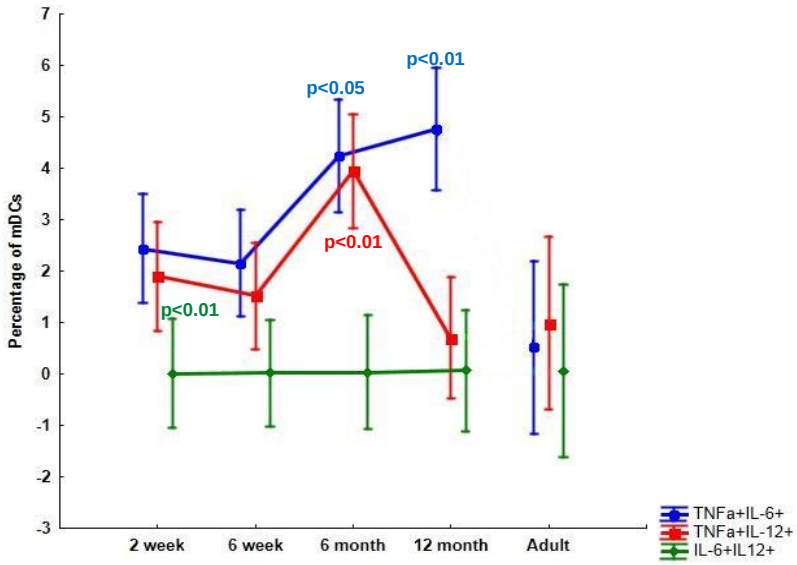

Figure 4.31 Percentage of double cytokine producers in mDCs in response to LPS. Figure illustrates TNF-α+IL-6+, TNF-α+IL-12+ and IL-6+IL-12+ double producers in infants versus adults. Vertical bars denote 95% confidence intervals.

In response to TLR4 stimulation, mDCs demonstrated high expression of TNF-α+IL-6+ and TNF-α+IL-12+ in infants and adults, but not IL-6+IL-12/23p40. At the two week and six week time points, no significant differences were observed in the percentages of TNF-α+IL-6+ mDCs between the infant and adult groups. Infants showed significantly higher percentages of TNF-α+IL-6+ mDCs compared to the adults at the six month ($p<0.05$) and twelve month time points ($p<0.01$).

No significant difference in the percentage of TNF-α+IL-12/23p40+ mDCs was observed between the infants and adults at the two and six weeks time points. At six months, infants showed a significantly higher percentage of TNF-α+IL-12/23p40+ mDCs when compared to the adult group ($p<0.01$). At 12 months, no significant difference was observed in the percentage of TNF-α+IL-12/23p40+ mDCs between infant and adult groups.

The percentage of IL-6+IL-12/23p40+ mDCs in infants at the two week time point was significantly lower than the adult group ($p<0.05$). No significant differences were observed for any other the subsequent time points when compared to the adult group.

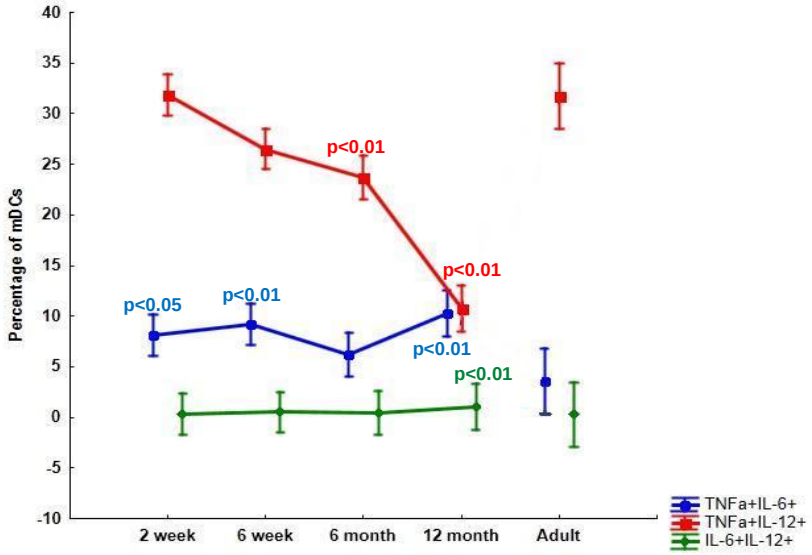

Figure 4.32 Percentage of double cytokine producers in mDCs in response to R-848. Figure illustrates TNF-α+IL-6+, TNF-α+IL-12+ and IL-6+IL-12+ double producers in infants versus adults. Vertical bars denote 95% confidence intervals.

In response to TLR7/8 stimulation, mDCs demonstrated high expression of TNF-α+IL-6+ and TNF-α+IL-12/23p40+ and low expression of IL-6+IL-12/23p40+ in both infant and adult groups. The percentages of TNF-α+IL-6+ mDCs were significantly higher in infants at two week ($p<0.05$) and 6 week ($p<0.01$) time points compared to the adult group. At six months, no significant differences wer observed in the percentage of TNF-α+IL-6+ mDCs between infants and adults. At 12 month a significant increase in the percentage of TNF-α+IL-6+ mDCs was observed in infants when compared to the adults ($p<0.01$).

The percentage of TNF-α+IL-12/23p40+ mDCs in infants at the two-week and six week time points showed no statistical significance when compared to the adult group. At 6 months of age, infants showed significantly lower percentages of TNF-α+IL-12/23p40+ mDCs in comparison to the adults, with a p-value of less than 0.01. This significant decline was also observed at 12 months of age when compared to the adult group with a p-value of less than 0.01.

The percentage of IL-6+IL-12/23p40+ mDCs between infants and adults showed no significant difference in infants at 2 weeks, 6 weeks and 6 months. At 12 months, infants showed a significantly higher percentage of IL-6+IL-12/23p40+ mDCs compared to the adults ($p<0.01$).

4.3.2.3. Triple cytokine expression

Table 4.8 Percentage of triple cytokine producers in mDCs at basal level. Table illustrates mean and SD of TNF-α+IL-6+IL-12+ triple producers in infants and adults as well as the p-values for the infant-adult comparison. Significant values are denoted in bold.

mDCs		Mean ± SD	Infant vs Adult (p-value)
TNF-α+IL-6+IL-12+ % positive	**2 wk**	0.01 ± 0.02	0.1632
	6 wk	0.02 ± 0.04	0.2686
	6 mo	0.08 ± 0.21	0.6075
	12 mo	0.01 ± 0.02	0.1621
	Adult	0.07± 0.07	

At basal level, low level expression of TNF-α+IL-6+IL-12/12p40+ was observed in both infant and adult mDCs. At each time point, no significant difference was observed in the infants at two weeks, six weeks, six months and twelve months in comparison to the adult group.

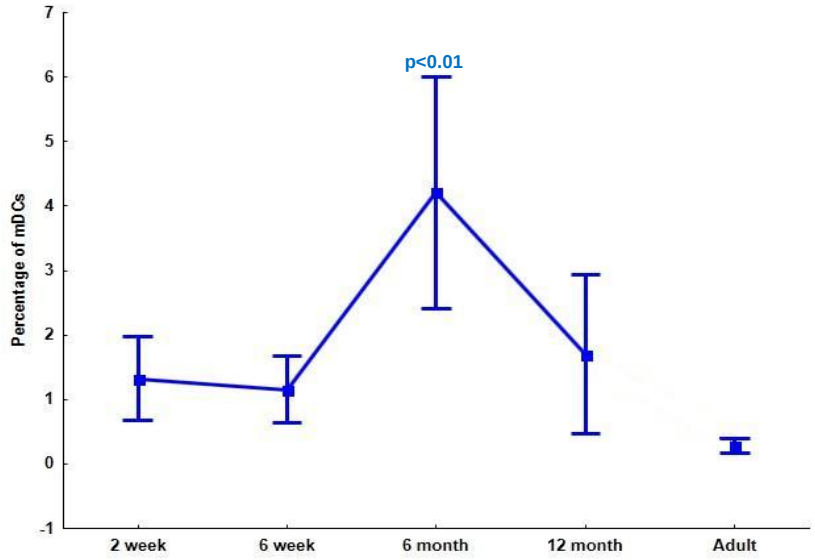

Figure 4.33 Percentage of triple cytokine producers in mDCs in response to LPS. Figure illustrates the expression of TNF-α+IL-6+IL-12+ mDCs in infants versus adults. Vertical bars denote 95% confidence intervals.

In response to TLR4 stimulation, low production of TNF-α+IL-6+IL-12/23p40+ was observed in infant and adult mDCs. No significant differences were observed in the percentage of TNF-α+IL-6+IL-12/23p40+ mDCs in infants at the two week and six week time points in comparison to the adult group. At six months, a significant increase in the percentage of TNF-α+IL-6+IL-12/23p40+ mDCs were observed compared to adult group ($p<0.01$). At 12 months, no significant difference was observed in the percentage of TNF-α+IL-6+IL-12/23p40+ mDCs between the infants and adults.

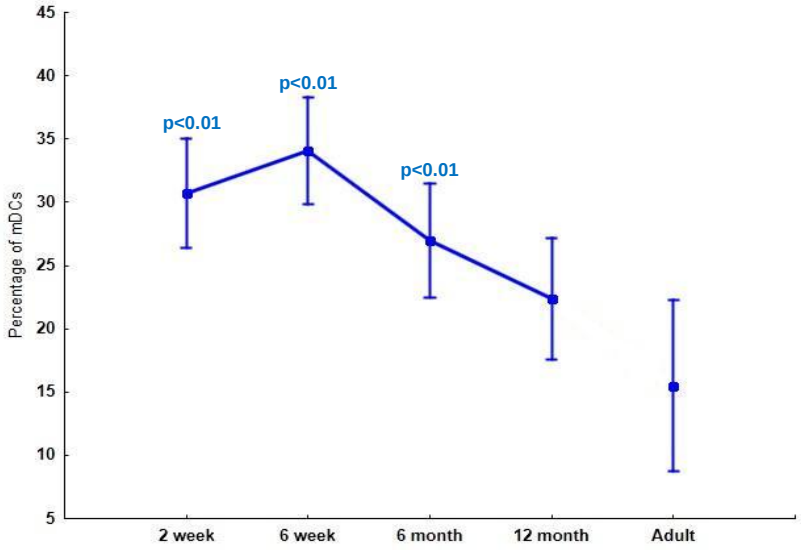

Figure 4.34 Percentage of triple cytokine producers in mDCs in response to R-848. Figure illustrates the expression of TNF-α+IL-6+IL-12+ producers in infants versus adults. Vertical bars denote 95% confidence intervals.

In response to TLR7/8 stimulation, robust production of TNF-α+IL-6+IL-12/23p40+ was observed in infant and adult mDCs. Significantly higher percentages of TNF-α+IL-6+IL-12/23p40+ mDCs was observed in infants at two weeks, six weeks and six months when compared to the adults ($p<0.01$). At 12 months of age, no significant difference in the percentage of TNF-α+IL-6+IL-12/23p40+ mDCs was observed between infants and adults.

4.3.3. Plasmacytoid dendritic cells

4.3.3.1. Single cytokine expression

Table 4.9 Percentage of single cytokine producers in pDCs at basal level. Table illustrates mean and SD of TNF-α+ and IFN-α single producers in infants and adults as well as the p-values for the infant-adult comparison. Significant values are denoted in bold.

pDCs		Mean ± SD	Infant vs Adult (p-value)
TNF-α+ % positive	**2 wk**	0.22 ± 0.44	0.4238
	6 wk	0.40 ± 0.59	0. 1453
	6 mo	0.78 ± 1.33	**0.0060**
	12 mo	0.17 ± 0.35	0.5605
	Adult	0.00 ± 0.00	
IFN-α+ % positive	**2 wk**	2.05 ± 5.32	0.9688
	6 wk	1.25 ± 2.01	0.5036
	6 mo	1.87 ± 1.60	0.9062
	12 mo	1.38 ± 1.92	0.5930
	Adult	2.00 ± 0.90	

At basal level, low level expression of TNF-α+ and IFN-α+ was observed in infant and adult pDCs. The percentage of TNF-α+ pDCs showed no significant difference in infants at the two week and six week time points in comparison to the adult group. The six-month time point revealed a significantly higher percentage of TNF-α+ pDCs in infants compared to the adult group ($p<0.01$). At 12 months of age, no significant difference was observed in the percentage of TNF-α+ pDCs in infants compared to the adults.

The percentage of IFN-α+ pDCs showed no significant differences in infants at two weeks, six weeks, six months and twelve months in comparison to the adult group.

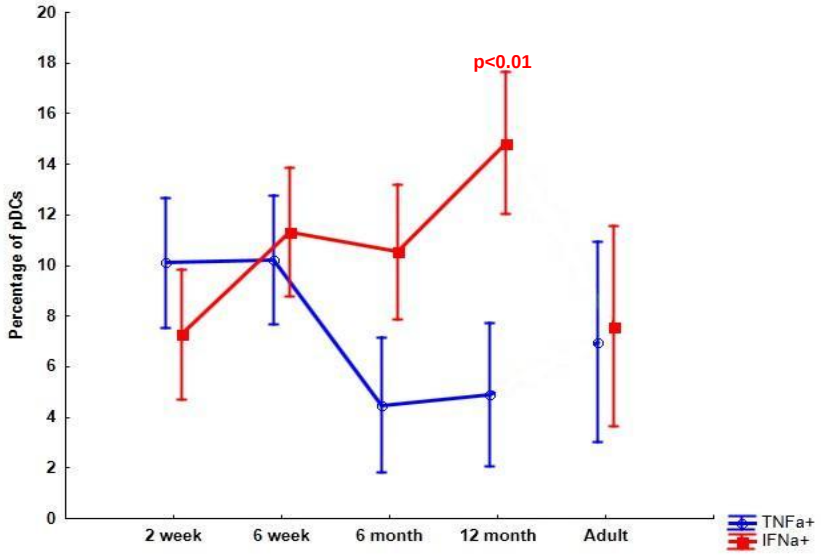

Figure 4.35 Percentage of single cytokine producers in pDCs in response to R-848. Figure illustrates the expression of TNF-α+ and IFN-α+ pDCs in infants versus adults. Vertical bars denote 95% confidence intervals.

In response to TLR7/8 stimulation, low expression of TNF-α+ and IFN-α+ was observed in infant and adult pDCs. The percentage of TNF-α+ pDCs showed no significant difference in infants at two weeks, six weeks, six months and twelve months of age in comparison to the adult group.

The percentage of IFN-α+ pDCs showed no significant differences in infants at two weeks, six weeks and six months in comparison to the adult group. At 12 months, infants showed a significantly higher percentage of IFN-α+ pDCs was observed in infants compared to the adult group ($p<0.05$).

