DEVELOPMENT OF THE NEONATAL IMMUNE SYSTEM IN THE PREMATURE INFANT

by

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Abstract

Newborns lack educated adaptive immunity and therefore rely on innate immune defenses to protect themselves from infections. Premature babies are at high risk of severe infections due to the immaturity of their immune system. Umbilical cord blood is readily accessible to study the premature neonate's immune system, but it does not capture important maturation events that may occur during the neonatal period. The overall goal of my PhD was to investigate the immune system of premature infants during the neonatal period.

In chapter 2, I examined the whole blood response to immune stimulation of two prototypic Toll-like receptors: TLR4 and TLR7/8, in preterm infants aged 1-42 days in the neonatal intensive care unit. I identified major functional deficits in pro-inflammatory cytokine levels compared to term cord blood, which were not due to a lack of immune cells. These findings support previous observations made from preterm cord blood studies. To the best of our knowledge, we were the first at the time to study functional TLR responses during this critical development period.

In chapter 3, I used RNA-sequencing methods to investigate how these responses are developmentally regulated. Using deconvolution algorithms, I found that preterm cord blood is distinct from preterm postnatal blood and term cord blood, with a gradual transition from an immature immune system enriched in hematopoietic stem cells, myeloid and erythroid progenitor cells, to a more mature immune cell composition in term cord blood. I also provide the first data directly linking immaturity of the preterm immune system to the risk of sepsis.

In chapter 4, I examined innate-like characteristics of naïve CD4 T cells in term cord blood, as a means whereby newborns may compensate for a lack of adaptive memory immune cells. I demonstrate that activation of the antigen presenting cell is important to enhance innate-

like IL-8 production in naïve CD4 T cells. The work within this thesis further characterized the immune function of newborns across the gestational age spectrum, providing novel insights into their immune development and factors underlying the high susceptibility of premature infants to infection.

Lay Summary

Premature infants are at high risk for infections. However, we know little about how their immune system develops during the newborn period. At birth preterm infants produces less of the proteins required for a successful immune response. In my research, I provide the first evidence that the premature newborn's immune system remains immature during the first month after birth. I demonstrate a gradual maturation of responses reaching levels in term infants by about 2-4 weeks of age. One main reason for this is an abundance of immature cell-types, immune cell progenitors, and stem cells. Finally, I studied newborn naïve T cells that can respond rapidly to pathogens, which may be an adaptation to a lack of memory immune cells at birth. Overall, my research sheds important light on the changes that occur in the immune system of premature babies and their susceptibility to infections in the neonatal period.

Preface

Chapter 1:

I performed the literature review and wrote all of the text presented in this chapter. No part has been previously published.

Chapter 2:

This chapter has been published in its entirety:

Marchant EA, Kan B, Sharma AA, van Zanten A, Kollman TR, Brant, R, Lavoie PM. Attenuated innate immune defenses in very premature neonates during the neonatal period. *Pediatric Research*. 2015; 78(5):492-497. doi:10.1038/pr.2015.132.

The cohort study was designed by myself and Dr. Lavoie based on a Canadian Institutes of Health Research Operating Grant obtained by Pascal Lavoie in 2012. I coordinated consent obtained by two research nurses: Alice van Zanten and Kristie Finley. Peripheral blood samples were drawn by Pascal Lavoie. Processing of these blood samples was done primarily by me with help from Mihoko Ladd, Bernard Kan, and Kelsey Lee. I performed the ELISA experiments with help from M. Ladd, K. Lee and B. Kan. I completed the flow cytometry with help from Ashish A. Sharma. I completed the simple, descriptive statistical analysis with help from P. Lavoie. The more complex statistical modeling was performed by Rollin Brant. I wrote the original manuscript and P. Lavoie and I edited all iterations together.

Chapter 3:

The cohort and blood samples were from Chapter 2. I reviewed the subjects' charts and mined the clinical data. I performed the extraction of RNA for RNA-sequencing, with help from Constantin Popescu, and completed all RNA quality checks. RNA sequencing and preliminary data analysis was performed by our collaborators Slim Fourati and A. A. Sharma at Case Western Reserve University (Rafick Sekaly's lab). Figures 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 3.10, 3.11, 3.12, 3.13 and Table 3.5, 3.7, 3.8 were generated by A. A. Sharma, all other figures and tables were generated by me. I reviewed the literature and selected the Kalikstad data set for validation with assistance from Christina Michalski, and A. A. Sharma applied the analysis to the public data set. I wrote the whole chapter with technical consultation from C. Michalski.

Chapter 4:

I conceived the experiments in this chapter with supervision from P. Lavoie, and in consultation with A. A. Sharma and John Priatel. All blood is a shared resource in our lab and samples used in this chapter were collected by K. Lee, Hamid Reza Razzaghian, Hong Li, Shannon Tang, Zohreh Sharafianardakani, C. Michalski, and I. The preparation of cells for FACS sorting was performed by K. Lee and H. R. Razzaghian. Naïve T cell FACS sorting was performed by Lisa Xu (BCCHRI flow core). I analyzed the Gibbons et al (2014) gene expression data set and A. A. Sharma analyzed the Tuomela et al. (2012) and Razzaghian et al. (manuscript in preparation) data sets to identify neonate specific T cell receptors. I adapted the neutrophil migration assay (from Himmel et. al, 2011) and subsequent runs of the assay were performed with technical help from M. Ladd, C. Popescu and Charlotte Alary. I designed and developed the APC-T cell crossover experiments in consultation with P. Lavoie. Assistance in cell purification and assay

set up was provided by S. Tang and Z. Sharafianardakani. Hong Li, Zohreh Sharafianardakani, S. Tang and A. A. Sharma assisted with phenotyping experiments. I designed the fluorophore panels, ran and analyzed the flow cytometry data in this chapter. The CD21 (CR2) phenotyping antibody panel was provided by Jessi Tuengel (Soren Gantt's lab, University of British Columbia) and she assisted in adapting the panel to include other markers of interest. I completed the data analysis for this chapter. I wrote the entire chapter with critical input from P. Lavoie.

All work conducted in this thesis was approved by The University of British Columbia Children's & Women's Research Ethics Board (#H07-02681).

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Acknowledgements

First and foremost, I must thank my supervisor, Pascal Lavoie and all past and present members of the Lavoie Lab for making this work possible. As a smaller lab we have always worked together and supported each other. I especially need to thank all our co-op students who assisted with cord blood collection, a rite of passage for all lab members. Mihoko Ladd and Kelsey Lee trained me when I first joined the lab and I appreciate the good lab practices instilled in me that I have shared with other students. Bernard Kan provided humour, a listening ear and excellent scientific and lunch room discussions throughout this journey. Ashish Sharma taught me about the intricacies and the frustrating subjectivity of flow cytometry. Christina Michalski has been a great friend and I could not have finished this dissertation without her support and technical and editorial comments. I would also like to thank the members of the Levings lab who supported me with visits and dinners after my skiing accident and provided friendship and antibodies throughout my PhD. I must also thank my sister, Claire Marchant, who cared for me after my accident.

During my PhD the field of immunology has continued to grow, with other groups discovering new phenotypes and functions of neonatal immune cells. Sometimes these discoveries have answered my questions and other times they have scooped my own results. This is the nature of scientific research; people are always asking questions and performing experiments to answer them. While I have not made ground breaking discoveries that will change the world today, my hope is that this work will answer questions for other researchers, so they can continue to advance our understanding of the human immune system.

Dedication

I dedicate my PhD to my parents, Anne and Tim Marchant, who have instilled in me their love of science and the pursuit of knowledge. They listened to the trials and tribulations of my experiments and rejoiced in my successes. I will always remember to agitate the phagocytes.

Chapter 1: Introduction

1.1 Newborns' susceptibility to infections

The duration of a normal pregnancy is 40 weeks (~ 9 months). The end of the second trimester of pregnancy (>22-24 weeks) marks the limit of viability with current medical care. With the advancement of medicine, a greater proportion of the youngest, most immature premature babies are given a chance to survive. Before the 1970s preterm infants rarely survived below 27 weeks, now the survival of those on the cusp of viability (24 weeks of gestation) is about 60% in the developed world. However, these infants require extensive medical support at birth and they can develop serious complications that can greatly increase their risk of long-term morbidities, including a 25% risk of severe infection. As more of these infants survive, we need to better understand how the immune system develops and their susceptibility to infections, with the hope that this knowledge will inform new ways to treat or prevent infections in babies and adults.

1.2 Neonatal infections and sepsis

Each baby born prematurely has unique circumstances of birth, pathologies, and medical requirements, but systemic bacterial infection, as traditionally defined by a positive blood culture, is universally more common in babies born at lower gestational age and exponentially increases below 33 weeks of gestation. Infection risk peaks during the second week of life, suggesting a window of opportunity for interventions in the youngest preterm infants (Lavoie 2009). To develop appropriate preventive measures, it is essential to identify risk factors and causes of neonatal sepsis. Common sources of infections are medical devices such as intravenous catheters, mechanical ventilation and surgeries. Central venous catheters are the leading cause of

coagulase-negative staphylococci (CoNS) infections in preterms (Lepainteur et al. 2013). During the postnatal period the baby is also colonized by potential pathogens in the environment. A hospital environment may have an abundance of antibiotic-resistant bacterial strains, and some pathogens, such as *Candida albicans,* can be colonizers of healthy populations. Many of these microbes can become pathogenic when they enter a sterile site such as the bloodstream. Even before birth the fetus can be exposed to microbes by the mother.

Infections of the mother's genital tract can ascend to the uterus infecting the amniotic fluid and fetus, and are strongly associated with premature birth (Helmo et al. 2017). Premature rupture of the amniotic membranes (PROM) surrounding the baby is associated with intrauterine infections often leading to premature birth. Intrauterine infections can be caused by a multitude of organisms, with the most prevalent organisms being group B Streptococcus and *Escherichia coli* (*E.coli*) (Seliga-Siwecka & Kornacka 2012). Sexually transmitted infection such as Trichomonas vaginalis, Chlamydia trachomatis and Neisseria gonorrhoeae can also cause premature birth (Helmo et al. 2017). It is unclear if it is the pathogen itself or the associated infection and inflammation that lead to PROM and premature birth.

Intrauterine infections are a main cause of premature birth. Infections, especially sepsis, in neonates can be difficult to diagnose. In the neonatal intensive care unit (NICU), doctors rely on non-specific clinical signs of an infection, including tachycardia, bradycardia, hypothermia, hyperthermia, lethargy, feeding intolerance, low blood sugar, and breathing problems (Camacho-Gonzalez et al. 2013). As a precaution many premature neonates are prescribed antibiotics when there is a suspected infection, and treatment may be continued for a set duration even in the absence of a positive blood culture. Neonatal sepsis is divided into two groups depending on when the infection occurs. Early-onset sepsis (EOS) is defined as occurring within 72 hours after

birth and is often an infection that is present at or prior to birth. Frequently, EOS is due to maternal genital tract, intrauterine or intra-amniotic infection (Camacho-Gonzalez et al. 2013; Simonsen et al. 2014). EOS is caused by both Gram-positive and Gram-negative pathogens with group B Streptococcus and *E. coli* having the greatest incidence, respectively (Hornik et al. 2012; Vergnano et al. 2011; Stoll et al. 2005). As premature birth can be due to infection, it is standard care to administer prophylactic antibiotics to most preterm babies at birth. Late-onset sepsis (LOS) occurs after the first 72 hours and is acquired from the environment. Many LOS infections in the NICU are caused by coagulase-negative staphylococci, *Enterobacteriaceae* and *Staphylococcus aureus*, with *Candida albicans* as the major fungal LOS pathogen (Dong & Speer 2015; Vergnano et al. 2011; Isaacs et al. 1996; Marchant et al. 2013). LOS is more prevalent in neonates of lower gestational age and lower birth weight, and in those who experience more medical interventions (Downey et al. 2010). LOS infections cause close to 30% mortality in very low birth weight (VLBW) infants (Hornik et al. 2012).

1.3 Neonatal immunology

The immune system has the complex task of discriminating foreign invaders from the body's own tissues and self-antigens. Learning this self/non-self discrimination begins early in gestation and continues throughout life. Production of hematopoietic cells first occurs in the yolk sac, although definitive hematopoiesis begins in the aorta-gonad-mesonephros (AGM) with the formation of the first hematopoietic precursors from pluripotent hematopoietic stem cells that then migrate to the liver. Hematopoiesis in the bone marrow begins after 10-20 weeks (reviewed in Kan et al. 2016). Myelopoiesis, begins as early as week 3, while lymphopoeisis, the production of lymphoid cells, begins around week 8 (Kan et al. 2016).

1.3.1 Physical and mucosal barriers

The first lines of defense against pathogens are the physical barriers of the skin and mucosa. Neonatal skin is protected at birth by the vernix caseosa, a lipid-enriched layer able to retain moisture and the pH of the skin, containing antimicrobial proteins and peptides (Collins et al. 2018). The lungs are coated with antimicrobial substances (surfactant, lysozymes, lactoferrin, and defensins), and the epithelial cells express pattern recognition receptors (PRRs) and interact with tissue resident alveolar macrophages (Georgountzou & Papadopoulos 2017). However, in preterm neonates, lung development has not been completed at birth and surfactant production is absent, making preterm neonates susceptible to lung infections (Li & Tullus 2002; van Well et al. 2017). Similarly, the vernix caseosa is formed during the last trimester, meaning this physical barrier is lacking in extremely preterm neonates (Visscher et al. 2005; Larson & Dinulos 2005).

Birth is a major event in the development of the immune system. The exit of the neonate through the birth canal is usually the first encounter with the mother's microbial flora (Jennewein et al. 2017), although recent data suggest that microbial particles may cross the placenta to prime the fetal immune system (Ganal-Vonarburg et al. 2017). Maternal microbiota can be transferred to offspring in mice during gestation together with maternal antibodies (Ganal-Vonarburg et al. 2017). Intrauterine infection and prolonged/premature rupture of the membranes are both factors that can induce premature birth (Holst & Garnier 2008; Pinto et al. 2018). There is evidence that the mode of delivery directs which microbes will colonize the baby vaginal deliveries will lead to neonatal flora being similar to the rectovaginal flora of the mother (Blaser & Dominguez-Bello 2016), and babies delivered by C-sections are colonized with the maternal skin flora (Dominguez-Bello et al. 2010). The neonate that must reside in the NICU for medical support may also be exposed to nosocomial pathogens.

1.3.2 Innate immune cells and response

If a microbe breaches the physical barrier the next line of defense are the resident immune cells, which can respond to signals of tissue damage from epithelial cells. Innate immune cells are then recruited to the site of infection. The recruitment of neutrophils and other innate immune cells through the upregulation of adhesion ligands and receptors on endothelial cells, which will slow and recruit other immune cells to the site of infection. The extravasation of immune cells into the infected tissue is directed by a chemokine gradient produced by local cells. Subsequently, the neutrophils recruited to the site will produce additional chemokines to recruit more cells. The innate immune response is made up of circulating and tissue resident cells. The circulating cells, such as monocytes, immature dendritic cells (DCs) and neutrophils act as sentinels in the blood and migrate to sites of infection or inflammation when directed. In addition to innate cells circulating through the blood and lymph nodes, there are tissue resident innate immune cells, such as Langerhans cells, microglia, Kupffer cells, and alveolar macrophages that respond to pathogens or inflammation locally (Mass et al. 2016).

1.3.2.1 Pattern recognition receptors

The innate immune response uses receptors to identify conserved components of pathogens called Pathogen-Associated Molecular Patterns (PAMPs). These PAMPs are recognized by Pattern Recognition Receptors (PRRs) mainly on immune cells. PRRs enable innate immune cells to recognize the same pathogen upon subsequent exposure without developing adaptive-like memory cells. These receptors are specific to conserved pathogen components, for example, TLR4 recognizes lipopolysaccharide (LPS), a component in the outer membrane of Gram-negative bacteria. The response of immune cells is measured by the

intracellular production or extracellular secretion of cytokines. These proteins measure the capacity of an immune cell to respond to PRR stimulation. Each pathogen (PAMP/PRR interaction) leads to the production of different cytokines to activate other cells. The fine-tuned ability of these PRRs to direct the cell to produce the correct cytokines is important for a successful immune response.

1.3.2.1.1 PAMPS, DAMPs and their PRRs

Intracellular PRRs detect viral DNA/RNA and extracellular PRRs detect ligands of bacterial and fungal cell wall components. Once a PRR is stimulated the cell undergoes activation and downstream signaling resulting in the nuclear localization of transcription factors that transcribe the genes of specific cytokines to be produced and secreted to elicit an immune response. PAMPs have an important role in the activation of antigen presenting cells (APCs) to produce the cytokines that will instruct the differentiation of naïve CD4+ T cells (Jain & Pasare 2017).