4.3.3.2. Double cytokine expression

Table 4.10 Percentage of double cytokine producers in pDCs at basal level. Table illustrates mean and SD of TNF-α+IFN-α double producers in infants and adults as well as the p-values for the infant-adult comparison. Significant values are denoted in bold.

pDCs		Mean ± SD	Infant vs Adult (p-value)
TNF-α+ IFN-α+ % positive	**2 wk**	0.00 ± 0.01	0.9899
	6 wk	0.04 ± 0.09	0.8285
	6 mo	0.45 ± 0.99	**0.0116**
	12 mo	0.00 ± 0.00	1.0000
	Adult	0.00 ± 0.00	

At basal level, low expression of TNF-α+IFN-α+ was observed in infant and adult pDCs. No significant differences were observed in the percentage of TNF-α+IFN-α+ pDCs in infants at the two-week and six-week time points in comparison to the adults. At the six-month time point, a significantly higher percentage of TNF-α+IFN-α+ pDCs was observed in infants in comparison to the adult group ($p<0.05$). At the 12-month time point, no significant difference was observed in infant group in comparison to the adult group.

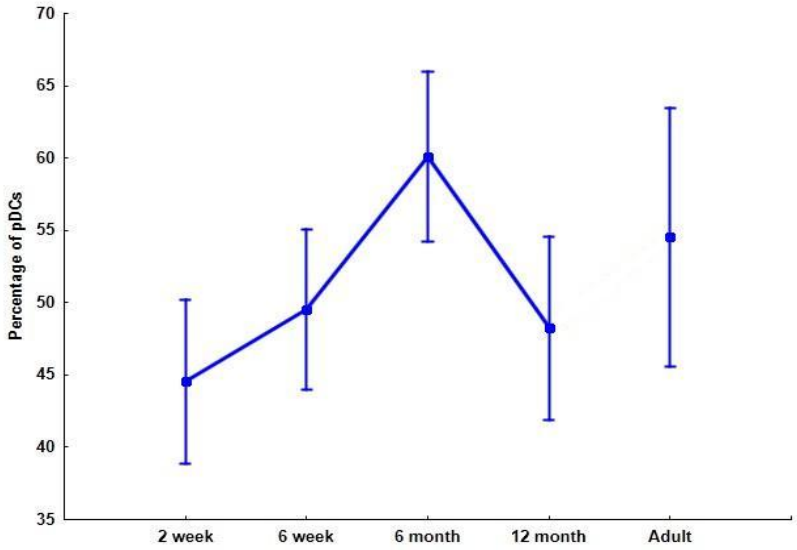

Figure 4.36 Percentage of double cytokine producers in pDCs in response to R-848. Figure illustrates the expression of TNF-α+IFN-α+ pDCs in infants versus adults. Vertical bars denote 95% confidence intervals.

In response to TLR7/8 stimulation, robust expression of TNF-α+IFN-α+ was observed in infant and adult pDCs. No significant differences were observed in the percentage of TNF-α+IFN-α+ pDCs in infants at two weeks, six weeks, six months and twelve months compared to the adults. The highest expression in infants was observed at the six-month time point and the lowest expression was observed at the two-week time point.

4.3.3.3. Triple cytokine expression

Table 4.11 Percentage of triple cytokine producers in pDCs at basal level. Table illustrates mean and SD of TNF-α+IFN-α+IL-6+ triple producers in infants and adults as well as the p-values for the infant-adult comparison. Significant values are denoted in bold

pDCs		Mean ± SD	Infant vs Adult (p-value)
TNF-α+IFN-α+IL-6+ % positive	**2 wk**	2.06 ± 5.32	0.9894
	6 wk	1.36 ± 2.17	0.8532
	6 mo	2.51 ± 2.66	**0.0197**
	12 mo	1.38 ± 1.92	1.0000
	Adult	2.04 ± 1.03	

At basal level, low level expression of TNF-α+IFN-α+IL-6+ was observed in infant and adult pDCs. No significant differences were observed in the percentage of TNF-α+IFN-α+IL-6+ pDCs in infants at the two-week and six-week time points in comparison to the adults. At the six month time point, a significantly higher percentage of TNF-α+IFN-α+IL-6+ pDCs was observed in infants in comparison to the adult group ($p<0.05$). At the 12-month time point, no significant difference was observed between the infant and adult groups.

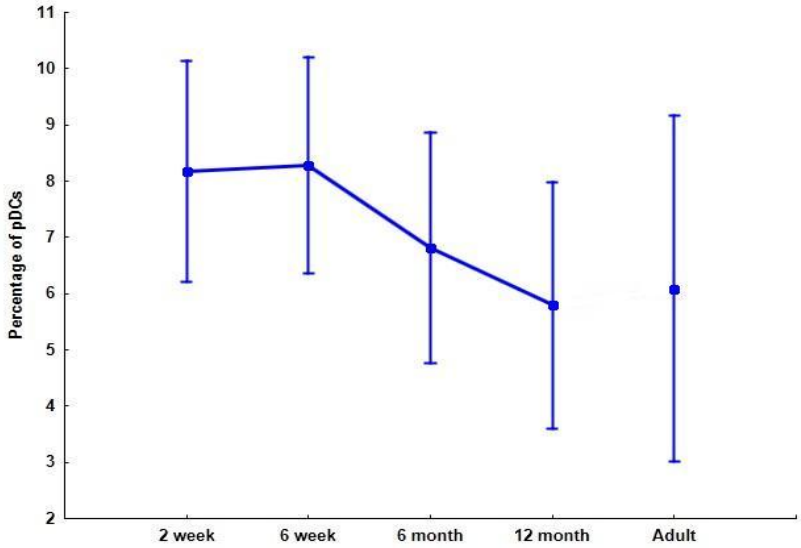

Figure 4.37 Percentage of triple cytokine producers in pDCs in response to R-848. Figure illustrates the expression of TNF-α+IFN-α+IL-6+ pDCs in infants versus adults. Vertical bars denote 95% confidence intervals

In response to TLR7/8 stimulation, low expression of TNF-α+IFN-α+IL-6+ was observed in infant and adult pDCs. No significant differences were observed in the percentages of TNF-α+IFN-α+IL-6+ pDCs in infants at the two week, six week, six month and twelve month time points when compared to the adults. The highest expression in infants was observed at the 2-week time pointand the lowest expression was observed at the 12 month time point.

Chapter Five

Discussion

5.1. Introduction

Neonates and young infants rely predominantly on their innate immune system for host defence against microbial invasion. Given the importance of the TLR system in innate immunity, several have described postnatal TLR-mediated innate immune function in neonatal cord blood versus adults (De Wit *et al*., 2003; Levy *et al*., 2004; Levy *et al*., 2006a; Levy *et al*., 2006b; Angelone *et al*., 2006; Belderbos *et al*., 2009b; Renneson *et al*., 2009). However, only a limited number of studies have investigated the longitudinal development of postnatal TLR-mediated responses in early childhood (Upham *et al*., 2002; Nguyen *et al*., 2010; Corbett *et al*., 2010). Moreover, very few studies have documented TLR-mediated cytokine responses in resource-limited or low-income countries (Burl *et al*., 2011; Teran *et al*., 2011); hence, very little information exists on these responses in a more pathogen-exposed, less resourced and therefore much more vulnerable infant population.

In South Africa, 16% of all deaths are caused by the environmental burden of disease, with diarrhoeal and respiratory infections being the major contributors to mortality (South African Environmental Burden of Disease, 2009). Intestinal infections and influenza/pneumonia infections, were found to be the leading causes of childhood deaths in South Africa, with a mortality rate of 17.4% and 11.7% within the first year, respectively (Statistics South Africa, 2009). Bacterial contamination of water sources has been found to be a major cause of enteric infections in South African rural communities (Momba *et al*., 2010). A study performed in the Eerste River/Kuils River demonstrated unacceptably high levels of faecal coliforms, of which 85% were *E.Coli* (Fourie, 2005), resulting in increased susceptibility to diarrhoeal disease. Another South African study observed, among others, increased incidence of diarrhoeal disease that was linked to densification of low-cost housing settlements, with higher incidences found in people living in main houses and in children less than 10 years of age (Govender *et al*., 2011). With the high burden of environmental and infectious disease, it is imperative to address the issue of maturation of specific TLR responses of the infants in this setting with a high disease burden.

In our study, we aimed to describe the ontogeny of TLR4- and TLR7/8-mediated *in vitro* cytokine response in South African infants to determine the development and stage at which infant's responses reach maturity in a country with a higher infectious disease burden. To our knowledge, this is the first study to document the longitudinal development of the cell-specific (monocytes, mDCs and pDCs) TLR-mediated cytokine responses in within the first year of life in a resource-limited setting. The longitudinal follow-up of our infant cohort during first year of life coincides with EPI schedule implemented in South Africa, depicted in Figure 5.1. (Department of Health, 2009) and the age at which infants have an increased susceptibility to infectious disease (Thaver & Zaida 2009). This is advantageous as it allows for the evaluation of TLR-mediated innate responses in relation to vaccination and against a background of increased microbial exposure, which is a common characteristic in developing countries.

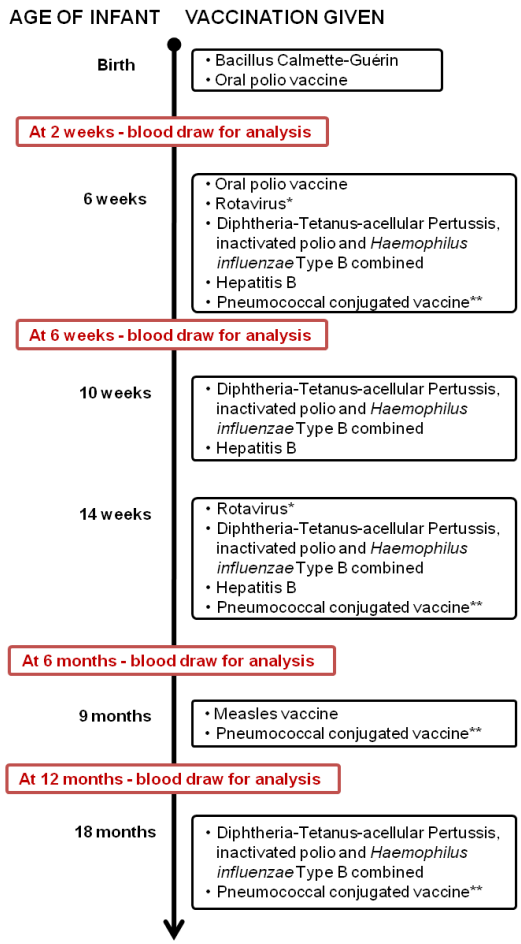

Figure 5.1 EPI vaccine schedule for study infants from birth to 18 months as well as blood draw time points. The Rotavirus vaccine and pneumococcal vaccine adminstration were not yet implemented at the time of the study, so all infants missed these two vaccinations.

5.2. Maturation of cytokine response in monocytes and mDCs

5.2.1. Cytokine-specific responses at basal level

Monocytes and mDCs populations are known to have a similar cytokine profile and will therefore be discussed together under this heading. Overall, monocyte and mDC populations produced low TNF-α, IL-6 and IL-12/23p40cytokine responses at basal level, in comparison to the responses to TLR4 and TLR7/8 stimulants.

Infant monocytes showed slightly lower, but not significant, TNF-α response at two and six weeks compared to the adult response, whereas in contrast to this mDCs showed significantly higher cytokine production when compared to the adult mDCs. This finding was in line with the data observed in the Gambian infant cohort, which found a significantly lower induction of TNF-α production at basal level (unstimulated control) in comparison to all the other TLR ligands, including TLR4 and TLR7/8 (Burl *et al.,* 2011). Similar findings to our study were seen in a Belgian infant cohort study published by Nguyen *et al* (2010). No robust production of TNF-α production was observed in cord blood stimulated at basal level (unstimulated control) compared to the adults. The IL-6 responses in monocytes and mDCs were similar in both infant and adult groups. Similar to the adults, low percentages of IL-6-producing were observed in infants at each time point, which indicates no robust IL-6 response at basal level. This finding in infants at two- and six weeks of age was in line with the Gambian cohort, which observed significantly low induction of IL-6 at basal level, in comparision to all the TLR stimulants, including TLR4 and TLR7/8 (Burl *et al*., 2011).

No robust IL-12/23p40 response was observed in infant or adult monocytes. The IL-12/23p40 responses in infant monocytes and mDCs were noticeably higher in infants at two weeks of age, though not statistically significant. Although, to our knowledge, no data has been published in response to the IL-12/23p40 cytokine response at basal level, we speculate that the probable cause of this response is due to spontaneous cytokine responses in some infants at this time point. Furthermore, Vanden Eijnden *et al* (2006) observed no significant difference in the IL-12/23p40 mRNA in neonatal and adult dendritic cells at basal level, suggesting no difference in IL-12/23p40 production between the two age groups. This is in contrast to our data at 2 weeks of age. The increase in IL-12/23p40 could suggest an increase in the production of IL-23 and consequently IL-17, indicating a response to BCG vaccination administered at birth. A previous study has observed an increase IL-12/23p40 production in

mice dendritic cells post-BCG exposure (Rothfuchs *et al.*, 2009), which together with IL-23p19 subunit, results in the production of IL-23, an important component of the differentiation of T_H-0 naïve cells to the T_H-17 pathway (Kollmann *et al*., 2009). This spontaneous increase could provide evidence of early protective T_H-17 responses to BCG vaccination.

At six months of age, infant monocytes showed no significant increase in the TNF-α response in comparison to the adults, whereas infant mDCs showed a significantly lower and higher TNF-α response respectively. At 12 months of age, the monocyte population showed no significant difference in the percentage of TNF-α-producing cells compared to the adults, though the response in mDCs remained significantly lower in the infants at 12 months of age compared to adults. In comparison to the Belgian infant cohort, the findings at six and twelve months of age were in line their data (Nguyen *et al*., 2010). No robust TNF-α production was observed in their infant groups at 6-9 months and 12 months of age, though no statistical significance was determined between their infant and adult groups at any of the time points

With respect to the Belgian infant study, our study findings were also comparable to their stimulation at basal level, as no robust IL-6 production was seen in their infants at birth, 3 months, 6-9 months and 12 months of age, in comparision to the adults (Nguyen *et al*., 2010). The same trend was observed for the IL-12/23p40 response in infant monocytes and mDCs at 6 weeks, 6 months and 12 months of age were similar to the adults. Unfortunately, no literature was found to compare our findings.

5.2.2. Age–dependant differences in infants and adult responses to LPS (TLR4)

The cytokine responses in monocytes and mDCs were overall similar in both cell types for infants as well as adults in response to LPS. We thus discuss them together here. We found an amplified capacity to produce TNF-α and IL-6 in neonatal monocytes and mDCs at two and six weeks of age in response to stimulation. In response to LPS, the IL-12/23p40 response was decreased in monocytes and similar in mDCs when compared to the adult group at both two and six weeks of age.

Various studies have observed diminished proinflammatory cytokine responses in cord blood, which were in contrast when compared to our findings in neonates at two weeks of age.