There are several classes of PRRs, RIG-I-like receptors (RLR), C-type lectin receptors (CLR), NOD-like receptors (NLR) and Toll-like receptors (TLR) (all PRRs reviewed in Patin et al, 2018). These detect both intracellular (endosomal and cytosolic) and extracellular PAMPs (Kawai & Akira 2010). RIG-I-like receptors (RLRs) detect RNA viruses and include RIG-I, LGP2 and MDA5 (Yoneyama & Fujita 2009; Patin et al. 2018). RLRs are intracellular receptors sensing foreign viral RNA (Yoneyama & Fujita 2009). Preterm neonates are significantly impaired in their production of pro-inflammatory cytokines, Interleukin (IL)-1β, IL-6, tumor necrosis factor α (TNF-α) and IL-12/23p40, compared to term neonates in response to RIG-I stimulation of mononuclear cells (Sharma et al. 2014; Marr et al. 2014). C-type lectin receptors

(CLRs) are extracellular PRRs mainly expressed on myeloid cells (Patin et al. 2018). There are three subtypes of CLRs: type I consist of DEC205 and MMR, type II includes Dectin-1, -2, Mincle, DC-SIGN and the final type is soluble mannose-binding lectin (Patin et al. 2018). The expression and function of CLRs on preterm neonatal immune cells has not yet been completely characterized. Nod-like receptors (NLRs) recognize cytosolic pathogen components. NOD1 and NOD2 recognize peptidoglycan degraded in the cytosol, while NLRP3 when activated can form the inflammasome complex resulting in the maturation of caspase-1 and the cleavage of IL-1β and IL-18 (Kawai & Akira 2010). NOD1 and NOD2 have equal expression and function in preterm monocytes as in term neonates and adults, suggesting that the preterm's increased susceptibility to invasive infections is not due to attenuated NOD signaling (Granland et al. 2014).

The best characterized PRRs are the Toll-like receptors (TLRs) found on the extracellular surface of the cell (TLR 2, 4, 5, and 6) and intracellularly in endosomes (TLR3, 4, 7/8, and 9). At birth in the term infant, TLR4 is present at adult levels on mononuclear cells (Yan et al. 2004; Levy et al. 2004). There is evidence that *ex vivo* TLR4 responses in term cord blood are higher than in adults, based on secreted cytokine levels (Nguyen et al. 2010). During the postnatal period the term infants' response to TLR4 stimulation showed increasing cytokine production in whole blood from levels at birth (Burl et al. 2011). The neonate born prematurely is at an earlier stage of development and Sharma et al. have shown that there is a hierarchical maturation of the TLR responses, with TLR7/8 stimulation in preterm neonates at comparable levels to term neonates, and extracellular TLR responses maturing during the third trimester (28-40 weeks of gestation) (Sharma et al. 2014). The described attenuated cytokine production in the preterm neonate is unlikely due to low receptor expression, as the levels of TLR 4 and TLR2 on

monocytes and neutrophils are comparable between neonatal cord blood (30-40 weeks gestation), preterms with sepsis (postnatal sample) and adults (Silveira-Lessa et al. 2016). TLRs are not restricted to innate immune cells and are present on other immune cells (e.g. T cells, neutrophils) and non-immune cells (e.g. mucosal epithelial cells) (Rahman et al. 2009; McClure & Massari 2014; Futosi et al. 2013). Multiple PRRs may respond to different components of the same pathogen, providing safeguards to ensure that the invader is detected, and an immune response is generated. This may depend on where the pathogen is detected, intracellularly or extracellularly. For example, *Salmonella* can activate intracellular NLR or extracellular TLR (Broz et al. 2012), and the beta-glucan of *Candida albicans* is recognized by the Dectin-1 receptor (a CLR) and TLR2 (Barreto-Bergter & Figueiredo 2014). These overlapping recognition receptors likely propel the arms race between host and pathogen, and without these backup systems humans would not survive as long.

Immune cells also respond to danger-associated molecular patterns (DAMPs), molecules released by the host as a result of tissue injury to indicate danger, which can induce inflammation in the absence of an infectious agent (Bianchi 2007). DAMPs can be intracellular proteins that are released by damaged cells into the extracellular environment, such as heat-shock proteins, high mobility group-box 1(HGMB1) and hyaluron fragments, or non-protein molecules including DNA, ATP and uric acid (Lamkanfi & Dixit 2014; Modlin 2012; Bianchi 2007). These DAMPs can also influence an immune response as they bind and activate PRRs on immune cells (Martinon et al. 2002). For example, exposure of a cell to exogenous ATP can lead to the assembly of the NLRP3 inflammasome, an NLR, and subsequently the cleavage of IL-1β and IL-18, pro-inflammatory cytokines, into their mature forms for secretion. Elevated levels of HGMB1in amniotic fluid are associated intra-amniotic infection/inflammation, preterm PROM

and premature birth (Romero et al. 2011). Currently, there is a lack of knowledge about the production of and response to DAMPs by preterm neonates.

1.3.2.1.2 Signaling in PRRs and cytokine production

PRR activation of an immune cell leads to downstream signaling of different pathways leading to the nuclear localization of transcription factors, such as NF-κB, that induce the transcription of pro-inflammatory cytokines, chemokines and type I interferons (Dowling & Mansell 2016). Downstream signaling of the TLRs is dependent on the intracellular adaptor protein myeloid differentiation primary response 88 (MyD88) or TIR-domain-containing adaptor-inducing interferon-β (TRIF) (reviewed in Dowling & Mansell 2016). TLR-1/2/6, 5, 7, 8, and 9 signal through MyD88 while TLR-3 signals through TRIF. TLR4 is unique in its use of both MyD88 and TRIF signaling pathways. Neonatal monocytes have reduced MyD88 protein (Yan et al. 2004) while others have shown that MyD88 mRNA is present in neonates (Sadeghi et al. 2007).

Cytokines act on immune cells in an autocrine or paracrine manner, inducing activation and cell recruitment. For example, TNF-α can induce the upregulation of adhesion receptors on the endothelium (Bevilaqua et al. 1989; Pober et al. 1987), recruit neutrophils and monocytes (Ming et al. 1987) and can activate them to mount an immune response at the site of infection. Signaling downstream of dectin-1 the PRR for β-glucan, a cell wall component of fungal pathogens, leads to the translocation of transcription factors NF-κB, AP-1, NFAT and the production of pro-inflammatory cytokines (Patin et al. 2018, in press; Geijtenbeek & Gringhuis 2009). Pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α are produced by monocytes and IL-12/23p40 is produced by activated DCs in response to PRR stimulation. IL-10 is the

traditional anti-inflammatory cytokine produced by monocytes and lymphocytes. It should be noted that IL-6 has been shown to have both pro- and anti-inflammatory properties (Scheller et al. 2011).

The presence of a receptor on a cell does not indicate that it is functional. To confirm the presence and the function of a receptor there needs to be measurement of an output. The measurement of downstream signaling proteins or the production of cytokines can indicate if a receptor is working. An example is the production of a certain cytokine after the stimulation with a PRR ligand. This type of outcome enables the quantification of the functional response of the receptor present on the cell. Neonatal plasmacytoid dendritic cells (pDCs) express TLR7 and TLR9 at adult levels but they fail to secrete IFN-α/β against viral infections (Schuller et al. 2013). The expression of TLR mRNA and surface proteins on neonatal monocytes are comparable to adults, but the production of TNF to TLR stimulation is reduced (Levy et al. 2004). At birth in the term infant, TLR4 is present at adult levels on monocytes (Yan et al. 2004).

Basal TLR levels in neonates are comparable to those in adults (Quinello et al. 2014; Levy 2007; Silveira-Lessa et al. 2016), but the ability to produce cytokines in response to TLR stimulation is different (Levy et al. 2004). It has been shown that the PRRs are present on preterm innate immune cells (Levy et al. 2004; Levy 2007; Kan et al. 2018) suggesting that the diminished cytokine secretion is due to other mechanisms. The cause could be impairments of signaling molecules downstream of the PRR, a lack of transcription of cytokine mRNAs or a failure to properly cleave the protein into its mature form, such as IL-1β.

After encountering PAMPs or DAMPs, the innate immune cells are then recruited to the site of infection. The recruitment of neutrophils and other immune cells is done by signaling to endothelial cells to upregulate ligands/receptors which will slow and signal to circulating cells to

attend an infection at a specific site. The extravasation of immune cells out of circulation is directed by a chemokine gradient produced by resident macrophages and the upregulation of selectins and integrins on the endothelial cells near the site of infection. Once the pathogen is under control, the immune response switches to an anti-inflammatory state to clear up the pro-inflammatory cytokines and DAMP signals that are present and begin the process of healing. Innate immune cells respond to the PAMPs they see and have the same response upon each subsequent exposure to the same pathogen.

1.3.2.2 Neutrophils

Neutrophils make up 50% to 70% of all circulating leukocytes making them an important cell type for the immune response (Mayadas et al. 2014). Originally believed to be short-lived (5-12h) these cells have been more recently shown to exist in circulation for up to 5 days (Pillay et al. 2010). It has been shown in mice that *S. aureus*-infected wounds provide signals to extend the half-life of recruited neutrophils through anti-apoptotic signals (Kim et al. 2011). Previous research indicated a limited role for neutrophils in the early phase of immune response to an infection, especially in phagocytosis and killing. Newer studies indicate a role for neutrophils in coordinating the interactions of APCs and adaptive immune cells, by their migration to draining lymph nodes in humans (Beauvillain et al. 2007; Beauvillain et al. 2011; Galli et al. 2011; Breedveld et al. 2017). Neutrophils are an important immune cell that may have a greater role than previously recognized.

1.3.2.2.1 Rolling and attachment

Neutrophils circulate patrolling for signals on the endothelium of blood vessels in the form of the expression of E-selectin, which is upregulated on inflamed endothelium (Levy 2007). This interaction with E-selectin reduces the velocity of rolling neutrophils along the endothelium to enable binding to other endothelial receptors like intercellular adhesion molecule 1(ICAM-1) (Chesnutt et al. 2006). The tethering, rolling and full arrest of the neutrophils along the inflamed endothelium enables extravasation and the subsequent migration of the neutrophils to the site of infection, (reviewed in Kolaczkowska & Kubes, 2013)(. Cytoskeleton rearrangement through actin polymerization of neutrophils for migration is reduced in neonatal neutrophils (Harris et al. 1993) and neonatal polymorphonuclear cells' (PMNs) orientation and reaction to chemotactic gradients is impaired (Anderson et al. 1984) suggesting that their recruitment to sites of infection may be hampered (Linderkamp et al. 1998). In addition to the upregulated adhesion receptors on the endothelium at the site of infection or inflammation neutrophils will migrate along a chemokine gradient.

1.3.2.2.2 Chemoattraction and migration

Neutrophils use chemokine gradients to direct them out of circulation and toward the site of infection or inflammation. The main chemokine for neutrophil recruitment is interleukin-8 (IL-8, also known as CXCL8). To recruit circulating neutrophils IL-8 is produced by local cells such as pericytes, endothelial cells, previously recruited monocytes that have differentiated into macrophages, or tissue resident macrophages (Kim & Luster 2015). IL-8 is a known chemoattractant for neutrophils, but also of other immune cells such as basophils and T cells monocytes, macrophages and lymphocytes to sites of inflammation (Evgenievich et al. 2017).

IL-8 is produced by neutrophils, monocytes/macrophages, some T cell subsets, endothelial and epithelial cells (Huber et al. 1991; Wilhelmsen et al. 2012). Endothelial cells (human umbilical vein endothelial cells) from extremely premature neonates (< 26 weeks GA) are able to secrete IL-8 with TNF-α, IL-1β and LPS stimulations, and they can upregulate cell adhesion molecules E-selectin, ICAM-1 and VCAM-1 (Wisgrill et al. 2018). The endothelium produces the IL-8 from their apical surface and up-regulates ICAM-1 to help slow circulating neutrophils (Huber et al. 1991). IL-8 stimulation of the neutrophil induces calcium flux, leading to chemotaxis and the release of elastase, a serine protease involved in pathogen destruction and activation of γδ T cells (Williams et al. 2005; Towstyka et al. 2018). Phagocytosing neutrophils have been shown to secrete IL-8, which could recruit more immune cells to the location of the invading pathogen (Bazzoni et al. 1991).

Different isoforms of IL-8 have been described in humans during the ontogeny. These arise from a 99kDa amino acid that is posttranslationally modified with multiple natural NH_2-terminal truncations (Mortier, Gouwy, et al. 2011; Mortier, Berghmans, et al. 2011). A 72 kDa form is produced mostly by immune cells, while the 77kDa form is from non-immune cells (Hébert et al. 1990; Huber et al. 1991). Previous research has found a greater amount of the 72 kDa form of IL-8 in the plasma of preterm and term neonates compared to adults (Maheshwari et al. 2009). The dominance of the immune cell form (72kDa) circulating in neonates led researchers to investigate if there is a special function of this form of IL-8 and whether it has an altered activity. Herbert et al. (1990) employed functional assays to examine proteolytic cleavage of IL-8 by proteases the differences in binding to neutrophils. They showed that the 72kDa form predominantly produced by T cells and monocytes, binds to neutrophils two-times more than the 77kDa form (Hébert et al. 1990). IL-8 itself can form dimers or monomers with both being

sufficient for chemotaxis or elastase release by neutrophils (Williams et al. 2005). The two receptors of IL-8, CXCR1 and CXCR2 (CD181 and CD182, respectively) were described by Hammond et al. (Hammond et al. 1995). They demonstrated that CXCR1 is the main receptor of IL-8 for chemotaxis, and CXCR2 is dispensable for IL-8-induced migration (Hammond et al. 1995). The two receptors can form homodimers or heterodimers for binding IL-8 (Evgenievich et al. 2017; Stillie et al. 2009). The CXCR1 receptor is found on multiple cell types including neutrophils, monocytes, macrophages and T cells (Evgenievich et al. 2017). IL-8 is not just a chemoattractant, its 72kDa form has also been shown to activate neutrophils, inducing degranulation (Topham et al. 1998), one mechanism of killing used by neutrophils.

1.3.2.2.3 Killing: Phagocytosis, degranulation and NETs

On arrival, neutrophils can fight pathogens using three different killing mechanisms: phagocytosis, degranulation and nuclear extracellular traps (NETs) (reviewed in Kolaczkowska & Kubes 2013). Phagocytosis of pathogens by neutrophils uses opsonic receptors to bind the opsonized extracellular pathogen and engulf the pathogen in the cell's phagosome. The phagosome containing the pathogen then fuses with cytoplasmic granules to kill it. In neonates there is evidence that neutrophils possess functional receptors and are capable of phagocytosis (Ortmann & Kolaczkowska 2018). The phagocytic capacity of circulating preterm PMNs is diminished compared to term cord blood and adult PMNs, but this may be due to a lack of maternal Ig and associated opsonization of pathogens (Källman et al. 1998).

Degranulation is the release of vesicles containing antimicrobial peptides, including defensins and cathelicidins. These peptides induce membrane permeabilization and disrupt bacterial biofilms by forming pores on microbes, (Mayadas et al. 2014). The process of NETosis,

the release of NETs, was first described by Zychlinsky and colleagues as a form of extracellular killing by neutrophils (Brinkmann et al. 2004). NETs are a combination of strands of DNA and granular proteins, including enzymes and antimicrobial peptides such as neutrophil elastase, LL-37, S100A and lactoferrin and myeloperoxidase (Mayadas et al. 2014). The production of NETs, granules and nuclear material extruded to snare microbes, in response to inflammatory agonists (LPS and platelet activating factor) is impaired in neonates (Yost et al. 2009). However, Byrd et al. (2016) showed that NETosis occurs in response to fungal pathogen stimulation in neonates, indicating that it is not a complete impairment of NETosis, but is pathogen specific (Byrd et al. 2016). Induction of NET formation by phorbol 12-myrisate 13-acetate (PMA) in term neonates is better than in preterms at birth (Lipp et al. 2017). Preterms have the capacity for NETosis by day 3 after birth, with full capacity reached within the first 2 weeks (Yost et al. 2016). It was thought that NETosis meant the death of the neutrophil, but it has been shown that even without a nucleus these cells can migrate and kill bacteria by other methods (Yipp et al. 2012).

1.3.2.2.4 Subsets of neutrophils

The maturation process of neutrophils involves the segmentation of the nucleus. Examination by microscopy has been the traditional method to identify immature (band-form) and mature neutrophils (multi-lobed nucleus). With the advent of flow cytometry there have been efforts to characterize the phenotype of these two cell populations (Pillay et al. 2013). This has only increased the identified subsets of neutrophils. There is debate in the neutrophil field as to whether there are different subsets of neutrophils, or if these subsets are activated states or phenotypes that develop due to the specific insult or inflammation (Pillay et al. 2013; Christoffersson & Phillipson 2018). Some subsets of activated granulocytes have been

demonstrated to have a suppressive effect on T cells in adults (Schmielau & Finn 2001), and in neonates a neutrophilic/granulocytic phenotype myeloid-derived suppressor cell type is immunosuppressive for NK and T cells (Rieber et al. 2013). Our understanding of these subsets is only beginning and the investigation into the function of neutrophils subsets in neonates requires significant research.