Decreased TNF-α responses was observed in cord blood monocytes and mDCs in Canadian infants (Kollmann *et al*., 2009; Corbett *et al*., 2010), although a previous Australian infant study reported similar TNF-α production (Yerkovich *et al*., 2007). Previous studies have also reported high IL-6 responses in cord blood monocytes (Yerkovich *et al*., 2007; Kollmann *et al*., 2009; Corbett *et al*., 2010) and mDCs (Kollmann *et al*., 2009; Corbett *at al*., 2010). Increased IL-12/23p40 cytokine responses was reported in cord blood monocytes (Yerkovich *et al*., 2007; Kollmann *et al*., 2009) as well as diminished IL-12/23p40 cytokine responses was observed in cord blood mDCs (Kollmann *et al*., 2009). Unexpectedly, our IL-12/23p40 responses were in agreement with previous Corbett *et al*, which reported higher percentages of IL-12/23p40-producing monocytes (Corbett *et al.,* 2010) and mDCs (Vanden Eijnden *et al*., 2006) in cord blood. In a Gambian infant cohort, neonates showed a decreased production of inflammatory cytokines at birth (cord blood) (Burl *et al*., 2011). Decreased TNF-α production was observed in response to TLR4 in cord blood, which was in contrast to the responses we observed at two weeks of age. However, Gambian infants showed a trend towards increased production of proinflammatory cytokines within the first month of life. At one month of age, these infants showed an increased TNF-α and production from birth (cord blood) (Burl *et al*., 2011). This was contradictory to our findings, as we observed similar TNF-α responses between two and six weeks of age, but an increased capacity to produce TNF-α at six weeks of age when compared to the adult group after TLR4 stimulation.

In our study, we observed an increased ability in expression of single cytokines in both monocytes and mDCs at two and six weeks of life compared to the adult group, namely with the TNF-α+ single-producing cells. There is a trend towards decreased expression of double and triple cytokine producing cells, TNF-α+IL-6+, TNF-α+IL-12+ and TNF-α+IL-6+IL-12+ by infant monocytes and mDCs. This finding was in line with the results published by Corbet *et al*, who found similar expression of single cytokine and polyfunctional cytokine producing monocytes and mDCs in cord blood as compared to the adults (Corbett *et al*., 2010).

At two and six weeks, we observed increased percentages of TNF-α-producing cells and percentages of IL-6-producing cells, with the percentage of monocytes and mDCs producing IL-6 was substantially lower compared to TNF-α. This was contradictory to previous studies performed on cord blood, where a high IL-6/TNF-α ratio was observed in resource-rich settings (Angelone *et al*., 2006; Nguyen *et al*., 2010) and was also reported in neonates from

Western Australia and Papua New Guinea (van den Biggelaar *et al*., 2009). This high IL-6/TNF-α ratio persisted within the first seven days of life, irrespective of exposure to infection *in utero* (Angelone *et al*., 2006). However, a Gambian infant study observed high IL-6/TNF-α ratios at birth, which at one month of age was in accordance with our study, with lower IL-6/TNF-α ratio observed due to the robust IL-6 production and increasing TNF-α production at one month of age (Burl *et al*., 2011).

The diminished cytokine responses to bacterial lipoproteins described in previous literature are due to inhibitory factors present in neonatal plasma. Neonatal plasma contains a 3-fold higher concentration of adenosine than adult plasma, which elevates intracellular cAMP levels. The elevated adenosine levels present in neonatal plasma and sensitivity of neonatal mononuclear cells to adenosine-induced cAMP accumulation consequently inhibits TNF-α production in response to bacterial lipoprotein stimulation (Levy *et al*., 2006ba). Adenosine receptor ligation has also been shown to suppress the LPS-induced TNF-α and IL-12 production upstream in the intracellular pathway, resulting in diminished cytokine production (Hasko *et al.*, 2000).

The observed hyperresponsiveness in our infant cohort at two and six weeks of age is may be due to the *Mycobacterium bovis* BCG vaccination administered after birth. Stimulation with BCG is known to elicit inflammatory responses through TLR2, TLR4 (Uehori *et al*., 2003) and TLR9 (Bafica *et al*., 2005) activation. Neonates have been shown to elicit an innate immune response to BCG stimulation. Exposure of cord blood to BCG antigens evokes an IFN-γ production from NK cells (Watkins *et al*., 2008; van den Biggelaar *et al*., 2009) and IL-12 production from CD14+ cells (Watkins *et al*., 2008). The expression of IL-12 by APCs activates NK cells, resulting in proliferation, cytotoxicity and cytokine production, such as IFN-γ (Borish *et al*., 2003; Watkins *et al*., 2008), as well as facilitating the differentiation of naïve CD4+ T lymphocytes into mature T_H-1 effector lymphocytes (Borish *et al*., 2003; Watkins *et al*., 2008). Additionally, neonatal DCs preferentially produce IL-12/23p40 and IL-23p19 in response to LPS stimulation (Vanden Eijnden *et al*., 2006), which together form the IL-23 heterodimer. Apart from IL-23, neonates also demonstrate an increased capacity to produce IL-6 and IL-1β, which are all key cytokines in the differentiation of naïve T-lymphocytes to the T_H-17 lineage (Kollmann *et al*., 2009). T_H-17 lymphocytes produce cytokines, such as IL-17 and IL-21, which trigger the release of antimicrobial peptides,

cytokines and chemokines that would result protection against extracellular bacteria and fungi by the production (Curtis & Way 2009). Immunisation against *Mycobacterium tuberculosis* has been demonstrated to confer protection via IL-17 cytokine production, which is critical for the regulation of the chemokines, CXCL9, CXCL10, and CXCL11. The IL-17 chemokine response initiates neutrophil and macrophage infiltration and recruitment of CD4+ T-lymphocytes producing IFN-γ, which subsequently restricts mycobacterial growth and controls infection (Khader *et al*., 2007; Curtis & Way 2009). Vaccination with BCG at birth could in turn favour the development of T_H-17 lineage for protection against pathogens in the postnatal period.

In response to BCG stimulation, neonates born in Papua New Guinea had a significantly higher production of IFN-γ, TNF-α and IL-6 at birth compared to neonates born in Western Australia (van den Biggelaar *et al*., 2009). This suggests a bias towards the early development of T_H-1 responses in neonates born in the low-income settings with high disease burden. The increased IL-10 production suggests the development of regulatory T-lymphocytes and immunosuppression in Papua New Guinea neonates (van den Biggelaar *et al*., 2009). Therefore, the production of IL-10 and proinflammatory cytokines to BCG and other antigens provides a balance between microbial defence and prevention of systemic inflammation in neonates exposed to an environment with increased microbial pathogen exposure.

A significantly heightened proinflammatory cytokine response was observed in our study in monocytes and mDCs of six-month-old infants, compared to two weeks, 6 weeks and 12 months as well as adult group. Our findings were in contrast to a Belgian infant study, which observed higher levels of TNF-α at six months compared to birth but similar to adult levels at this age. The IL-6 responses in this cohort were higher at birth but became comparable to adults at 3 months and remained stable at 6 months (Nguyen *et al*., 2010). In the Gambian infant study, the TNF-α and IL-6 levels peaked at one month of age and remained stable up to 12 months of age (Burl *et al*., 2011). Data from the single-, double- and triple cytokine expressing monocytes and mDCs followed the same trend with a significant increase in the expression of TNF-α+ and IL-12+ single cytokine producing cells as well as the TNF-α+IL-6+, TNF-α+IL-12+ and TNF-α+IL-6+IL-12+ polyfunctional cytokine producing cells. This is the first study to report on the polyfunctional expression of cytokines at six months.

Interestingly, the elevated LPS response in our study population coincided with the age of weaning and introduction of mixed feeding into the infants' diet as well as increased infectious morbidity observed at this time point.

In early life, infants receive passive immunity by breastfeeding through the transfer of immunomodulatory factors, including IgG, IgM and soluble IgA, cytokines (M'Rabet *et al*., 2008), as well as soluble TNF-α receptors and IL-1 receptor antagonists that suppress inflammatory activity in neonates (Buescher *et al*., 2001; M'Rabet *et al*., 2008). Apart from protective factors, human breast milk has the capacity to influence microbial recognition by modulation of the TLR-mediated response. Soluble TLR2 and CD14 receptors present in human milk augment the immune response (Labeta *et al*., 2000; M'Rabet *et al*., 2008). This has an enhancing effect on the binding and recognition of bacteria and could sensitise the neonatal gut to gram-negative bacteria. In addition, an 80-kDa protein identified in colostrum is associated with the enhanced TLR4 and TLR5 response observed in neonatal gut (LeBouder *et al*., 2006) aiding in the recognition of gram-negative and flagellated bacteria, which are common infectious agents in neonates and young infants (Zaidi *et al*., 2009).

Exclusive or predominant breastfeeding is associated with decreased risk of gastrointestinal and acute respiratory infections in infants, especially within low-income settings (Arifeen *et al*., 2001). The infants in our study were exclusively breastfed for up to a minimum of 12 weeks of age, with the gradual introduction of complementary foods and mixed feeding thereafter. This finding was in line with a WHO data bank survey performed in 2003-2004 where 11.8% of South African mothers exclusively breastfed their infants less than 4 months of age and 8.3% of mothers continued up to <6 months of age before mixed feeding or complementary food introduction (WHO Global Data Bank, South Africa). In contrast, a 2005-2006 survey revealed that 52.5% of Gambian mothers were found to breastfeed exclusively to <4 months of age and 40.8% of Gambian mothers exclusively breastfed to <6 months of age, after which complementary foods were introduced (WHO Global Data Bank, Gambia). Thus, South African infants are likely to exhibit lower levels of protective passive immunity acquired from breastmilk.

The incidence of diarrhoeal disease is shown to peak between 6-11 months of age, when infants are introduced to complementary foods and begin to explore their environment,

resulting in exposure to several infectious microbial pathogens (Dewey & Mayer 2011). However, subclinical infections can occur in infants due to the frequent exposure to pathogens. In developing countries, there is a high prevalence of a subclinical condition known as environmental enteropathy or tropical enteropathy. Environmental enteropathy is speculated to be caused by the chronic ingestion of pathogenic microorganisms, which leads to a high concentration of pathogens in the gastrointestinal tract, and consequently a continuous production of inflammatory cytokines in response to infection (Dewey & Mayer 2011).

The heightened inflammatory response to TLR4 stimulation at six months of age differed substantially from the Gambian infant cohort. This difference could be attributed to the different trends in breastfeeding of the mothers in the respective countries, with Gambian infants having better immunological protection through breastmilk. Consequently, with the increase in microbial load through mixed feeding as well as the environmental exposure at six months, infants within our setting demonstrated the development of an amplified inflammatory response and immune activation at this age as a result of exposure and/or infection. This phenomenon has adverse effects as during infection and immune activation nutrients are diverted away from growth, resulting in inadequate availability of nutrients to support growth and subsequent stunting during the infectious period (Dewey & Mayer 2011).

The LPS-induced TNF-α and IL-12/23p40 response in monocytes and mDCs reached maturation at 12 months of age, but the percentage of IL-6-producing cells remained distinctly higher than adults at 12 months of age. Our findings were in agreement with a Canadian infant cohort at one year of age with regards to IL-12/23p40 and IL-6 responses. The TNF-α response in their study differed from our findings as they observed significantly higher responses compared to the adults at one year of age (Corbett *et al.*, 2010). Similar to our results, Nguyen *et al* (2010) described the maturation of the TNF-α production in response to TLR4 at one year of age. The IL-6 response in our study was in disaccord with the abovementioned study, which reported maturation of IL-6 production at 3 months of age (Nguyen *et al.*, 2010). Burl *et al* (2011) describes continually high IL-6 production from birth to 12 months of age.

The single cytokine producing cells (TNF-α+, IL-6+, IL-12+) reached adult-like levels at 12 months of age in both monocytes and mDCs. This in contrast to what was observed by Corbett *et al* (2010), that reported diminished single cytokine production at one year compared to the adult group. Concerning the double-expressing polyfunctional cells, we observed maturation of TNF-α+IL-12+ but the expression of TNF-α+IL-6+ remained high compared to the adult group. In addition, the triple cytokine-expressing monocytes and mDCs remained higher than the adult group, but this was not significant. Corbett *et al* found that the double cytokine expressing cells were higher than the adults at one year of age for both cell types as well as a diminished ability to produce triple cytokine producing cells in response to LPS. This is in contrast with our data.

The early inflammatory responses and maturation of LPS-induced cytokine responses in South African infants demonstrates an enhanced ability to produce T_H-1 type cytokines. This observation may suggest that the early inflammatory maturation may be due to vaccination, increased microbial exposure and hence the necessity to develop protective immunity earlier in life compared to infants in a setting with lower disease burden. Also, these responses may support the hygiene hypothesis as infants with a background of increased microbial exposure have a decreased risk of developing allergies and atopic disease (Belderbos *et al*., 2009a). Furthermore, the robust responses to LPS in infancy suggests the suitability of TLR4 agonists, such as monophosphoryl lipid A (MPL-A), as vaccine adjuvants in infancy. MPL-A is derived from the lipopolysaccharide portion of *Salmonella minnesota*, is recognised by TLR4, and has been accepted as a low toxicity and efficient effective vaccine adjuvant able to elicit an immune response through TLR4 stimulation (Casella & Mitchell 2008).

In the Gambian study, high IL-6 and IL-1β production was observed from birth to 12 months of age (Burl *et al*., 2011). Apart from inducing T_H-17 lineage differentiation, IL-6 is a key inducer of acute phase proteins and a potent inflammatory inducer (with IL-1β) (Borish & Steinke 2003). Therefore, the infants may remain, in part, skewed towards the acute phase and T_H-17 differentiation responses.

5.2.3. Differential maturation in infants response to R-848 (TLR7/8)

R-848 is a potent immunostimulant known to activate host cells via TLR7/8 ligation. In monocytes and mDCs, R-848 stimulation induced strong cytokine responses in both the

infants and adults for all cytokines evaluated. This was similar to previous studies employing this or other TLR7/8 agonists (Levy *et al*., 2004; Kollmann *et al*., 2009; Corbett *et al*., 2010; Burl *et al*., 2011).

As with the LPS responses, a heightened TNF-α, IL-6 and IL-12/23p40 cytokine response to R-848 was observed in monocytes and mDCs of infants at two and six weeks of age compared to adults. This amplified response was higher compared to observations from previous studies, which observed lower percentages of TNF-α-, IL-6- and IL-12/23p40-producing cord blood monocytes and mDCs compared to adults to TLR7/8 (Kollmann *et al*., 2009; Corbett *et al*., 2010). However, one study observed impaired TNF-α production from cord blood monocytes for all TLRs evaluated, with the exception of TLR7/8. The variation in response to R-848 and TLRs activated by bacterial lipoproteins suggests different mechanisms of p38 MAP kinase phosphorylation upstream in the TLR activation pathway (Levy *et al*., 2004).

In infants at two and six weeks of age, we observed an increased ability in the single cytokine expression in monocytes, with TNF-α expression being the most prominent, as well as decreased single cytokine expression of TNF-α+ and IL-12+ mDCs compared to the adults. In comparison with a Canadian cohort, the monocyte responses were in contrast to their findings as they observed lower single cytokine expression in cord blood. However, the single cytokine expression in mDCs was similar to their findings in cord blood (Corbett *et al*., 2010). The expression of TNF-α+IL-6+ is significantly lower in infants at two and six weeks of age, while the expression of TNF-α+IL-12+, remains significantly higher at both two and six-week old infants compared to the adults. In a Canadian infant cohort, neonates demonstrated higher levels of double cytokine polyfunctionality in cord blood monocytes but lower levels of double cytokine polyfunctionality in cord blood mDCs in response to TLR7/8 stimulation (Corbett *et al*., 2010). The expression of triple cytokines TNF-α+IL-6+IL-12+ were higher in both cell types at two and six weeks of age, which was in agreement with Corbett *et al*, which observed similar results from neonatal monocytes and mDCs in cord blood (Corbett *et al*., 2010).