1.3.2.2.5 Functional interactions between neutrophils and T cells

It has been demonstrated that neutrophils migrate to the spleen and lymph nodes after encountering pathogens. Using mice, it has been shown that subcutaneously administered fluorescently-labelled chicken egg ovalbumin (OVA) particles are phagocytosed by neutrophils, which migrate via the lymphatics to draining lymph nodes (Maletto et al. 2006). In another murine study, antigen-positive neutrophils were detected in the lymph nodes and spleen suggesting a role beyond killing and clearance of pathogens at the site of infection (Hampton et al. 2015). Vono and colleagues demonstrated that neutrophils can act as APCs for antigen-specific memory CD4+ T cells in an HLA-DR-dependent manner, but professional APCs, monocytes and DCs, are better at presenting to CD4 T cells (Vono et al. 2017). The presence of the antigen and antigen-specific CD4 T cells induced by cytokines in the environment leads to the upregulation of major histocompatibility complex (MHC) class II and co-stimulatory molecules (CD80, CD86) on polymorphonuclear neutrophils (Radsak et al. 2000). In addition, neutrophils are able to suppress T cells via three mechanisms: arginase 1 secretion removes L-arginine required for T cell proliferation (Rodriguez et al. 2007), extracellular reactive oxygen species (ROS) production inhibits cytokine production by T cells, especially IFN-γ (Schmielau

& Finn 2001), and ROS expelled directly into the immune synapse suppresses T cell activation (Pillay et al. 2012).

1.3.2.3 Antigen presenting cells: bridging the innate and adaptive immune systems

Professional APCs consist mainly of B cells, monocytes, macrophages and DCs, which present processed peptides to T cells. The two sub-types of DCs are conventional DCs (cDC, previously myeloid DC) and plasmacytoid DCs (pDC) (Willems et al. 2009), which are considered immature while circulating in the blood during their migration to tissues. In order to present antigens professional APCs must phagocytose the pathogen or part thereof to process them into peptides, which can then be presented on the surface of the cell in association with MHC class I and class II molecules. As phagocytosis is critical for APC capacity, this function has been evaluated in preterm cord blood as well as postnatal blood (Strunk et al. 2012). Early research indicated that fewer monocytes and neutrophils in preterms were able to phagocytose than in term neonates but those that did had a higher capacity to phagocytose *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Escherichia coli* (Prosser et al. 2013). However, more recent work has shown that neutrophils and monocytes of healthy neonates regardless of gestational age were capable of phagocytosing *E. coli* and *S. aureus*, but at a lower frequency than in adults (Silveira-Lessa et al. 2016). The reduced phagocytosis of *E. coli* by monocytes in neonates is resolved by day 3 after birth (Filias et al. 2011). Recent work from our lab has shown that the metabolic requirements for phagocytosis of, for instance fixed clinical isolates of *Candida*, differ from the requirements for innate immune PRR activation in preterm monocytes (Kan et al. 2018). The production of ROS is not different in neonatal and adult phagocytes, but phagocytosis-induced cell death is lower in neonatal cord blood monocytes (Gille et al. 2009).

After activation of their PRRs, APCs secrete cytokines and upregulate co-stimulatory receptors in concert with the presentation of the foreign antigen to T cells to activate the adaptive immune system. The uptake and processing of antigens from phagocytosed or endocytosed pathogens enables the presentation of antigens mounted on MHC class I or II receptors, known as human leukocyte antigens (HLA) in humans. The major human MHC class I are HLA-A, -B, -C and the major MHC class II are HLA-DP, -DQ, -DR (Choo 2007). All cells except red blood cells express MHC class I molecules and can present peptides to CD8 T cells, while APCs can also express MHC class II molecules and present peptide antigens to CD4 T cells. The expression of HLA-DR on preterm monocytes is lower than in term neonates (Prosser et al. 2013; Hallwirth et al. 2004), potentially leading to reduced antigen presentation. The expression of CD80, a co-stimulatory molecule for CD4 T cells, on myeloid DCs and monocytes reaches adult levels by three months of age and HLA-DR on plasmacytoid DCs and monocytes by 6-9 months after birth (Nguyen et al. 2010) MHC class I and class II molecules usually present self-antigens, which T cells recognize as self. It is when these MHC molecules present foreign peptides in the context of self-MHC molecules that antigen-specific T cells will recognize as non-self and look for other signals to determine if there is an active infection and not a false alarm. The presentation of foreign peptide relies on the ability of APCs to phagocytose pathogens and degrade their constituent components for presentation. The APCs, if not tissue resident cells, must migrate out of circulation to the site of an infection. After encountering and phagocytosing the pathogen the activated APC migrates to a draining lymph node where it enters the paracortex and presents its antigens loaded onto MHC class I and class II molecules to the T cells.

The cytokines produced by the APCs can activate local immune cells at the site of infection and direct the differentiation of naïve T cells after the migration to the secondary lymphoid organs (SLO), making them essential components of both the innate and adaptive immune system. In humans, IL-12p70 is produced by cDCs and IFN-α/β is produced by pDCs after PRR stimulation, though production of this cytokine is generally very low in neonatal immune cells (Willems et al. 2009). We investigated the production of IL-1β by monocytes to understand why preterms secrete minimal amounts upon stimulation with lipopolysaccharide (LPS). Preterm monocytes produce comparable amounts of pro-IL-1β protein, but fail to secrete mature IL-1β to the same levels as terms and adults (Sharma et al. 2015). Further investigation into the maturation of IL-1β via the cleavage of pro-casepase-1 by the NLRP3 inflammasome showed low levels of mature caspase-1 in preterms (Sharma et al. 2015). Further work by Kan et al. shows that the inability of preterms to cleave IL-1β in response to fungal stimulation is due to a lack of MALT-1 protein. MALT-1 is a crucial part of the paracaspase complex required to cleave pro-caspase into its mature form resulting in the cleavage of pro-IL-1β into its mature, secreted form (Kan et al. 2018). Further, this work suggests that there is a disconnection between the production of IL-1β mRNA in response to PRR stimulation in innate immune cells and the production of cytokines. The poor or limited production of cytokines by APCs can have consequences not only for the early innate immune response, but also adaptive immunity. IL-12 secretion is required for the differentiation of naïve CD4 T cells into a Th1 cells. A reduced capacity to produce this cytokine could result in an inability of neonatal naïve T cells to differentiate into Th1 cells, required to combat intracellular infections, and instead turn into Th2 cells.

1.3.3 Adaptive immune system

The intersection of the innate and adaptive immune systems is the point where detecting a PAMP becomes the antigen-specific recognition of that individual strain of a pathogen. The function of adaptive immunity is to direct both antibody and cellular responses to help identify and capture pathogens and to kill infected cells. The adaptive immune system begins with the interaction between an APC presenting foreign antigens to the cognate T cell. A repertoire of 1 x 10^{15} T cell receptors (TCRs) can be generated through somatic rearrangement to recognize a diverse array of antigens (Arstila et al. 1999), and each T cell is searching for its cognate antigen recognized by its unique TCR. The interaction of the T cell with the APC occurs in secondary lymphoid organs, such as lymph nodes (LN). Activated DCs mature during migration to SLOs and present antigen to CD4 T cells. Once a T cell finds its cognate antigen under the right conditions, it will become activated and undergo clonal expansion and differentiate into a specific type of effector T cell. Effector T cells modify chemokine detecting receptors to enable migration out of the LN and homing to the site of inflammation/infection (Murphy 2012). Upon migration to the site of the infection effector cells produce cytokines to activate innate immune cells.

1.3.3.1 CD4 T cells

The adaptive arm of the immune system relies on antigen-presenting cells (especially DCs) migrating to secondary lymphoid organs and presenting the pathogen-specific antigens to CD4+ T cells, to find the T cell expressing pathogen-specific TCRs. Subsequently, a cognate-antigen specific T cell will become activated, proliferate and differentiate into a specific type of T helper cell capable of instructing the immune response. These effector T cells leave the LN

and migrate back to the site of infection secrete cytokines to support the function of innate immune cells in their responses to the infection. After the pathogen has been controlled, the number of effector T cells declines through apoptosis and a small long-lived population of antigen-specific memory T cells is generated. Memory T cells are important upon a secondary encounter with the same pathogen as they can mount a rapid response and immediately possess effector functions such as the rapid production of pro-inflammatory cytokines.

1.3.3.1.1 Development and thymic selection of T cells

Bone marrow-derived lymphoid progenitor cells migrate to the thymus where they undergo development into mature T cells (reviewed in Vacchio et al. 2016). In the thymus, immature T cells called thymocytes that lack expression of CD4 and CD8 co-receptors (termed double-negative) undergo rearrangement of the gene segments of the alpha and beta chains of the T cell receptor (TCR). Upon productive rearrangement and expression of its TCR at the surface, thymocytes expressing both CD4 and CD8 molecules (called double-positive) undergo the process of positive selection to ensure they recognize self-MHC molecules on the surface of thymic epithelial cells. Thymocytes that are positively selected (self-restricted) down modulate CD4 or CD8 and become CD4 or CD8 single positive (SP) depending on whether their TCR recognizes MCH class II or class I molecules respectively. To ensure developing T cells are tolerant of self-antigens, they undergo a second check point called negative selection in which thymocytes expressing TCRs that are strongly self-reactive undergo programmed cell death. Negative selection is thought to be mediated by the presentation of ubiquitous and tissue-restricted self-antigens by cortical epithelial cells, medullary epithelial cells and thymic DCs that instruct clonal deletion of the self-reactive (pathogenic) T cells. Once they have passed these

checkpoints, mature T cells exit the thymus and begin circulating through secondary lymphoid organs in search of their cognate antigen. Expression of chemokine receptors is important for directing the migration of newly minted T cells toward secondary lymphoid organs, such as lymph nodes. For example, CCR7 is highly expressed on naïve (antigen-inexperienced) T cells and its ligands CCL19 and CCL21 are produced by high endothelial venules of the peripheral lymph nodes (LN), instructing them to travel toward lymph nodes (Baekkevold et al. 2001). Naïve CD4 T cells that have recently exited the thymus are called recent thymic emigrants (RTEs). RTEs express CD31 and have higher levels of single joint TCR excision circle (sjTREC) than other naïve CD4 T cells that have been circulating in the periphery for a while (Scheible et al. 2018). The homeostatic proliferation of naïve CD4 T cells dilutes the amount of sjTREC within these cells during their extended time in the periphery (Naylor et al. 2005). With age, thymic involution results in diminished output of RTEs and the remaining naïve T cells continue to proliferate and decrease their sjTREC levels, whilst maintaining their CD31+ CD45RA+ phenotype (Kimmig et al. 2002; Kilpatrick et al. 2008).

1.3.3.1.2 T cell activation and signaling

Naïve CD4 T cells require interaction with their cognate antigen presented by an APC for T cell activation. To be fully activated three signals need to be received by the naïve CD4 T cell. The first signal is the interaction of the antigen loaded on MHCII of the APC with the TCR and CD4 on the T cell. The second signal is the interaction of the CD28 and other co-stimulatory receptors on the T cell with the CD80 and CD86 receptors on the APC. If the T cell does not receive both these signals, it may become anergic (unresponsive) or undergo apoptosis through activation-induced cell death. The third signal is the environment of cytokines secreted by the

APC and neighbouring cells that instructs the naïve T cell's differentiation program and the type of immune response initiated (Geginat et al. 2013). Moreover, cytokines will direct the naïve T cell to differentiate into the appropriate T helper cell to combat the specific pathogen. To increase the number of T cells specific to the pathogen, activated T cells undergo proliferation, and thus clonal expansion. The process of differentiation can take days and once completed, the expanded, activated T helper cell will return to circulation and migrate to the site of the infection. Once there, effector CD4 T cells secrete their designated cytokines, specific to the T helper subtype, to further direct the response of local immune cells against the invading pathogen. The role of the cytokines produced from the effector T cell is to help direct the activation and function of macrophages, DCs and NK cells to clear the infection and instruct B cells to undergo antibody class-switching. T helper cells regulate adaptive immunity by assisting B cells in immunoglobulin class-switching, providing IL-2 to support the expansion and function of cytotoxic CD8 T cells and by inducing the upregulation of co-stimulatory molecules on APCs (Murphy 2012).

1.3.3.1.3 Differentiation into effector cells

Once the naïve T cell has encountered its cognate antigen and received co-stimulatory signals from the APC, it utilizes cytokine cues in the local environment to direct its differentiation into a T helper subset. Currently, there are six types of described T helper cell subsets in humans (Th1, Th2, Th17, Tfh, Th9, Th22) (Sun 2014). The best studied are Th1, Th2, and Th17. IL-12 produced by APCs and other local cells direct a naïve cell to express T-bet and become a Th1 cell capable of targeting intracellular infections. Th1 cells respond to viruses and intracellular bacteria (e.g. salmonella and listeria). If the environment contains IL-4, IL-25, IL-33

and thymic stromal lymphopoietin, (Geginat et al. 2013; Paul & Zhu 2010), then T cells upregulate GATA-3 and become Th2 cells. Th2 cells are designed to combat extracellular bacteria and parasites. In the presence of IL-6, IL-23 and TGF-β, an activated naïve CD4 T cell will be programmed to differentiate into a Th17 cell, identified by production of IL-17 and the presence of transcription factor RORC2. Th17 cells are important for immune responses at epithelial barriers against bacterial and fungal pathogens (Cosmi et al. 2008).

There has been much in the literature indicating that neonatal CD4 T cells skew toward the Th2 phenotype (Adkins et al. 2003; Jacks et al. 2018). The intrinsic skewing of neonatal T cells towards producing Th2 cytokines was first described in neonatal mice (Adkins et al. 2003), but once research was conducted on human cord blood cells it was determined that the differentiation of naïve T cells in neonates is more complicated (Jacks et al. 2018). This apparent Th2-skewing could be an intrinsic default setting of the neonatal CD4 T cell, part of the reduction in pro-inflammatory responses as the Th1/Th17 cytokines, including IFN-γ and IL-17, could be detrimental to the developing fetus. A second reason for the skewing of neonates to Th2 could be that the cytokine milieu produced by APCs and other cells (signal 3), which drives the differentiation of the naïve T cell, are produced in greater abundance than those required for Th1/17 differentiation (Koch et al. 2011). Epigenetic examination of this Th2-skewing has shown the presence of permissive marks at the Th2 promoter in Th2 differentiated cells, and surprisingly a lack of repressive marks of the Th2 locus in Th1 differentiated cells (Webster et al. 2007).

1.3.3.1.4 Development of memory cells (post-effectors)

After the pathogen has been cleared, most effector T cells undergo apoptosis, while some remain in the tissues as memory effector cells, while others in SLO remain there as central memory T cells. Upon a secondary encounter with their cognate antigen, memory cells rapidly proliferate, produce pro-inflammatory cytokines and migrate to sites of infection. After each encounter with the antigen a subset of central memory T cells remain in SLO to maintain an army ready to become effector cells with the next encounter. It is the memory T cells that enable the rapid response (hours, not days) and in hosts that possess few memory T cells, such as newborns, each encounter requires a primary response and the adaptive immune response cannot be relied upon to rapidly help fight an infection.

In the neonate most of the T cell population is naïve, as the T cells have yet to encounter many antigens and have been in a tolerant and relatively sterile (low microbe) environment. With low numbers of effector and memory T cells the neonate must rely on its ability to fight infections using the innate immune system (Zhang et al. 2014) and contain pathogens until the effector response of T cells and the antibody production of B cells can occur. The ability to study the preterm neonatal immune system is often restricted to *in vitro* studies of their cord blood. One study examined the capacity to respond to polio virus antigen after vaccination to a secondary infection, comparing preterms (≤ 33wks) to terms (≥37wks) (Klein et al. 2010). They found polio-specific cell numbers in preterms were comparable to term infants, but the preterms had less proliferation of their T cells (Klein et al. 2010).

1.3.3.2 B Cells and antibodies (maternal transplacental antibodies)

B cells possess the B cell receptor (BCR) with machinery capable of generating a large BCR repertoire by recombination, similar to the TCR (Brack et al. 1978). The BCR recognizes antigens as whole proteins, not only peptides. Differentiated B cells become plasma cells that will produce antibodies (immunoglobulins, Ig) that can opsonize the pathogen for phagocytosis by other immune cells (Murphy 2012). The transfer of maternal antibodies via the placenta is a major source of antibody protection for the neonate; however, the majority of the IgG is transferred after 32 weeks of gestation leaving preterm infants born before 30 weeks vulnerable to vaccine preventable infections (van den Berg et al. 2011). The administration of vaccines to preterm neonates has been shown to provide protective antibody titer levels while others have shown that preterms have lower titers compared to term infants and this may be vaccine-specific, but the response is dependent on gestational age and birth weight (Gagneur et al. 2015). Antibody levels in circulation do not indicate the capability of the CD4 T helper cells to respond to the vaccination.