The significantly higher TLR7/8 response observed in our infant cohort at two and six weeks of age can be attributed to the scheduled EPI vaccines administered at birth. As part of the

EPI programme in South Africa, oral polio vaccine (OPV) and BCG is administered to neonates after birth. BCG is speculated to elicit protective responses by engagement of TLR8. Polymorphisms in the *TLR8* gene have been associated with increased susceptibility to pulmonary tuberculosis and decreased ability to induce TLR8 expression by macrophages after BCG exposure (Davila *et al*., 2008). OPV is also able to initiate an innate immune response by the stimulation of TLR7 and TLR8 on endosomal membranes. OPV, a live attenuated trivalent vaccine, is composed of three serotypes of poliovirus (Mueller *et al*., 2005; Nathanson & Kew 2010). The virus itself is an enterovirus with a genome composed of positive sense ssRNA (Mueller *et al*., 2005; Nathanson & Kew 2010) which is recognised by and innate immune response by activation through TLR7 and TLR8.

TLR8 is abundantly expressed in monocytes and mDCs (Gorden *et al*., 2005), and neonatal monocytes and mDCs preferentially generate robust TNF-α and IL-12p40/70 production via TLR8 stimulation. Therefore, the comparable inflammatory cytokine responses in between two and six week old infants and adults are attributable to two factors. Firstly, the efficacy of TLR8 agonist to elicit a response is through the mechanism of phosphorylating p38 MAP kinases and the prolonged degradation of IκB-α in the NF-κB activation pathway (Levy *et al*., 2006b). Secondly, due to the exposure of infant monocytes and mDCs to BCG and OPV at birth, infants have an amplified inflammatory cytokine response at two and six weeks of age with subsequent stimulation of TLR7/8.

At six months of age, divergent responses were observed in the infant monocyte and mDC populations. The TNF-α response in infant monocytes remained significantly higher at six months compared to the adults, whereas the TNF-α response in infant mDCs reached at state of maturity at this time point. The IL-6 response also remained noticeably higher in both cell types compared to the infant group. The IL-12/23p40 responses were considerably lower than early infancy (two and six weeks of age) and were comparable to the adults at six months for both monocyte and mDC populations. As this is the first study, to our knowledge, to evaluate cell-specific cytokine responses in infants at six months, we are unable to compare our findings to previous literature.

With the polyfunctional cytokine evaluation, differing responses were also observed between monocytes and mDCs at six months. The expression of TNF-α single cytokine in monocytes

reached maturation, though the TNF-α+ expression in mDCs remained lower than the adults at this time point. The IL-12+ single cytokine expression in monocytes at six months remained lower than adult levels but reached maturation in the infant mDC population at this age. The TNF-α+IL-6+ monocytes in infants at six months remained higher than the adult monocytes, however, the TNF-α+IL-6+ mDCs in infants at six months found to be comparable to the adult mDCs. The TNF-α+IL-12+ monocytes and mDCs were both significantly lower than the adult group at this age. TNF-α+IL-6+IL-12+ triple cytokine producing monocytes and mDCs at six months were significantly higher in infants compared to the adult monocytes and mDCs. Again, as this is the first study to evaluate cell-specific polyfunctional cytokine responses in infants at six months, we are unable to compare our findings to previous literature.

Interestingly, TNF-α response to R-848 in infant monocytes reached maturation at 12 months of age. This finding was different when compared to a previous Canadian cohort study where diminished TNF-α response showed at one year of life (Corbett *et al*., 2010). Moreover, the percentage of TNF-α-producing mDCs, as well as the percentage of IL-12/23p40-producing monocytes and mDCs showed a significant decline at 12 months when compared to the six-month time point. This observation was also in disaccord with Corbett *et al* (2010), which observed significantly lower percentages of TNF-α-producing monocytes, and significantly higher percentages of TNF-α-producing mDCs and IL-12/23p40-producing monocytes compared to adults. This study also observed maturation of IL-12/23p40 response in mDCs at one year of age (Corbett *et al*., 2010). In our study, the IL-6 responses in infant monocytes and mDCs remained consistently higher than adult monocytes and mDCs until the age of 12 months. This was only consistent with Corbett *et al* (2010) concerning the monocyte responses. Findings by Yerkovich *et al* (2007) also did not agree with our data, as they observed similar IL-6 production in cord blood and adult monocytes, which wanes by one year and two years of age.

The cytokine responses to TLR7/8 stimulation in infant monocytes and mDCs show fluctuation in maturation from six to 12 months of age. The TNF-α response in mDCs and IL-12/23p40 response in both monocytes and mDCs reached maturity at six months, whereas the TNF-α response in monocytes matured at 12 months of age. The decline in the adult-like proinflammatory cytokine responses from 6 months to 12 months of age suggests a trend

towards lower inflammatory profile in South African infants and may be an indication of early immune senescence due to the high inflammatory responses caused by the chronic exposure to pathogens. The IL-6 response in infant monocytes and mDCs did not reach maturity at 12 months. Corbett *et al* (2010) have observed increased IL-6 responses in infant monocytes and mDCs up to two years of age, thus possibly indicating maturation of IL-6 responses in later in childhood.

In the monocytes and mDCs, lower responses single cytokine-expressing TNF-α+ mDCs as well as IL-12+ monocytes and mDCs were observed in infants at 12 months, with the exception of TNF-α+ monocytes where maturation was reached at this time point. This is largely in agreement with the Corbett *et al* (2010) that observed lower single cytokine expression. The TNF-α+IL-6+ monocytes and mDCs at 12 months remained significantly higher than adults, while the TNF-α+IL-12+ monocytes and mDCs remained lower than the adults at 12 months. This was in contrast to the Canadian infant cohort as they observed similar double cytokine expression in monocytes and mDCs in infants at one year and adults (Corbett *et al*., 2010). Maturation of the TNF-α+IL-6+IL-12+ triple expressing monocytes and mDCs was observed at 12 months, which in monocytes were similar to the responses in the Canadian infant cohort at one year. However, this was in disagreement with the mDCs in this cohort as they observed higher triple cytokine responses at one year of age in response to TLR7/8 stimulation (Corbett *et al*., 2010).

Overall, infant monocytes and mDCs show early inflammatory cytokine responses and skewing towards T_H-1 cytokine maturation in response to R-848 stimulation. The early inflammatory response is possibly due to BCG and OPV immunisation, with early maturation of cytokine responses indicating development of adequate response by six months of age. However, the decline observed at 12 months suggests a trend to decreased inflammatory responses, which are as a result of regulatory mechanisms to prevention of systemic inflammation.

5.3. Maturation of the cytokine in infant pDCs

5.3.1. Cytokine-specific responses at basal level

Plasmacytoid DCs are the most potent cell type involved in antiviral defence and are the dominant producers of type I IFNs. In our study, we found that pDCs were the dominant source of IFN-α, as this was not produced by monocytes and mDCs in either adult or infant

groups. This was in agreement with Kollmann *et al* (2009) and Corbett *et al* (2010). The cytokine responses in pDCs at basal level showed an overall low cell-specific TNF-α, an IFN-α response in comparison to TLR7/8 stimulation.

Infant pDCs showed similar TNF-α and IFN-α cytokine responses at two and six weeks compared to the adult response. No robust expression TNF-α was observed in cells were observed at basal level. This finding was in agreement with in Danis *et al* (2008), which found no robust production TNF-α and IFN-α in purified cord blood pDCs at basal level.

At six months, however, a significantly higher TNF-α response was observed in infant pDCs, whereas the IFN-α response remained similar to the adult group. At 12 months of age, the TNF-α and IFN-α responses were similar to the adults group. For both the six months and twelve month time points, still no robust production was seen. Though this is an expected finding. No literature was found to enable us to compare our results to previously published data.

5.3.2. Maturation of the TLR7/8 response

Through activation of TLR7 and TLR9, pDCs secrete large amounts of type I IFNs during viral infection (Barchet *et al*., 2005; Cao & Liu 2007; Koyama *et al*., 2008). Neonatal and young infant pDCs, similar to monocytes and mDCs, show increased responsiveness to TLR7/8 agonist (R-848), though only produce TNF-α and IFN-α in response to stimulation. This finding was in agreement with Kollmann *et al* (2009) and Corbett *et al* (2010).The percentage of neonatal pDCs producing TNF-α and IFN-α at two weeks was similar to the adults. This was in contrast to previous studies performed on cord blood, where reduced production of TNF-α and IFN-α to TLR7 and TLR7/8 agonists was observed (Danis *et al*., 2008; Kollmann *et al*., 2009; Corbett *et al*., 2010). This trend continued in infant pDCs at six weeks.

The same trend was observed when comparing single cytokine TNF-α and IFN-α pDC responses. Comparable single cytokine responses were observed between the infants at two and six weeks of age and the adult group. This was in contrast to the findings from the Canadian infant cohort, which observed higher single cytokine expression in neonatal cord blood pDCs (Corbett *et al*., 2010). The double cytokine expression showed lower but

comparable TNF-α+IFN-α+ pDCs at two and six weeks in infants, which was also in contrast to the Canadian infant cohort, which found lower double cytokine expressing pDCs in neonatal cord, blood in response to R-848 (Corbett *et al*., 2010).

A previous study has associated defective IRF-7 translocation in neonatal pDCs with diminished IFN-α/β production, both during exposure to TLR7/8 agonist (R-848) and viral exposure (Danis *et al*., 2008). IRF-7 is expressed in large quantities in the cytosol of resting pDCs and translocates to the nucleus upon activation of TLR7 and TLR9, resulting in activation of IFN gene transcription and expression (Cao & Liu 2007; Danis *et al*., 2008). IRF-5 translocation and NF-κB activation is crucial for the production of inflammatory cytokines and upregulation of co-stimulatory molecules in pDCs (Cao & Liu 2007). The decreased inflammatory cytokine production described in previous literature indicates a maturational defect in IRF-5 pathway that is responsible for reduced TNF-α production in pDCs (Danis *et al*., 2008).

In our study, the heightened responsiveness in infant pDCs at two and six weeks of age is presumably due to administration of OPV at birth as part of the EPI programme in South Africa. OPV is able to elicit an inflammatory cytokine response in neonatal pDCs through activation of TLR7 on the endosomal membrane. Thus, subsequent stimulation of TLR7 by the TLR agonist, R-848, at the abovementioned time points results in the production of robust TNF-α and IFN-α responses observed in our cohort. This hyper-responsiveness of pDCs observed in early infancy in our cohort is an indication of the ability of neonates and young infants to mount robust antiviral responses to vaccination and possibly viral infection.

The TNF-α and IFN-α cytokine responses in infant pDCs reached maturation by the age of six months. At this time point, no significant difference was observed in the percentage of pDCs producing TNF-α and IFN-α between infants and adults. Although the TNF-α response showed a significant decline at 12 months, the IFN-α response in pDCs remained stable throughout the first 12 months of life. The maturation of the infant pDCs in our cohort reached maturity at an earlier time point. In comparison to the observations by Corbett *et al* (2010), the infant pDCs in our cohort reached maturity at an earlier time point compared to a Canadian infant cohort (Corbett *et al*., 2010). In comparison to adults, in this study they

observed equivalent TNF-α response and greater IFN-α+ response at one year of age (Corbett *et al*., 2010).

Maturation of the single and double-expressing pDCs were observed at 6 months of age, with little change observed at 12 months of age in infants. In the Canadian infant cohort, they observed adult-like responses at one year of age in infant pDCs, which is similar to our findings (Corbett *et al*., 2010).

5.4. The use of LPS and R-848 as vaccine adjuvants

The marked T_H-2 polarisation in neonatal immune responses presents a distinct problem concerning the vaccination. The innate immune response is crucial in vaccine efficacy as this is responsible for the antigen presentation and instruction of the adaptive immune function (Philbin& Levy, 2009). The use of vaccine adjuvants is to stimulate the immune system in order to enhance the efficacy of vaccines and is a crucial component in vaccine development (Casella & Mitchell, 2008).

5.4.1. LPS response

Within our study, we observed robust proinflammatory cytokine production in response to LPS stimulation, with similar or higher T_H-1 cytokine responses in infants. Infants showed early maturation of these LPS-induced responses within the first six months of life compared to the European and Canadian cohorts. This finding may suggest the efficacy and use of TLR4 agonists as vaccine adjuvants in infancy.

LPS is known to be potent immunostimulant due to its lipid-A component (Akira *et al.*, 2006). An attenuated production of LPS derived from *Salmonella Minnesota*, MPL-A has been recognised as a clinical adjuvant (Casella & Mitchell, 2008). Studies have revealed that MPL-A has a 0.08% lethality of LPS, while maintaining the immunogenicity of its parent (Casella & Mitchell, 2008). A previous study has found that MPL-A demonstrates dramatically less toxicity whilst still enhancing CD4+ clonal expansion, and reducing the levels of inflammatory cytokine (IL-1β, IL-6 and TNF-α) production (Thompson *et al*., 2008), thereby reducing the risk of systemic inflammation. Although TLR4 activation is observed with administration of certain vaccines, including BCG and *Haemophilus influenza* type B, further investigation is required to ascertain the efficacy of the MPL-A adjuvant for neonatal and infant adjuvant use.

5.4.2. R-848 response

Both the infant and adult group showed robust cytokine production upon R-848 exposure. At each time points, substantially higher TNF-α, IL-6 and IL-12/23p40 responses were observed with this specific TLR7/8 agonist. As with the LPS response, early maturation of proinflammatory responses were observed in the infants, which were in contrast to infant cohort studies in Europe and Canada, indicating the sensititvity of infants cells to the agonist and its efficacy in eliciting an immune response.

The robust T_H-1-polarisation in infant and neonatal pDCs to R-848 indicates a potential of this agonist as a candidate for vaccine adjuvant in neonatal and infant vaccines. The exposure of pDCs to imidazoquinolines, such as imiquimod (TLR7 agonist) and R-848 is shown to boost maturation of this cell type by the induction of robust proinflammatory cytokine and type I IFN production, upregulation of co-stimulatory marker expression as well as B-lymphocyte activation (Gibson *et al*., 2002). The efficacy of R-848 as a vaccine adjuvant has been established either alone or in combination with another TLR agonist. R-848 was successful as an adjuvant when covalently linked to HIV Gag protein in a Rhesus macaque model *in vivo*, with enhanced T_H-1 cellular and antibody responses (Wille-Reece *et al*., 2005). The combination of R-848 and TLR9 agonist CpG ODN forms a potent adjuvant for hepatitis B antigen (HBsAg) vaccination in mice. The TLR7/8/9 adjuvant consequently resulted in the T-lymphocyte differentiation into HBsAg-specific effector memory T-lymphocyte phenotype and IFN-γ-producing T-lymphocytes, as well as in higher HBsAg-specific antibody production (Ma *et al*., 2007). Thus, the success of this agonist in producing strong T_H-1 immune responses provides strong motivation for use in neonatal vaccination. However, the safety of this adjuvant in infancy requires further investigation.

5.5. Novel aspects and limitations of the study

To the best of our knowledge, this is the first study to characterise the longitudinal cell-specific TLR-mediated cytokine response of infants in a resource-limited setting. Here, we describe the progression of the TLR4- and TLR7/8-mediated responses in three major APCs (monocytes, mDCs and pDCs) at a single-cell level, using the ICS method described in previous studies (Jansen *et al*., 2008; Kollmann *et al*., 2009; Corbett *et al*., 2010). Preceeding studies have evaluated the ontogeny of the TLR-mediated cytokine response in supernatant

(Corbett *et al*., 2010; Nguyen *et al*., 2010), including resource-limited settings (Burl *et al*., 2011; Teran *et al*., 2011). The evaluation of cytokine production in supernatant provides a global comparison of cytokine levels, but does not allow for measurement of cell-specific cytokine production. In our study, we were able to provide information on precise, single-cell-specific cytokine production in response to TLR stimulation by using ICS.

Our study described the evolution of these responses within the first year of life as compared to the adults in a similar environment. This is of considerable value as previous studies have concluded that infants from countries with a higher disease burden may exhibit noticeably different innate immune profiles, including TLR-mediated responses influenced by BCG vaccination (van den Biggelaar *et al*., 2009). Thus, characterising the ontogeny of TLR responses in an African cohort is essential for the development and selection of appropriate vaccine adjuvants for neonates and infants in this setting.

Presently, this is the second study describing the maturation of the TLR-mediated responses within an African infant cohort. Several differences exist between our study and the Gambian infant cohort (Burl *et al*., 2011). The previously mentioned cross-sectional study evaluated the cytokine production to TLRs 1-9 in whole blood culture. The Gambian study recruited 120 infants into eight different age groups: birth and 1-, 2-, 3-, 4-, 6-, 9- or 12- months of age with 15 infants per age group. The main limitation of the Gambian study was that it only focused on the measurement of total cytokine secreted in cell-free supernatants, and did not evaluate the cell-specific cytokine production in response to TLR ligation. The total secreted cytokine measurements do not provide any insight into the various cell populations as per our current study.

Though limited to TLR4 and TLR7/8 responses, our study was able to delineate cell-specific cytokine responses in response to TLR ligation in the primary APCs. This allows the evaluation of cell-specific TLR responses, as several other leukocytes express TLRs and respond to ligation. For example, neutrophils (Hayashi *et al*., 2003; Iwasaki & Medzhitov, 2004) and NK cells (Hornung *et al*., 2002; Iwasaki & Medzhitov, 2004) are able to respond to TLR stimulation and therefore looking at cytokine production in supernatant may not provide an accurate representation of the APC function in response to TLRs. Furthermore, we

specifically focused on TLR4 and TLR7/8 responses due to the high incidence of infections caused by gram-negative and viral pathogens in infants in resource-limited settings.

Unfortunately, as a limitation, we were unable to assess the maturation of the anti-inflammatory cytokine responses or cytokine production in supernatant. Several other studies have reported higher production of IL-10 in the first few months of life (Belderbos *et al*., 2003; Nguyen *et al*., 2010; Burl *et al*., 2011), which remained elevated at 12 months of age (Nguyen *et al*., 2010). The bias towards high IL-10 production, especially to BCG, may aid in dampening the exuberant inflammatory responses to antigenic exposure in early life (Madura Larsen *et al*., 2007). This aspect was not evaluated in the current study. Therefore, in our study we speculate that the innate responses are skewed to T_H-1, due to the hyperresponsiveness in the infants. Our speculation that the innate responses of neonates are skewed towards a T_H-1 bias could neither be confirmed nor refuted.

Another disparity between our study and the Gambian cohort is the longitudinal follow up our infant cohort from 2 weeks to 12 months of age, evaluating their TLR-mediated cytokine response at each time point. This allowed us to document the development of these responses in the same infant cohort over time, thereby eliminating inter-patient variability that arises with recruitment of several infants within each age group. The only limitation in our follow-up was that we were unable to obtain cord blood from the infants to evaluate the TLR4 and TLR7/8 responses at birth. In addition, by inclusion of a comparative adult group, we were able to establish a baseline TLR-mediated response for South African infants as well as determine when these infants' responses reached a state of maturity within the first year of life. A summary of the findings in our study is presented is hereunder in Table 5.1.

5.6. Future work

Our study revealed different TLR-mediated inflammatory responses in our cohort compared to industrialised countries. This study was the first study to evaluate the TLR4 and TLR7/8 responses in APCs (monocytes, mDCs and pDCs) in an African cohort. However, in order to address the limitations in our study, further investigation is required. An important aspect for future research is evaluation of TLR2/1, TLR3, TLR5, TLR2/6, and TLR9 responses within this cohort. This will provide a more comprehensive evaluation of the infant's innate response to infectious agents and for the selection of appropriate adjuvants in settings with

higher incidence of disease. Furthermore, as this was a small cohort, a larger cohort study is necessary to validate our findings.

Table 5.12 Summary of the main results

Parameter measured	Response at 2 weeks compared to adult	Response reaches adult level at
LPS response		
Monocytes		
TNF-α	▲	12 months
IL-6	▲	>12 months
IL-12/23p40	▲	12 months
mDCs		
TNF-α	▲	>12 months
IL-6	▲	>12 months
IL-12/23p40	▲	12 months
R-848 response		
Monocytes		
TNF-α	▲	12 months
IL-6	▲	>12 months
IL-12/23p40	▲	6 months
mDCs		
TNF-α	▲	6 months
IL-6	▲	>12 months
IL-12/23p40	▲	6 months
pDCs		
TNF-α	▲	6 months
IFN-α	▲	6 months

In addition, the comparison of our findings to other cohorts globally will provide us with a better understanding of the disparate cytokine responses in our cohort to other countries and impact of environmental and social factors on these responses.

An area of future interest is the parallel measurement of both T_H-1 and T_H-2 cytokines. This will enable the differentiation and quantification cell-specific proinflammatory and anti-inflammatory cytokine production in APCs. Jansen *et al* (2008) has previously described the ICS method employed in our innate immune analysis, which is adaptable to include several

markers of interest, specifically T_H-2 type cytokines such as IL-10 and IL-4. By employment of this modified multiparameter flow cytometric platform, it would provide a basis to determine the maturation of both proinflammatory and anti-inflammatory responses within the first year of life.

A much broader assessment of both cell-specific and secreted cytokines is the coupling of the multiparameter flow cytometric ICS method with multiplexed cytokine bead array. This platform was employed in the Canadian infant cohort studies (Kollmann *et al*., 2009; Corbett *et al*.,2010) to allow simultaneous analysis of cytokine production by extracellular secretion (via multiplexed bead array) and cell-specific production by multiparameter flow cytometry (via ICS) to assess both T_H-1 and T_H-2 mediated cytokine production. This robust platform would provide a more comprehensive comparison of the maturation of infant's innate response to TLR stimulation over time and provide a better understanding of the innate immune response in infants and adults alike.

In the evaluation of adaptive immunity, further investigation is required to assess the vaccination responses in this setting. According to the literature, vaccinated infants had a low capacity to mount T_H-1 cytokine responses to diphtheria, tetanus (Upham *et al*., 2006) and polio antigens (Vekemans *et al*., 2002) even with adequate IgG production (Upham *et al*., 2006). However, Upham *et al* concluded that this reduced response was primarily due to the diminished antigen presentation in infants up to 12 months. With the increased inflammatory responses in infants within our setting, evaluation of the vaccine-specific T-lymphocyte responses would be beneficial to delineate the vaccine-specific response in this inflamed background.

Taken as a whole, the further characterisation of the innate or adaptive immune responses in South Africa will provide a comprehensive description of the infant's innate response to infectious agents as well as aiding in the selection of appropriate vaccine adjuvants in settings with higher incidence of disease.

Chapter Six
Conclusion

In recent years, a substantial amount of evidence has accumulated of significant differences between adult and neonatal innate immune responses and the possible mechanisms that could explain these observations. To the best of our knowledge, this study represents the first longitudinal, cell-specific study describing the TLR-mediated innate immune profile of infants within the first year of life in a resource-poor setting. The findings in our present study differ significantly from most published literature in resource-rich countries. Instead, South African infants appear to have increased or adult-like inflammatory cytokine profile within the first six weeks of life to both TLR4 and TLR7/8 stimulation. These robust TLR4- and TLR7/8-mediated innate responses in infants may be related to BCG and OPV vaccinations administered at birth, suggesting an adequate T_H-1 responsiveness to neonatal vaccination in South African neonates.

A novel finding in our study when compared to the Gambian, Canadian and European cohorts is that infants in our setting tend to have an increased inflammatory response at six months of age. This may be caused by the subsequent exposure to and infection with pathogenic microorganisms at the time of breastmilk weaning in this high disease burden setting. Maturation of most cytokine responses was reached within the first year of life. We speculate that infants in our setting display an early T_H-1 polarisation and maturation of proinflammatory responses in this cohort reflects the impact of environmental exposure to pathogens, thereby priming the infants to develop adequate T_H-1 responses for protective immunity to infection. This needs to be ascertained withT_H-2 cytokine investigation Furthermore, as with the Gambian infant study, the efficacy of TLR4 and TLR7/8 agonists to produce robust responses in infants advocates the use of these agonists as adjuvants for childhood vaccine.

Therefore, in our study with the currently acquired knowledge, on the developmental changes in TLR4 and TLR7/8-mediated responses in South African infants, we conclude that infants in this high disease burden setting show increased capacity to develop T_H-1 responses from birth, due to vaccination and environmental exposure, with maturation seen earlier in life. This response suggests a mechanism to conferring protection through T_H-1 and/or T_H-17 pathway and possibly adequate adaptive immunity. These observations should be taken into consideration during the development of age-specific interventions for vaccination or selection of vaccine adjuvants for this group of highly susceptible infants in early life.

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Addendum A

Ethical approval

Longitudinal follow-up of the infant cohort

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot • your knowledge partner

13 March 2009 **MAILED**

Dr M Esser
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P O Box 19063
Tygerberg
7505

Dear Dr Esser

"Pilot Study of Innate Immune Abnormalities in HIV exposed Uninfected infants."

ETHICS REFERENCE NO: N08/10/289

RE : URGENT

At a meeting of the Committee for Human Research that was held on 10 November 2008 the above project was approved on condition that further information that was required, be submitted.

This information was supplied and the project was finally approved on 12 March 2009 for a period of one year from this date. This project is therefore now registered and you can proceed with the work. We do howeever urgentley request that you submit the following outstanding documents:
- The signed MTA form (before 1 April 2009)
- Translated Xhosa Information Consent Form (when available)
- Please place a note on the Information Consent Form that the local anesthetic (a cream) should be applied 20-30 min before venepuncture - not "if necessary'. (submit changed information consent form to this office before 1 April 2009)

Please quote the above-mentioned project number in ALL future correspondence.

Please note that a progress report (obtainable on the website of our Division: www.sun.ac.za/knowledgepartner/committees_CHR.htm) should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly and subjected to an external audit.
Translations of the consent document in the languages applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239
The Committee for Human Research complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Kind regards

13 March 2009 11:51 Page 1 of 2

Fakulteit Gesondheidswetenskappe · Faculty of Health Sciences

Verbind tot Optimale Gesondheid · Committed to Optimal Health
Afdeling Navorsingsontwikkeling en -steun · Division of Research Development and Support
Posbus/PO Box 19063 · Tygerberg 7505 · Suid-Afrika/South Africa
Tel.: +27 21 938 9075 · Faks/Fax: +27 21 931 3352

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jou kennisvennoot · your knowledge partner

Approval Date: 12 March 2009 Expiry Date: 12 March 2010

Yours faithfully

MRS MERTRUDE DAVIDS
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 938 9207 / E-mail: mertrude@sun.ac.za
Fax: 021 931 3352

Fakulteit Gesondheidswetenskappe · Faculty of Health Sciences

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11 June 2009

MAILED

Dr M Esser
Department of Pathology
Head of Immunology
Med Microbiology, 9th floor
Tygerberg
7505

Dear Dr Esser

"Pilot Study of Innate Immune Abnormalities in HIV exposed Uninfected infants."

ETHICS REFERENCE NO: N08/10/289

RE : AMENDMENT

Your letter dated 05 June 2009 refers.

The Chairperson of the Health Research Ethics Committee approved the amended documentation in accordance with the authority given to him by the Committee.

Yours faithfully

MRS EL ROHLAND
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 938 9677 / E-mail: elr@sun.ac.za
Fax: 021 931 3352

11 June 2009 11:23

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Inclusion of the adult samples for comparative analysis

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16 March 2011 **MAILED**

Dr M Esser
Department of Pathology
Head of Immunology
Med Microbiology, 9th floor
Tygerberg
7505

Dear Dr Esser

"Pilot Study of Innate Immune Abnormalities in HIV exposed Uninfected infants."

ETHICS REFERENCE NO: N08/10/289

RE : AMENDMENT

Your letter dated 18 February 2011 refers.

The Chairperson of the Health Research Ethics Committee approved the amended documentation in accordance with the authority given to him by the Committee.

The following amendments were approved:
1. Waiver of consent for use of adult samples is granted.
2. Request to perform HIV testing on stored plasma samples is granted.

Yours faithfully

MRS MERTRUDE DAVIDS
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 938 9207 / E-mail: mertrude@sun.ac.za
Fax: 021 931 3352

Addendum B

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM
AMENDMENT AND STUDY EXTENSION, SEPTEMBER 2009

REFERENCE NUMBER: **N08/10/289**

PRINCIPAL INVESTIGATOR: **Dr M. Esser**
CONTACT NUMBER: **021 938 4032**

ADDRESS: **Department of Pathology, Immunology Unit, 9th Floor, Core Laboratory, Tygerberg Hospital**
STUDY NURSE PHONE: **0765623119**

You are being invited to take part in this research project with extension to 2, 6,12 weeks, 6, 12 and 18 months visits which will also include questions relating to the food that your baby drinks and eats. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part. Your child will still be given best treatment.

This study has been approved by the **Committee for Human Research at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

- The study will be conducted at Tygerberg Hospital. We are looking at new blood tests to explain why some babies whose parents are HIV positive have severe infections even though they are not infected with the virus.
- 5ml blood – about one teaspoon full, will be taken from your child's vein at each of the visits in the KID CRU research ward by a person experienced in drawing blood from babies. Soothing and pain relief will be provided with a local anaesthetic cream (which makes pain less felt).
- Nothing will be injected into your baby.
- Different laboratory tests will be done on different parts of your baby's blood (whole blood, serum, plasma) to evaluate the general health and functioning of the immune system of your baby.

- You may withdraw your baby from the study at any time without any negative impact on the future treatment of your baby at this or any other medical facility. Please inform the study nurse if you wish to do so.

What about future investigations on current or new samples?

- *If you give additional consent, any unused blood will be stored for future analysis to test for **new markers in the immune system that may help us predict the chance of getting infections more easily**, after obtaining extended approval from the Committee for Human Research. These tests will not increase the volume of blood collected from your child.*
- *This consent is not linked to the primary participation in the study and you may refuse the storage of your baby's samples without withdrawing from the primary study.*
- *You may also be recalled for additional samples if the volume collected at the routine visit seems to be insufficient or if blood clots appear in the routine sample.*
- *The unused samples will be stored for a period up to 10 years and all unused samples will be destroyed after this time. All samples will be stored at the Immunology and/or Virology laboratory, Tygerberg Hospital.*
- *Only the investigators will have access to your child's information. Your child will receive a unique code number when enrolling in the study. Any reference to your child will only be via the code number.*

Why have you been invited to participate?