1.3.3.3 Innate-like T cells and other novel functions

The discovery of innate-like and innate lymphocytes has modified our understanding of the adaptive immune system. Lymphoid cells that can respond in an effector T cell manner by undergoing clonal expansion and producing cytokines in response to non-MHC-directed stimulation indicates a new function for lymphoid cells. These cells include γδ (gammadelta) T cells, mucosal associated invariant T cells (MAIT), and innate-lymphoid cells (ILCs) which tend to be tissue resident cells and react to pathogen components or cytokines (Liuzzi et al. 2015; Verykokakis et al. 2014; Wencker et al. 2014; Bedoui et al. 2016). γδT cells produce more IFN-γ

than αβT cells, and may compensate for the impaired αβT cell responsiveness at birth in both terms and preterm (Gibbons et al. 2009). Cytokines produced from γδT and MAIT cells have been shown to induce an APC-like phenotype in neutrophils increasing their survival and enabling their cross-presentation of soluble antigens to CD4 and CD8 T cells (Davey et al. 2014). The continued study of innate-lymphoid cells suggests that current categorization of T cells is too narrow. More subsets of T cells may have specialized functions with beneficial capabilities for neonates.

1.4 Understanding the postnatal immune system of premature neonates

To understand the development of preterm immune responses during the postnatal period it is imperative that we study premature babies during their time in the NICU when they are not experiencing any type of infection. It must be noted that these babies may have been exposed to infections *in utero* as infections are a main cause of premature birth. However, assessing comparatively healthy preterms across various gestational ages will provide a baseline of developmental changes. It will inform the timing of immune responses reaching term levels after premature birth, indicating if birth and exposure to the *ex utero* environment can itself speed up development that continues during the postnatal period.

1.4.1 Objectives

The immune system of neonates is developmentally regulated, and premature birth interrupts this process making these infants more vulnerable to infections. The objective of this research is to determine how the immune responses of preterm neonates change after birth (during the period they are hospitalized) compared to their term counterparts or to adults. To investigate this, we prospectively recruited preterm neonates sampling at birth (cord blood) or during their time in the NICU. Term cord blood and adult peripheral blood were recruited and collected as controls. The overall goal of the thesis is to study the maturation of immune responses of premature neonates during the postnatal period.

Objective 1 (Chapter 2): To investigate TLR responses in premature neonates during the postnatal period.

Objective 2 (Chapter 3): To characterize postnatal maturation events using transcriptomic profiles of uninfected preterm infants.

Objective 3 (Chapter 4): To investigate innate-like production of the IL-8 cytokine in neonatal naïve CD4 T cells.

Chapter 2: Attenuated innate immune response in premature neonates during the neonatal period

2.1 Background

Despite improvements in neonatal health care, mortality from infections has continued to increase in preterm neonates over the last two decades, owing to the improved survival of younger and more vulnerable infants (Berrington et al. 2012). However, the underlying immunological basis of their high morbidities and high vulnerability to sepsis remains poorly understood (Sharma et al. 2012). To defend themselves against infection, neonates rely on first-line, innate immune defense mechanisms. These mechanisms include the detection of specific microbial molecular structures called Pathogen-Associated Molecular Patterns (PAMP) through specialized receptors termed Pattern Recognition Receptors (PRR), but also other defense mechanisms such as phagocytosis and antigen presentation to the immunologically naïve adaptive immune system.

It is widely stated that the high risk of neonatal sepsis observed in these infants is due to immature immune defense mechanisms. However, data are lacking on the development of immune functions beyond measures performed on umbilical cord blood. Recent studies have reported reduced antigen-presenting receptor expression during the first week of age in infants born below 33 weeks of gestation (Azizia et al. 2012; Palojarvi et al. 2013). In contrast, phagocytic functions in preterm monocytes and neutrophils are more comparable to term infants (Prosser et al. 2013). Toll-like receptors (TLRs) play a predominant role as a major family of PRR that act as essential “detectors” in the recognition of microbial pathogens by the innate

immune system. We and others have shown that TLR-induced cytokine immune reactivity is profoundly attenuated in cord blood of preterm neonates and develops asynchronously over the last trimester of gestation (Strunk et al. 2012; Lavoie et al. 2010; Sharma et al. 2014; Förster-Waldl et al. 2005; Sadeghi et al. 2007). These infants also display sustainably high levels of systemic inflammation, indicating a potential immune dysregulation that has also been associated with worsened clinical outcomes (Chang et al. 2011). On the other hand, cord blood responses may not adequately reflect important developmental adaptation occurring during the first few weeks after birth. Also, it is unclear to what extent specific perinatal events may induce an earlier maturation of PRR responsiveness during the neonatal period in these infants, resulting in an hyper-reactive state (Paananen et al. 2009).

In this study, we evaluated anti-microbial TLR-induced cytokine responses in preterm neonates over the first 28 days of age. We confirmed that these cytokine responses remain markedly attenuated during the neonatal period using whole blood measures of secreted cytokines and production within innate immune cell subsets. These data provide indispensable support to the premise that reduced innate immunity may contribute to the increased risk of neonatal sepsis in this age group.

2.2 Materials and methods

2.2.1 Study population

Neonates were prospectively enrolled at the British Columbia Children's & Women's Health Centre (Vancouver, Canada) between June 2012 and March 2014, after parental informed consent. Preterm neonates born below 30 weeks of gestation admitted to the Neonatal Intensive Care Unit were sampled once, according to a gestational age-matched schedule where blood was taken during either periods of 2 to 14, or 15 to 28 days of age in order to distribute subjects evenly across postnatal days. All peripheral blood samples were coordinated with other early morning routine blood taking. Also, samples were scheduled to be collected at least 48 hours away from a blood product transfusion, or from postnatal corticosteroid administration. Infants who were actively diagnosed with suspected or proven sepsis or under antibiotic treatment were excluded. For external validation, the amount of exposure to supplemental oxygen, mechanical ventilation, parenteral nutrition, and use of intravenous sedation (either morphine or midazolam) at time of blood sampling in preterm neonates is also provided in **Table 2.1**. To further confirm generalizability of our findings, white blood cell composition was determined using a diagnostic automated cell count for neutrophils, monocytes and lymphocytes in the subgroup of preterm neonates throughout the study period, as part of routine clinical care and was within expected normal ranges (**Figure 2.1**). Cord blood samples were obtained from healthy control neonates delivered at term by elective Caesarean section and from peripheral blood of healthy adults using identical protocols and reagent batches. All blood samples were collected in sodium heparin BD Vacutainer tubes (Becton, Dickinson, Mississauga, Canada) and processed within one hour of collection. To avoid an experimental historical bias during recruitment, a proportional number of preterm, term, and adult subjects were tested over the course of the study. Chorioamnionitis was

defined as infiltration of leukocytes by histological examination as previously described (Chang et al. 2011). Antenatal corticosteroid and magnesium sulfate exposure were defined as occurring within 72 and 24 hours prior to delivery, respectively. SNAP scores were determined as described (Richardson et al. 2001). The study was approved by the University of British Columbia Children's & Women's Research Ethics Board (#H07-02681).

Table 2.1 Clinical characteristics of preterm neonates on sampling day for each group

Clinical characteristics	Cord blood (Day 0)	Day 2 to 14	Day 15 to 28
Mechanically ventilated (%)	N/A	50	29
Supplemental oxygen (%)	N/A	81	76
Parenteral nutrition (%)	N/A	81	24
Use of sedation[a] (%)	N/A	50	18

[a] either morphine (most commonly used) or midazolam (only occasionally used)