You have been asked to participate, because your baby may have been in contact with HIV during your pregnancy or you may have been selected for the control study group if your baby was not exposed to HIV in pregnancy. Some children who have been born to HIV positive mothers suffer from severe infections in the early years of life even though the tests for HIV are negative. We aim to look at blood tests which may predict the onset of such infections earlier so that babies can be treated and protected better from infections.

It may be that your child may not yet be able to fight infections normally and our tests may help to show what this abnormality is by testing the immune system very early in life. We hope that children may benefit in future from the findings of this study through guidelines for improved care, even if your child may not benefit directly now.

What will your responsibilities be?

- *You are not required to do anything when you give consent.*

Will you benefit from taking part in this research?

Your child will be examined carefully and we may be able to advise you on additional medication and healthy feeding practices which may help your child.

Are there in risks involved in your taking part in this research?

- *There may be a bit of bleeding or bruising where the blood has been drawn.*

If you do not agree to take part, what alternatives do you have?

- *You do not have to partake in this study and your child will receive the best available treatment. You may also withdraw your child from one or both parts of the study at any stage without impacting on your child's future treatment at this or any other medical facility.*

Who will have access to your medical records?

- *The medical records of your child will at all times be kept with the doctors looking after your child. Any part of the study that may be communicated to other doctors, meetings or medical journals will be confidential and the name of the patient remains unknown.*

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

- *Other than a small amount of blood oozing from the vein there should be harm to your child.*

Will you be paid to take part in this study and are there any costs involved?

- *A compensation of R70.00 will be given for each visit for partaking in the research project. There will be no costs involved for you, if you do take part. Your transport costs will be covered and your child will receive a free medical examination by a qualified child care trained doctor at each visit. Also free HIV counselling is provided at each visit if you would like to make use of this.*

Is there any thing else that you should know or do?

- *You can contact Dr M Esser at Tel 021-938 4032 or 082 883 4216 if you have any further queries or encounter any problems.*
- *You can contact the Committee for Human Research at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.*
- *You will receive a copy of this information and consent form for your own records.*

<u>Declaration by participant</u>

1. By signing below, I ……..…….……. agree to take part in a research study entitled "PILOT STUDY OF INNATE IMMUNE ABNORMALITIES IN HIV EXPOSED UNINFECTED INFANTS".

And / or

2. By signing below, I ……..…….……. agree that my child's blood samples may be stored and analysed further at a later stage as set out in the informed consent above.

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....…….. on (*date*) ……...……..

…….. ……..

Signature of participant **Signature of witness**

Declaration by investigator / person obtaining consent

I *(name)* ……..…… declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use an interpreter. (*If a interpreter is used then the interpreter must sign the declaration below)*

Signed at (*place*) .....…….. on (*date*) ……...……..

…….. ……..

Signature of person obtaining consent **Signature of witness**

…….

Signature of investigator

Declaration by interpreter

I *(name)* ……..…… declare that:

- I assisted the investigator (*name*) …… to explain the information in this document to (*name of participant*) …….. using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .....…..…….. on (*date*) ……....……...

…… ……..

Signature of interpreter **Signature of witness**

Addendum C

Innate Immune Abnormalities in HIV Exposed Uninfected Infants

PILOT STUDY

(MATERNAL)

Study Number: _____

Hospital folder number: _____ **Ward:** _____

Maternal visit **Date:** _____/_____/2009

1. Date of birth _____/_____/_____
2. Age at delivery _____ years
3. Highest schooling level achieved:
   - Primary school (grade 1 - 7)
   - Some high school (grade 8 - 11)
   - Grade 12
   - Tertiary education, specify: _____
4. House currently living in:
   - Formal
   - Informal
5. How many people live in the mother's household (including the mother)? _____
6. How many people share the bedroom with the baby and the mother? _____
7. Is there access to running water (tap) inside or outside the house? _____
   - Inside
   - Outside
8. If no, what is the source of water? _____
9. What is the main source of income for the mother's household?
   - Salary /
   - Any government grant / Other, specify: _____
10. Current marital status?
    - Single / unmarried
    - Married / living as married

11. Did the mother smoke during pregnancy?
    - ☐ Yes, if yes, how many per day: _____
    - ☐ No
12. Did the mother use alcohol during this pregnancy?
    - ☐ Yes, _____ drinks per day / week / month (circle indicator)
    - ☐ No

Medical and Obstetric History

13. Gravida: Para: Miscarriage: Ectopic ____:____:____:____
14. Gestational age (GA) at booking: _____weeks
15. Obstetric history

a. Year	b. Gestation	c. Delivery Method	d. Weight	e. Sex	f. Outcome	g. Complications

16. Last Normal Period ___/___/20__
17. Ultrasound (date & gestation) ___/___/20__ ___w___d

18. Medication during pregnancy (1 = yes; 2 = no) _____
    If yes, specify:
    _____
19. Chronic illness _____
    (No = 0; Hpt = 1; DM = 2; Anaemia = 3; Heart disease = 4; Psychiatric = 5; TB = 6; Other = 7)
20. Pregnancy complications _____
    (No = 0; PE = 1; Hpt = 2; DM = 3; Anaemia = 4; APH = 5; PROM = 6; PPROM = 7; PTL = 8; Placenta praevia = 9; Other = 10)
21. Total antenatal visits _____
22. HIV diagnosis
    a. Positive = 1; Negative = 2 (if 2, go to Q23) _____
    b. Diagnosed during current pregnancy = 1; prior to pregnancy = 2) _____
    a. If 22b = 1, what was the GA at diagnosis? _____weeks
23. CD4 count _____
24. Was mother on dual therapy (1) or HAART (2) _____

25. Date therapy started
    ___/___/20__

26. GA when therapy started
_____weeks

27. Diagnosed with genital infections during pregnancy?
☐ Yes, specify: _____
☐ No

28. Has the mother been treated for any of the following conditions prior to delivery?
a. Tuberculosis, treated for ≥ 2/12 ☐ Yes ☐ No (if treated for <2/12, baby not eligible)
b. Syphilis, on treatment ≥4 weeks ☐ Yes ☐ No (if treated for <4/52, baby not eligible)

29. Has the mother been hospitalized during this pregnancy?
☐ Yes, _____ time(s)
☐ No

Give primary reason and dates for admissions:
a. _____
b. _____
c. _____
d. _____

Addendum D

INNATE IMMUNE ABNORMALITIES IN HIV EXPOSED UNINFECTED INFANTS

PILOT STUDY

(INFANT)

Study number: _____

Hospital folder number: _____

Visit 1 (BIRTH)

1. Date of birth

   _____/_____/2009

2. GA at birth: _____ weeks

2a. Determined by: □ obstetric history / □ LMP / □ ultrasound

3. Mode of delivery _____

   (NVD = 1; Breech = 2; Forceps = 3; Vacuum extraction = 4; C/S = 5)

   If 3, 4 or 5, specify reason:

   _____

4. Duration of ROM: _____ h_____ min

5. Birth weight

   _____

5a. □ Large for GA (>90%),

   □ Appropriate for GA

   □ SGA (<10%)

   □ Not evaluated

6. APGAR

   ___:___:___

7. Birth Length:

   _____cm

8. Head Circumference:

   _____cm

9. Was there a break/cut in the skin?

   □ Yes □ No

10. Gender:

□ Male □ Female

11. Racial group

□ African (specify, _____) / □ Coloured / □ White / □ Asian

12. Any minor congenital anomalies / birth defects identified at birth?

□ Yes □ No (e.g. extra digits – no functional impairment)

If yes, specify:

_____

13. PHYSICAL EXAMINATION OF INFANT

Examination	Normal	Abnormal	Comment
HEENT [head,eyes,ears,nose,throat]			
Cardiovascular			
Chest lungs			
Abdominal			
Liver			
Spleen			
Musculoskeletal			
Genitourinary / rectal			
Skin			
Lymph nodes			
Extremities			
Neurological function			
Other			

14. Did infant receive any medication?

☐ Yes (complete table below) ☐ No

Medication name	Start date	Stop date / ongoing	Indication

15. Feeding method

a. Formula
b. Breast
c. Mixed feeding (including solids)

Signature: _____ **Date:** _____/_____/2009

Addendum E

INNATE IMMUNE ABNORMALITIES IN HIV EXPOSED UNINFECTED INFANTS

PILOT STUDY

(INFANT)

Visit __

1. Weight: _____ grams or □ not done
2. Length: _____ cm or □ not done
3. Head circumference: _____ cm or □ not done
4. Temperature: _____ °C or □ not done
   Method: □ Axillary; □ Oral; □ Rectal; □ Tympanic; □ Other: _____
5. Heart Rate: _____ pm or □ not done
6. Respirations: _____ pm or □ not done
7. PHYSICAL EXAMINATION OF INFANT

Examination	Normal	Abnormal	Comment
HEENT[head,eyes,ears,nose,throat]			
Cardiovascular			
Chest lungs			
Abdominal			
Liver			
Spleen			
Musculoskeletal			
Genitourinary / rectal			
Skin			
Lymph nodes			
Extremities			

Neurological function			
Other			

8. Did infant receive any medication?

☐ Yes (complete table below) ☐ No

Medication name	Start date	Stop date / ongoing	Indication

9. Feeding method

☐ Formula

☐ Breast

☐ Mixed feeding (including solids)

10. Blood obtained for PCR?

☐ Yes ☐ No, give reason:

_____

11. PCR result

a. ☐ Positive ☐ Negative

b. Mother informed on _____/_____/20__

c. If baby positive, where was he / she referred to?

_____

Signature: _____ **Date:** _____/_____/2009

Addendum F

MIFlowCyt standard compliant information for submitted flow cytometric data.

1. Experiment overview.

1.1. 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 from 2 weeks to 12 months of life in South African infants born to non-HIV infected mothers (HIV unexposed; UE). Comparison to adult peripheral blood was performed to give an idea as to the relationship between adult and changing early life TLR responses. This was done in whole blood to reduce the any possible processing artifact. We hypothesized that–given the changes in environment and ontogeny–that 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.

1.2. Keywords:

1.3. Organization:

1.3.1. Kollmann Lab, University of British Columbia

1.3.2. 950 W28th Ave. Vancouver, British Columbia, V5Z4H4, Room: A5-1147

1.4. Primary Contact:

1.4.1. P.I. Dr. Tobias Kollmann tkollmann@cw.bc.ca

1.4.2. Graduate Student. Ms Rozanne Adams 14914085@sun.ac.za

1.5. Date: Experiments were **set up from June 2009 to June 2010 and stained from August 2010 to December 2010.**

1.7. Quality Control Measures:

Unstipulated 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.

2. Flow Sample/Specimen Description

2.1. Sample/Specimen Material

2.1.1. Biological Samples:

2.1.1.1. Biological Sample Name

Whole blood obtained by peripheral blood venipuncture.

2.1.1.2.Biological Sample Source: Healthy human peripheral blood; obtained and processed within < 4h from.

2.1.1.2.1. Biological Sample Source Organism:

2.1.1.2.1.1. 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

2.1.1.2.1.2. Age:

2 weeks to 12 months of age

2.1.1.2.1.3. Gender:

Male and Female

2.1.1.2.1.4. Phenotype:

healthy (none); UE for infant group only. All adults were UE.

2.1.1.2.1.5. Genotype:

not applicable

2.1.1.2.1.6. Treatment:

Whole blood diluted 1:1 in RPMI.

2.1.2. Environmental Samples: not applicable

2.1.3. 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.

2.1.4 Sample Treatment Description

Cells were plated in a 96 well plate and cultured for a total of 6 hrs. Cells were stimulated with either nothing, 0111:B4 LPS (TLR4, InVivogen) and R848 (TLR7/8, InVivogen) in the presence of Brefeldin A (Sigma) at 2mg/ml. After culture, cells were treated with a final concentration of 2mM EDTA for 15 min at 37°C, then centrifuged @400g for 5min @22°C and resuspended in 100ul of 1x BD FACS Lysing solution (BD 349202) for 10 minutes at room temperature before being frozen at -80°C.

3. Fluorescence Reagent Description:

Table 13.

	Characteristic Being Measured	Antibody Name *Clone Name*	Vendor cat# *dilution used*
VIOLET			
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*
PerCPCy5.5	Cell Surface Protein	MHCII *TU36*	BD#custom *1:100*
PE-Cy7	Cell Surface Protein	CD14 (M5E2)	BD #557742 *1:50*
PE	Cell Surface Protein	CD123 (6H6)	eBio #121239

Instrument Details:

3.1. Manufacturer:

BD Biosciences

3.2. Model:

BD FACSAria Flow 3 Laser, Blue/Red/Violet serial # P22300055

3.3. Instrument Configuration and Settings:

All lasers, filters and mirrors were manufactured by BD Biosciences. The machine has not been altered.

3.3.1. 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 488 detectors detect light that has been scattered 90 o, 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 11.25o.

Table 14.

Laser	Detector Name (Type)	Filter 1 (LP)	Filter 2 (BP)	Parameter detected	Detector voltage	Amplification Type
Blue Laser (488 nm)	SSC (PD)	na	488/10 BP	SSC-A	440	LINEAR
	488 A (PMT)	735 LP	780/60 BP	PE-Cy7-A	605	LOG
	488 B (PMT)	655 LP	695/40 BP	PerCP-Cy5.5-A	585	LOG
	488 C (PMT)	595 LP	610/20 BP	PE-TexRed	na	
	488 D (PMT)	556 LP	575/26 BP	PE	498	LOG
	488 E (PMT)	502 LP	530/30 BP	FITC	480	LOG
Violet Laser (407 nm)	407 A (PMT)	502 LP	530/30 BP	Alexa-430-A	na	
	407 B (PMT)	blank	450/40 BP	Pacific Blue-A	530	LOG
Red Laser (633 nm)	633 A (PMT)	755 LP	780/60 BP	APC-Cy7-A	605	LOG
	633 B (PMT)	685 LP	720/40 BP	Alexa700-A	515	LOG
	633 C (PMT)	blank	660/20 BP	APC-A	492	LOG

4. Data Analysis

4.1. FCS Data File:

To request raw data please contact Dr. Tobias Kollmann tkollmann@cw.bc.ca

4.1.1. Total Count of Events:

Recorded within individual FCS files, as keyword $TOT, 200,000.

4.2. Compensation Description:

Compensation was done in FlowJo using BDCompBeads as single stain controls.

A representative compensation matrix for one of the samples is shown below.

	FITC	PE	PerCP-Cy5-5	PE-Cy7	APC	APC-Cy7	Pacific Blue	Alex 700
FITC-A		29.11	1.847	0.2719	0	-0.1527	0.6106	-0.4128
PE-A	0.6607		6.615	1.07	-0.249	-0.3758	0.3544	-0.6247
PerCP-Cy5-5-A	0.1683	0.2436		33.73	2.026	11.66	1.175	30.06
PE-Cy7-A	0.1653	1.982	0.3268		0	15.32	0.1029	0.5033
APC-A	0.1415	0.1788	1.576	0.4223		20.5	0.9705	65.71
APC-Cy7-A	0.08945	0.119	0.3078	2.183	12.56		0.3553	13.26
Pacific Blue-A	0.1281	0.1833	0	0	0.05744	-0.172		-0.2704
Alex 700-A	0.1859	0.208	1.44	0.9827	0.2883	29.7	0.662	

4.3. Gating (Data Filtering) Description:

4.3.1. Gate Summary Information:

4.3.1.1. -4.3.1.3 Gate Descriptions/subpopulations/statistics:

		Gate Statistics (% Parent Gate)	
Gate Description:	**Qualitative Description of the Subpopulation**	**Unstim (red)**	**R848 stim (blue)**
Live Cells	High cell density excluding lower left corner population	61.0	62.9
Monocytes	CD14 high, MCHII high	8.63	4.61
Other MHCII+ cells	MHCII high, CD14 mid to low	6.24	7.02
Myeloid Dendritic Cells (mDCs)	MHCII high, CD11c high, CD123 low	41.8	30.7
Plasmacytoid Dendritic Cells (pDCs)	MHCII high, CD11c low, CD123 high	2.76	2.62
Monocyte TNF+ IL-6	"Monocyte" TNFa high, IL-6 low	0.145	27.5
Monocyte TNF+ IL-6+	"Monocyte" TNFa high, IL-6 high	0.0104	65.6
Monocyte TNF- IL-6+	"Monocyte" TNFa low, IL-6 high	0.28	0.306
Monocyte TNF- IL-6	"Monocyte" TNFa low, IL-6 low	99.5	6.65
Monocyte TNF+ IL-12	"Monocyte" TNFa high, IL-12 low	0.149	64.3
Monocyte TNF+ IL-12+	"Monocyte" TNFa high, IL-12 high	0.00346	28.7
Monocyte TNF- IL-12+	"Monocyte" TNFa low, IL-12 high	0.0588	0.174
Monocyte TNF- IL-12	"Monocyte" TNFa low, IL-12 low	99.8	6.78
Monocyte TNF+ IFNa	"Monocyte" TNFa high, IFNa low	0.0934	92.1
Monocyte TNF+ IFNa+	"Monocyte" TNFa high, IFNa high	0.0554	0.917
Monocyte TNF- IFNa+	"Monocyte" TNFa low, IFNa high	0.166	0.535
Monocyte TNF- IFNa	"Monocyte" TNFa low, IFNa low	99.7	6.38
mDC TNF+ IL-6	"mDC" TNFa high, IL-6 low	0.194	40.1
mDC TNF+ IL-6+	"mDC" TNFa high, IL-6 high	0	52.7
mDC TNF- IL-6+	"mDC" TNFa low, IL-6 high	0.343	0.446
mDC TNF- IL-6	"mDC" TNFa low, IL-6 low	99.5	6.79
mDC TNF+ IL-12	"mDC" TNFa high, IL-12 low	0.194	43.0
mDC TNF+ IL-12+	"mDC" TNFa high, IL-12 high	0	49.8
mDC TNF- IL-12+	"mDC" TNFa low, IL-12 high	0.0915	2.9
mDC TNF- IL-12	"mDC" TNFa low, IL-12 low	99.7	4.34
mDC TNF+ IFNa	"mDC" TNFa high, IFNa low	0.194	92.7
mDC TNF+ IFNa+	"mDC" TNFa high, IFNa high	0	0.0297
mDC TNF- IFNa+	"mDC" TNFa low, IFNa high	0	0.0149
mDC TNF- IFNa	"mDC" TNFa low, IFNa low	99.8	7.19
pDC TNF+ IL-6	"pDC" TNFa high, IL-6 low	0	70.0
pDC TNF+ IL-6+	"pDC" TNFa high, IL-6 high	0	5.23
pDC TNF- IL-6+	"pDC" TNFa low, IL-6 high	0	0
pDC TNF- IL-6	"pDC" TNFa low, IL-6 low	100	25.1
pDC TNF+ IL-12	"pDC" TNFa high, IL-12 low	0	73.7
pDC TNF+ IL-12+	"pDC" TNFa high, IL-12 high	0	1.57
pDC TNF- IL-12+	"pDC" TNFa low, IL-12 high	0	0.174
pDC TNF- IL-12	"pDC" TNFa low, IL-12 low	100	24.9
pDC TNF+ IFNa	"pDC" TNFa high, IFNa low	0	5.92
pDC TNF+ IFNa+	"pDC" TNFa high, IFNa high	0	69.2
pDC TNF- IFNa+	"pDC" TNFa low, IFNa high	0	15.0
pDC TNF- IFNa	"pDC" TNFa low, IFNa low	100	10.1

4.4. 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.

Addendum G

Flow cytometric comparison of percentage of single-, double- and triple cytokine expressing cells expressing cytokines for longitudinal infant and adult responses to LPS and R-848 stimulation and basal stimulation. Analysis of the mean percentage of monocytes, mDC, or pDC expressing a particular cytokine. Statistically significant differences between age groups are indicated in bold.

			LPS (TLR4)						R-848 (TLR7/8)					
	Cytokine		Mean ± SD			p-value			Mean ± SD			p-value		
Monocytes														
	TNF-α+	**2 wk**	4.46 ± 3.45					**2 wk**	13.07 ± 7.09					**2 wk**
	% positive	**6 wk**	3.47 ± 2.77				**6 wk**	0.4324	10.70 ± 6.97				**6 wk**	0.2154
		6 mo	8.43 ± 6.44			**6 mo**	**0.0002**	**0.0029**	8.83 ± 5.06			**6 mo**	0.3380	**0.0329**
		12 mo	4.24 ±5.63		**12 mo**	**0.0029**	0.5636	0.8720	10.78 ± 8.75		**12 mo**	0.3500	0.9691	0.2630
		Adult	2.79 ± 1.06	**Adult**	0.4063	**0.0013**	0.6850	0.3232	10.99 ± 3.41	**Adult**	0.9335	0.4010	0.9063	0.4164
	IL-6+	**2 wk**	0.39 ± 0.40					**2 wk**	0.00 ± 0.28					**2 wk**
	% positive	**6 wk**	0.27 ± 0.36				**6 wk**	0.3209	0.00 ±0.45				**6 wk**	0.8763
		6 mo	0.21 ± 0.41			**6 mo**	0.5652	0.1270	0.00 ± 0.16			**6 mo**	0.8756	0.9966
		12 mo	0.44 ± 0.52		**12 mo**	0.0585	0.1610	0.6403	0.96 ± 2.50		**12 mo**	**0.0064**	**0.0032**	**0.0054**
		Adult	0.02 ± 0.04	**Adult**	**0.0075**	0.2172	0.0907	**0.0162**	0.00 ± 0.04	**Adult**	**0.0247**	0.9325	0.9726	0.9343
	IL-12/23p40+	**2 wk**	0.15 ± 1.45					**2 wk**	-4.63 ± 14.00					**2 wk**
	% positive	**6 wk**	0.38 ± 0.44				**6 wk**	0.5580	0.62 ± 1.00				**6 wk**	**0.0091**
		6 mo	1.33 ± 1.59			**6 mo**	**0.0182**	**0.0040**	0.64 ± 0.90			**6 mo**	0.9933	**0.0111**
		12 mo	0.71 ± 1.08		**12 mo**	0.1454	0.4247	0.1822	1.78 ± 2.58		**12 mo**	0.5986	0.5830	**0.0031**
		Adult	1.37 ± 0.92	**Adult**	0.2205	0.9394	0.0572	**0.0205**	7.20 ± 2.51	**Adult**	**0.0500**	**0.0159**	**0.0139**	**0.0000**
mDCs														
	TNF-α+	**2 wk**	8.92 ± 7.02					**2 wk**	15.35 ± 6.34					**2 wk**
	% positive	**6 wk**	7.71 ± 6.75				**6 wk**	0.5449	13.18 ± 7.59				**6 wk**	0.2537
		6 mo	12.11 ± 8.50			**6 mo**	**0.0325**	0.1219	14.11 ± 6.09			**6 mo**	0.6342	0.5236
		12 mo	6.11± 7.35		**12 mo**	**0.0075**	0.4747	0.2979	13.31 ± 8.05		**12 mo**	0.7011	0.9494	0.3155
		Adult	4.95 ± 1.93	**Adult**	0.6506	**0.0089**	0.2979	0.1380	21.57 ± 3.04	**Adult**	**0.0021**	**0.0044**	**0.0012**	**0.0159**
	IL-6+	**2 wk**	0.23 ± 0.22					**2 wk**	0.16 ± 0.22					**2 wk**

	% positive	**6 wk**	1.36 ± 0.22				**6 wk**	0.1925	0.32 ± 0.44				**6 wk**	0.3124
		6 mo	0.06 ± 0.19			**6 mo**	0.3489	**0.0298**	0.32 ± 0.41			**6 mo**	0.9881	0.3202
		12 mo	0.35 ± 0.36		**12 mo**	**0.0002**	**0.0039**	0.0917	1.30 ± 1.00		**12 mo**	**0.0000**	**0.0000**	**0.0000**
		Adult	0.16 ± 0.18	**Adult**	**0.0484**	0.3259	0.7793	0.4838	0.22 ± 0.32	**Adult**	**0.0000**	0.6244	0.6263	0.7844
	IL-12/23p40+	**2 wk**	0.00 ± 2.27					**2 wk**	1.16 ± 9.29					**2 wk**
	% positive	**6 wk**	0.30 ± 0.41				**6 wk**	0.0564	3.60 ± 2.56				**6 wk**	0.1194
		6 mo	0.78 ± 0.77			**6 mo**	0.2059	**0.0023**	7.23 ± 2.76			**6 mo**	**0.0243**	**0.0002**
		12 mo	0.18 ± 1.21		**12 mo**	0.1386	0.7577	0.1379	5.34 ± 5.70		**12 mo**	0.2665	0.2952	**0.0137**
		Adult	0.12 ± 0.14	**Adult**	0.9157	0.1920	0.7213	0.2786	9.84 ± 1.37	**Adult**	**0.0385**	0.2170	**0.0031**	**0.0000**
pDCs														
	TNF-α+	**2 wk**	Undetectable						10.24 ± 8.27					**2 wk**
	% positive	**6 wk**	Undetectable						10.30 ± 7.58				**6 wk**	0.9674
		6 mo	Undetectable						5.06 ± 3.46			**6 mo**	**0.0032**	**0.0039**
		12 mo	Undetectable						5.36 ± 3.93		**12 mo**	0.8722	**0.0072**	**0.0086**
		Adult	Undetectable						7.27 ± 0.66	**Adult**	0.4185	0.3390	0.1815	0.1941
	IFN-α+	**2 wk**	Undetectable						7.26 ± 8.05					**2 wk**
	% positive	**6 wk**	Undetectable						11.42 ± 8.37				**6 wk**	**0.0370**
		6 mo	Undetectable						10.36 ± 5.71			**6 mo**	0.5970	0.1306
		12 mo	Undetectable						14.61 ± 6.44		**12 mo**	**0.0500**	0.1309	**0.0074**
		Adult	Undetectable						7.62 ± 2.93	**Adult**	**0.0117**	0.3062	0.1488	0.8923

			Basal Stimulation					
	Cytokine		Mean ± SD		p-value			
Monocytes								
	TNF-α+	**2 wk**	0.32 ± 0.40					**2 wk**
	% positive	**6 wk**	0.47 ± 0.32				**6 wk**	0.3988
		6 mo	0.59 ± 0.55			**6 mo**	0.5076	0.1441
		12 mo	0.74 ± 1.22		**12 mo**	0.4668	0.1656	**0.0325**
		Adult	0.61 ± 0.17	**Adult**	0.6233	0.9317	0.5515	0.2254
	IL-6+	**2 wk**	0.21 ± 0.20					**2 wk**
	% positive	**6 wk**	0.34 ± 0.41				**6 wk**	0.083
		6 mo	0.25 ± 0.25			**6 mo**	0.2532	0.5894
		12 mo	0.11 ± 0.30		**12 mo**	0.1190	**0.0074**	0.2762
		Adult	0.17 ± 0.07	**Adult**	0.5869	0.4784	0.1107	0.7588
	IL-12/23p40+	**2 wk**	6.53 ± 16.22					**2 wk**
	% positive	**6 wk**	0.40 ± 0.62				**6 wk**	**0.0070**
		6 mo	0.52 ± 0.51			**6 mo**	0.9582	**0.0110**
		12 mo	0.92 ± 1.22		**12 mo**	0.8727	0.8287	**0.0219**
		Adult	1.76 ± 1.03	**Adult**	0.7904	0.6885	0.6525	0.1166
mDCs								
	TNF-α+	**2 wk**	0.28 ± 0.15					**2 wk**
	% positive	**6 wk**	0.49 ± 0.33				**6 wk**	0.1353
		6 mo	0.79 ± 0.77			**6 mo**	**0.0406**	**0.0007**
		12 mo	0.54 ± 0.67		**12 mo**	0.1173	0.7214	0.0839
		Adult	1.38 ± 0.41	**Adult**	**0.0000**	**0.0031**	**0.0000**	**0.0000**
	IL-6+	**2 wk**	0.30 ± 0.22					**2 wk**
	% positive	**6 wk**	0.38 ± 0.41				**6 wk**	0.5412
		6 mo	0.35 ± 0.45			**6 mo**	0.2963	0.1064
		12 mo	0.21 ± 0.22		**12 mo**	0.0646	0.3565	0.7252
		Adult	0.38 ± 0.35	**Adult**	0.1537	0.9697	0.4456	0.2274

	IL-12/23p40+	**2 wk**	4.25 ± 10.29					**2 wk**
	% positive	**6 wk**	0.65 ± 0.67				**6 wk**	**0.0138**
		6 mo	0.50 ± 0.55			**6 mo**	0.9169	**0.0137**
		12 mo	1.30 ± 1.98		**12 mo**	0.6187	0.6776	0.0601
		Adult	1.60 ± 0.80	**Adult**	0.8815	0.5796	0.6262	0.1754
pDCs								
	TNF-α+	**2 wk**	0.22 ± 0.44					**2 wk**
	% positive	**6 wk**	0.40 ± 0.59				**6 wk**	0.3847
		6 mo	0.78 ± 1.33			**6 mo**	0.0667	**0.0097**
		12 mo	0.17 ± 0.35		**12 mo**	**0.0074**	0.2852	0.8048
		Adult	0.00 ± 0.00	**Adult**	0.05605	**0.0060**	0.1453	0.4238
	IFN-α+	**2 wk**	2.05 ± 5.32					**2 wk**
	% positive	**6 wk**	1.25 ± 2.01				**6 wk**	0.3435
		6 mo	1.87 ± 1.60			**6 mo**	0.4734	0.8375
		12 mo	1.38 ± 1.92		**12 mo**	0.5954	0.8926	0.4605
		Adult	2.00 ± 0.90	**Adult**	0.5930	0.9062	0.5036	0.9688

			LPS (TLR4)						R-848 (TLR7/8)					
	Cytokine		Mean ± SD			p-value			Mean ± SD			p-value		
Monocytes														
	TNF-α+IL-6+	**2 wk**	1.31 ± 1.32					**2 wk**	18.41 ± 10.85					**2 wk**
	% positive	**6 wk**	0.90 ± 0.59				**6 wk**	0.5646	19.75 ± 11.00				**6 wk**	0.6729
		6 mo	3.00 ± 3.87			**6 mo**	**0.0045**	**0.0226**	14.57 ± 10.43			**6 mo**	0.1116	0.2408
		12 mo	2.50 ± 3.64		**12 mo**	0.5209	**0.0354**	0.1185	19.72 ± 15.10		**12 mo**	0.1385	0.9921	0.7007
		Adult	0.09 ± 0.07	**Adult**	**0.0155**	**0.0030**	0.3978	0.2013	2.52 ± 2.09	**Adult**	**0.0001**	**0.0058**	**0.0001**	**0.0003**
	TNF-α+IL-12+	**2 wk**	0.88 ± 1.01					**2 wk**	20.59 ± 9.02					**2 wk**
	% positive	**6 wk**	0.62 ± 0.59				**6 wk**	0.6445	15.39 ±7.53				**6 wk**	0.05531
		6 mo	3.59 ± 3.67			**6 mo**	**0.0000**	**0.0000**	19.06 ± 11.49			**6 mo**	0.1837	0.5815
		12 mo	1.18 ± 1.57		**12 mo**	**0.0001**	0.3481	0.6164	11.06 ± 8.62		**12 mo**	**0.0074**	0.1312	**0.0013**
		Adult	2.60 ± 1.47	**Adult**	0.070	0.1899	**0.0092**	**0.0024**	44.33 ± 12.39	**Adult**	**0.0000**	**0.0000**	**0.0000**	**0.0000**
	IL-6+IL-12+	**2 wk**	0.02 ± 0.03					**2 wk**	0.04 ± 0.07					**2 wk**
	% positive	**6 wk**	0.02 ± 0.02				**6 wk**	0.6107	0.05± 0.10				**6 wk**	0.5981
		6 mo	0.02 ± 0.06			**6 mo**	0.7069	0.9305	0.01 ± 0.04			**6 mo**	0.1956	0.4383
		12 mo	0.07 ± 0.10		**12 mo**	**0.0025**	**0.0027**	**0.0114**	0.16 ± 0.19		**12 mo**	**0.0002**	**0.0010**	**0.0002**
		Adult	0.01 ± 0.01	**Adult**	**0.0064**	0.4768	0.6631	0.4160	-0.01 ± 6.54	**Adult**	**0.0001**	0.6560	0.1482	0.2948
mDCs														
	TNF-α+IL-6+	**2 wk**	2.42 ± 2.54					**2 wk**	8.18 ± 4.19					**2 wk**
	% positive	**6 wk**	2.15 ± 2.26				**6 wk**	0.8124	9.19 ± 4.70				**6 wk**	0.4524
		6 mo	4.24 ± 7.13			**6 mo**	0.0744	0.1229	6.20 ± 4.24			**6 mo**	**0.0304**	0.1519
		12 mo	4.77 ± 7.13		**12 mo**	0.6651	**0.0315**	0.0551	10.26 ± 6.68		**12 mo**	**0.0063**	0.4528	0.1497
		Adult	0.54 ± 0.24	**Adult**	**0.0803**	**0.0175**	0.2862	0.2163	3.59 ± 1.91	**Adult**	**0.0005**	0.1530	**0.0022**	**0.0115**
	TNF-α+IL-12+	**2 wk**	1.89 ± 1.82					**2 wk**	31.78 ± 6.84					**2 wk**
	% positive	**6 wk**	1.52 ± 1.71				**6 wk**	0.5511	26.45 ± 7.63				**6 wk**	**0.0137**
		6 mo	3.95 ± 3.88			**6 mo**	**0.0003**	**0.0023**	23.76 ± 9.61			**6 mo**	0.2181	**0.0004**
		12 mo	0.70 ± 1.12		**12 mo**	**0.0000**	0.2342	0.0841	10.76 ± 6.76		**12 mo**	**0.0000**	**0.0000**	**0.0000**
		Adult	1.01 ± 0.52	**Adult**	0.7276	**0.0009**	0.5544	0.3020	31.93 ± 4.86	**Adult**	**0.0000**	**0.0054**	0.0552	0.9589
	IL-6+IL-12+	**2 wk**	0.01 ± 0.03					**2 wk**	0.34 ± 0.19					**2 wk**

	% positive	**6 wk**	0.01 ± 0.02				**6 wk**	0.7405	0.52 ± 0.58				**6 wk**	0.1787
		6 mo	0.03 ± 0.04			**6 mo**	0.2312	0.1328	0.43 ± 0.34			**6 mo**	0.5078	0.5142
		12 mo	0.06 ± 0.11		**12 mo**	0.0942	**0.0047**	**0.0019**	1.02 ± 0.72		**12 mo**	**0.0000**	**0.0005**	**0.0000**
		Adult	0.05 ± 0.02	**Adult**	0.6447	0.3601	0.0655	**0.0382**	0.27 ± 0.14	**Adult**	**0.0000**	0.3747	0.1590	0.6921
pDCs														
	TNF-α+ IFN-α+	**2 wk**	Undetectable						44.49 ± 19.05					**2 wk**
	% positive	**6 wk**	Undetectable						49.47 ± 11.08				**6 wk**	0.2127
		6 mo	Undetectable						59.99 ± 11.92			**6 mo**	**0.0108**	**0.0002**
		12 mo	Undetectable						48.12 ± 16.81		**12 mo**	**0.0072**	0.7492	0.3955
		Adult	Undetectable						54.53 ± 3.03	**Adult**	0.2450	0.3115	0.0611	0.3955
	TNF-α+IL-6+	**2 wk**	Undetectable						0.57 ± 0.77					**2 wk**
	% positive	**6 wk**	Undetectable						0.69 ± 0.73				**6 wk**	0.4611
		6 mo	Undetectable						0.12 ± 0.13			**6 mo**	**0.0004**	**0.0051**
		12 mo	Undetectable						0.18 ± 0.21		**12 mo**	0.7306	**0.0023**	**0.0178**
		Adult	Undetectable						0.18 ± 0.12	**Adult**	**0.0258**	**0.0109**	0.8857	0.6822
	IFN-α+IL-6+	**2 wk**	Undetectable						0.06 ± 0.13					**2 wk**
	% positive	**6 wk**	Undetectable						0.07 ± 0.19				**6 wk**	0.8636
		6 mo	Undetectable						0.02 ± 0.07			**6 mo**	0.2642	0.3467
		12 mo	Undetectable						0.12 ± 0.17		**12 mo**	**0.0313**	0.2469	0.1914
		Adult	Undetectable						0.00 ± 0.00	**Adult**	**0.0314**	0.6401	0.1829	0.2311

			Basal Stimulation					
	Cytokine		Mean ± SD		p-value			
Monocytes								
	TNF-α+IL-6+	**2 wk**	0.04 ± 0.09					**2 wk**
	% positive	**6 wk**	0.03 ± 0.02				**6 wk**	0.9314
		6 mo	0.11 ± 0.17			**6 mo**	**0.0379**	**0.0479**
		12 mo	0.07 ± 0.22		**12 mo**	0.2512	0.4101	0.4606
		Adult	0.14 ± 0.07	**Adult**	0.1732	0.6386	**0.0387**	**0.0460**
	TNF-α+IL-12+	**2 wk**	0.05 ± 0.07					**2 wk**
	% positive	**6 wk**	0.05 ± 0.07				**6 wk**	0.9633
		6 mo	0.13 ± 0.10			**6 mo**	0.0672	0.0764
		12 mo	0.17 ± 0.32		**12 mo**	0.5036	**0.0149**	**0.0176**
		Adult	0.26 ± 0.07	**Adult**	0.1458	**0.0439**	**0.0006**	**0.0008**
	IL-6+IL-12+	**2 wk**	0.02 ± 0.02					**2 wk**
	% positive	**6 wk**	0.01 ± 0.01				**6 wk**	0.7064
		6 mo	0.05 ± 0.06			**6 mo**	**0.0007**	**0.0024**
		12 mo	0.02 ± 0.05		**12 mo**	**0.0045**	0.7211	0.9951
		Adult	0.07 ± 0.02	**Adult**	**0.0006**	0.2027	**0.0003**	**0.0003**
mDCs								
	TNF-α+IL-6+	**2 wk**	0.03 ± 0.04					**2 wk**
	% positive	**6 wk**	0.06 ± 0.05				**6 wk**	0.6305
		6 mo	0.16 ± 0.33			**6 mo**	0.6608	**0.0232**
		12 mo	0.07 ± 0.18		**12 mo**	0.1377	0.8136	0.4979
		Adult	0.17 ± 0.20	**Adult**	0.1795	0.8620	0.1122	0.0539
	TNF-α+IL-12+	**2 wk**	0.03 ± 0.04					**2 wk**
	% positive	**6 wk**	0.06 ± 0.05				**6 wk**	0.6088
		6 mo	0.16 ± 0.29			**6 mo**	**0.0194**	**0.0053**

		12 mo	0.08 ± 0.15		**12 mo**	0.1016	0.5685	0.3000
		Adult	0.24± 0.09	**Adult**	**0.0127**	0.2099	**0.0024**	**0.0007**
	IL-6+IL-12+	**2 wk**	0.02 ± 0.03					**2 wk**
	% positive	**6 wk**	0.03 ± 0.04				**6 wk**	0.9246
		6 mo	0.09 ± 0.0.23			**6 mo**	**0.0457**	**0.0386**
		12 mo	0.01 ± 0.03		**12 mo**	**0.0197**	0.6102	0.6757
		Adult	0.07 ± 0.07	**Adult**	0.2019	0.5498	0.3514	0.3190
pDCs								
	TNF-α+ IFN-α+	**2 wk**	0.00 ± 0.01					**2 wk**
	% positive	**6 wk**	0.04 ± 0.09				**6 wk**	0.7849
		6 mo	0.45 ± 0.99			**6 mo**	**0.0019**	**0.0011**
		12 mo	0.00 ± 0.00		**12 mo**	**0.0019**	0.7852	0.9875
		Adult	0.00 ± 0.00	**Adult**	1.0000	**0.0116**	0.8285	0.9899
	TNF-α+IL-6+	**2 wk**	0.01 ± 0.02					**2 wk**
	% positive	**6 wk**	0.01 ± 0.04				**6 wk**	0.7751
		6 mo	0.08 ± 0.14			**6 mo**	**0.0008**	**0.0004**
		12 mo	0.00 ± 0.02		**12 mo**	**0.0007**	0.7248	0.9349
		Adult	0.00 ± 0.00	**Adult**	**0.8694**	**0.0034**	0.6509	0.8137
	IFN-α+IL-6+	**2 wk**	0.01 ± 0.05					**2 wk**
	% positive	**6 wk**	0.06 ± 0.15				**6 wk**	0.1862
		6 mo	0.12 ± 0.15			**6 mo**	0.0628	**0.0025**
		12 mo	0.00 ± 0.00		**12 mo**	**0.0012**	0.0988	0.6864
		Adult	0.04 ± 0.13	**Adult**	0.3612	0.0836	0.7199	0.5335

Cytokine		LPS (TLR4) Mean ± SD			p-value			R-848 (TLR7/8) Mean ± SD			p-value		
Monocytes													
TNF-α+IL-6+IL-12+	**2 wk**	0.65 ± 0.74					**2 wk**	41.03 ± 14.74					**2 wk**
% positive	**6 wk**	0.53 ± 0.48				**6 wk**	0.8512	47.06 ± 14.95				**6 wk**	0.1746
	6 mo	3.64 ± 4.01			**6 mo**	**0.0000**	**0.0000**	49.90 ± 14.41			**6 mo**	0.5308	0.0541
	12 mo	2.09 ± 2.91		**12 mo**	**0.0314**	**0.0265**	**0.0423**	36.06 ± 19.82		**12 mo**	**0.0049**	**0.0208**	0.2952
	Adult	0.46 ± 0.23	**Adult**	0.0749	**0.0005**	0.9414	0.8309	24.31 ± 13.80	**Adult**	0.0570	**0.0004**	**0.0001**	**0.0055**
mDCs													
TNF-α+IL-6+IL-12+	**2 wk**	1.32 ± 1.58					**2 wk**	30.48 ± 9.66					**2 wk**
% positive	**6 wk**	1.14 ± 1.28				**6 wk**	0.8014	33.95 ±10.22				**6 wk**	0.2575
	6 mo	4.21 ± 4.15			**6 mo**	**0.0000**	**0.0001**	27.00 ± 11.06			**6 mo**	**0.0279**	0.2715
	12 mo	1.69 ± 0.04		**12 mo**	**0.0013**	0.4596	0.6178	22.54 ± 13.89		**12 mo**	0.1837	**0.0006**	**0.0169**
	Adult	0.28 ± 0.15	**Adult**	0.1456	**0.0000**	0.3530	0.2668	15.90 ± 7.65	**Adult**	0.1187	**0.0084**	**0.0000**	**0.0005**
pDCs													
TNF-α+IFN-α+IL-6+	**2 wk**	Undetectable						8.12 ± 5.93					**2 wk**
% positive	**6 wk**	Undetectable						8.28 ± 4.79				**6 wk**	0.9086
	6 mo	Undetectable						6.64 ± 3.55			**6 mo**	0.2473	0.3002
	12 mo	Undetectable						5.73 ± 4.96		**12 mo**	0.5473	0.0848	0.1087
	Adult	Undetectable						5.84 ± 5.10	**Adult**	0.9534	0.6698	0.1864	0.2189

			Basal Stimulation					
	Cytokine		Mean ± SD		p-value			
Monocytes								
	TNF-α+IL-6+IL-12+	**2 wk**	0.01 ± 0.02					**2 wk**
	% positive	**6 wk**	0.01 ± 0.01				**6 wk**	0.9666
		6 mo	0.04 ± 0.06			**6 mo**	**0.0004**	**0.0046**
		12 mo	0.02 ± 0.05		**12 mo**	**0.0339**	0.5867	0.5634
		Adult	0.07 ± 0.02	**Adult**	**0.0003**	**0.0398**	**0.0000**	**0.0000**
mDCs								
	TNF-α+IL-6+IL-12+	**2 wk**	0.01 ± 0.02					**2 wk**
	% positive	**6 wk**	0.02 ± 0.04				**6 wk**	0.6872
		6 mo	0.08 ± 0.21			**6 mo**	**0.0348**	**0.0137**
		12 mo	0.01 ± 0.02		**12 mo**	**0.0172**	0.6514	0.9391
		Adult	0.07± 0.07	**Adult**	0.1621	0.6075	0.2686	0.1632
pDCs								
	TNF-α+IFN-α+IL-6+	**2 wk**	2.06 ± 5.32					**2 wk**
	% positive	**6 wk**	1.36 ± 2.17				**6 wk**	0.8184
		6 mo	2.51 ± 2.66			**6 mo**	**0.0039**	**0.0026**
		12 mo	1.38 ± 1.92		**12 mo**	**0.0041**	0.8159	0.9867
		Adult	2.04 ± 1.03	**Adult**	1.0000	**0.0197**	0.8532	0.9894