N/A: not applicable

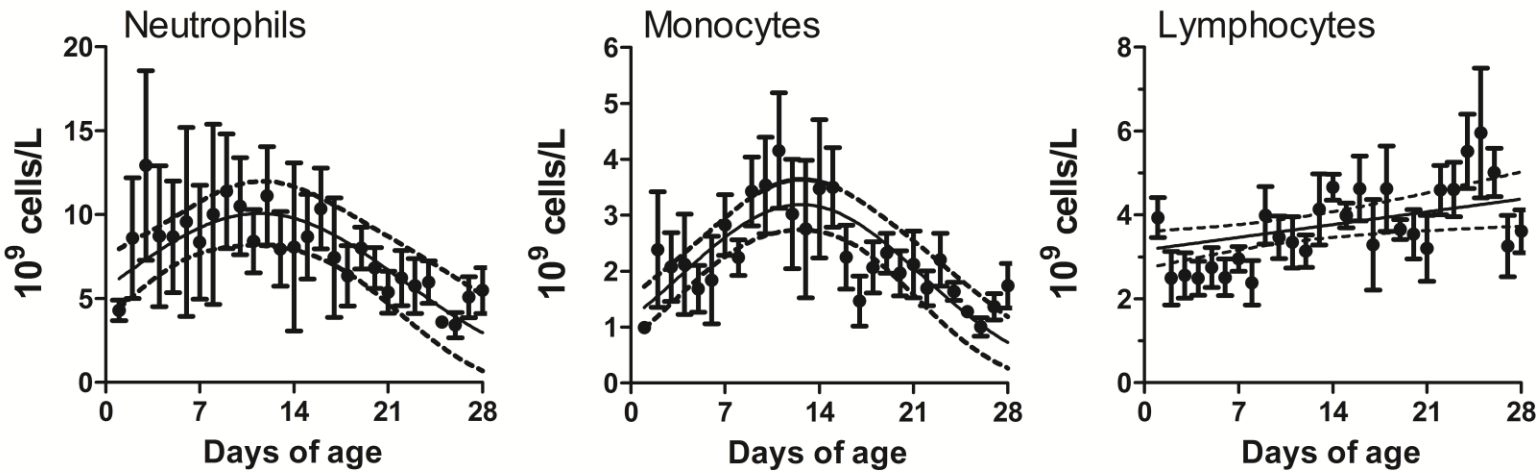


Figure 2.1 Complete blood counts in preterm neonates

Cell counts for neutrophils, monocytes and lymphocytes from complete blood cell counts from routine hospital testing of all sampled preterm subjects. Acquired within 12 hours of birth and continuing throughout the hospital stay. Graph represents mean + SEM in addition to the nonlinear fit of the mean. The increasing and decreasing trends of neutrophil and monocytes numbers are consistent with the normal patterns in preterm babies.

2.2.2 Immune stimulation and cytokine measures

To limit experimental variability in our assays, TLR agonists were pre-diluted in batches at the beginning of the study in quantities enough for six month periods in accordance with pre-study stability tests done of all main reagents used in the study (data not shown). To ensure stability of the reagents, each ligand batch was also tested against a group of adult subjects at regular intervals (not shown). Whole blood was diluted 1:2 with RPMI 1640 medium with L-glutamine (Gibco, Grand Island, NY) and stimulated with saturating doses of the TLR4 agonist lipopolysaccharide (LPS, 100 ng/ml; InvivoGen, San Diego, CA), the TLR7/8 agonist R848 (1 μg/ml; InvivoGen), or unstimulated, for 24 hours at 37°C in 5% CO_2 atmosphere in pre-diluted aliquots round-bottom 96-well microplates. The concentration of TLR agonists used in this study corresponded to peak stimulating concentrations, as determined in our previous studies (Lavoie et al. 2010; Jansen et al. 2008). Following stimulation, cell supernatants were stored at -80°C for batches analyses. Cytokine measurements were performed in duplicates using commercially available enzyme-linked immunosorbent assays (ELISA) for the following cytokines: IL-1β, IL-6, IL-10 and TNF-α (eBioscience, San Diego, CA) and IL-12p40 (BioLegend, San Diego, CA), as described (Lavoie et al. 2010). Mean intra-assay coefficients of variability for all cytokine-stimulated levels were consistently below 15%. In each age group, half of the subjects were also randomly sampled in parallel for use in whole blood immune cell composition and intracellular cytokine production assays, using flow cytometry as described (Lavoie et al. 2010). For flow cytometry analyses, the following antibodies were used: Alexa Fluor 700-conjugated anti-CD11c, PE-Cy7-conjugated anti-CD14, eVolve605-conjugated anti-CD19 and PerCP-Cy5.5-conjugated anti-HLA-DR (eBioscience), and PE-conjugated anti-CD3 (BD Biosciences, Mississauga, Canada) for identification of monocytes (HLA-DR+, CD14+), myeloid dendritic

cells (HLA-DR+, CD11c+), B cells (CD19+), T cells (CD3+) and polymorphonuclear cells / granulocytes (by forward scatter and side scatter), as described (Lavoie et al. 2010). For intracellular cytokine detection, cells were incubated in the presence of brefeldin A (10 μg/μl, Sigma Aldrich, St. Louis, MO) and the following additional antibodies were used (after permeabilization): Pacific Blue-conjugated anti-CD3 (instead of PE-conjugated, BioLegend), APC-conjugated or FITC-conjugated anti-IL-6, PE-eFluor610-conjugated TNF-α and PE-conjugated-IL-12/23p40 (eBioscience), as described (Lavoie et al. 2010). No significant cytokine production was detected in unstimulated polymorphonuclear cells (not shown). In all flow cytometry analyses, cells were also stained using FITC-conjugated anti-glycophorin A to allow gating out of any residual red blood cells in the sample and gates were adjusted using fluorescence-minus one controls (for cell surface markers) and unstimulated control samples (for cytokine detection). Data was analyzed using the FlowJo software (FlowJo vX 10.0.7r2; FlowJo LLC, Ashland, Oregon).

2.2.3 Statistical analysis

Differences between stimulated and baseline cytokine responses were compared between age groups using non-parametric testing (Mann-Whitney U) were all highly significant ($p<0.0001$ without correcting for multiple testing; data not shown). In initial analyses, responses within the whole group of preterm neonates were assessed using linear regressions and were shown to be independent of whether samples were collected at the time of birth from cord blood (labeled as day 1), or from postnatal peripheral blood. This finding was then confirmed using analysis of covariance incorporating postnatal age as a continuous variable, where cytokine responses were then compared between cord and peripheral blood (data not shown); therefore, all

cord and peripheral blood samples were combined in subsequent analyses. To determine the impact of postnatal age, differences between groups were compared using a one-way ANOVA, as a function of postnatal age (categorized as preterm cord blood, preterm day 2-14 and preterm day 15-28, term) and using a post-test Dunn's correction for multiple testing ($p<0.05$). In subsequent analyses, the relative contributions of gestational age and the independent effect of postnatal age were confirmed within the subgroup of preterm neonates, using multiple linear regressions where both parameters (gestational and postnatal age) were included as continuous variables. The residual contributions of perinatal factors (chorioamnionitis: yes/no, antenatal corticosteroids: yes/no, presence of labour: yes/no, mode of delivery of the infant: vaginal/C-section and use of magnesium sulfate: yes/no) were individually assessed using partial correlations, with adjustment for gestational age (as a continuous variable). Associated 95% confidence intervals were obtained using the bootstrap.

2.3 Results

2.3.1 Attenuated postnatal immune responses in premature neonates

Clinical characteristics of preterm subjects are provided in **Table 2.2**. To determine the extent to which preterm neonates were able to respond to PAMP during the neonatal period, whole blood cytokine responses to two prototypic TLR agonists were compared between preterm neonates (n = 50) on both cord and postnatal blood, term infants (n = 30) and adult controls (n = 25). Expectedly, cord blood LPS and R848 responses were markedly reduced in preterm neonates (**Figure 2.2**). When comparing TLR responses in preterm infants as a function of postnatal age, significant increase was also observed over the first 28 days of age (**Figure 2.2**). The distribution of gestational age in preterm infants was identical between time periods,

indicating little effect from the degree of maturation at birth (**Figure 2.3**). However, to more fully account for an effect due to gestational age, postnatal TLR reactivity was analyzed in preterm neonates according to postnatal days used as a continuous variable, in regression models. Estimates of effects are presented in **Table 2.3** and quantify the increase in TLR responsiveness for each increase in postnatal days independent of gestational age, within the group of preterm neonates**.** These results confirmed a large effect of postnatal age on most LPS and R848 cytokine responses. Indeed, significant residual influences of postnatal age were detected for IL-6 responses (using R848), TNF-α (for both LPS and R848) and IL-12/23p40 (for both LPS and R848) ($p<0.05$).

Table 2.2 Clinical characteristics of all preterm neonates

Clinical characteristics	N = 50
Gestational age (weeks; mean +/- SD)	27.2 +/- 2.1
Birth weight (g; mean +/- SD)	1051 +/- 370
Sex (% male) (95% CI)	67 (54, 81)
Received antenatal corticosteroids (%) (95% CI)	87 (72, 101)
Presence of labour (%) (95% CI)	70 (56, 83)
Received antenatal MgSO4 (%) (95% CI)	76 (62, 90)
Born by caesarian section (%) (95% CI)	70 (56, 83)
Histological chorioamnionitis (%) (95% CI)	49 (34, 63)
SNAP score at birth (median, IQR)	26, 14 – 49

SD: standard deviation; CI: confidence interval; IQR: inter-quartile range; $MgSO_4$: magnesium sulfate; SNAP scores were recorded within 12 hours of birth

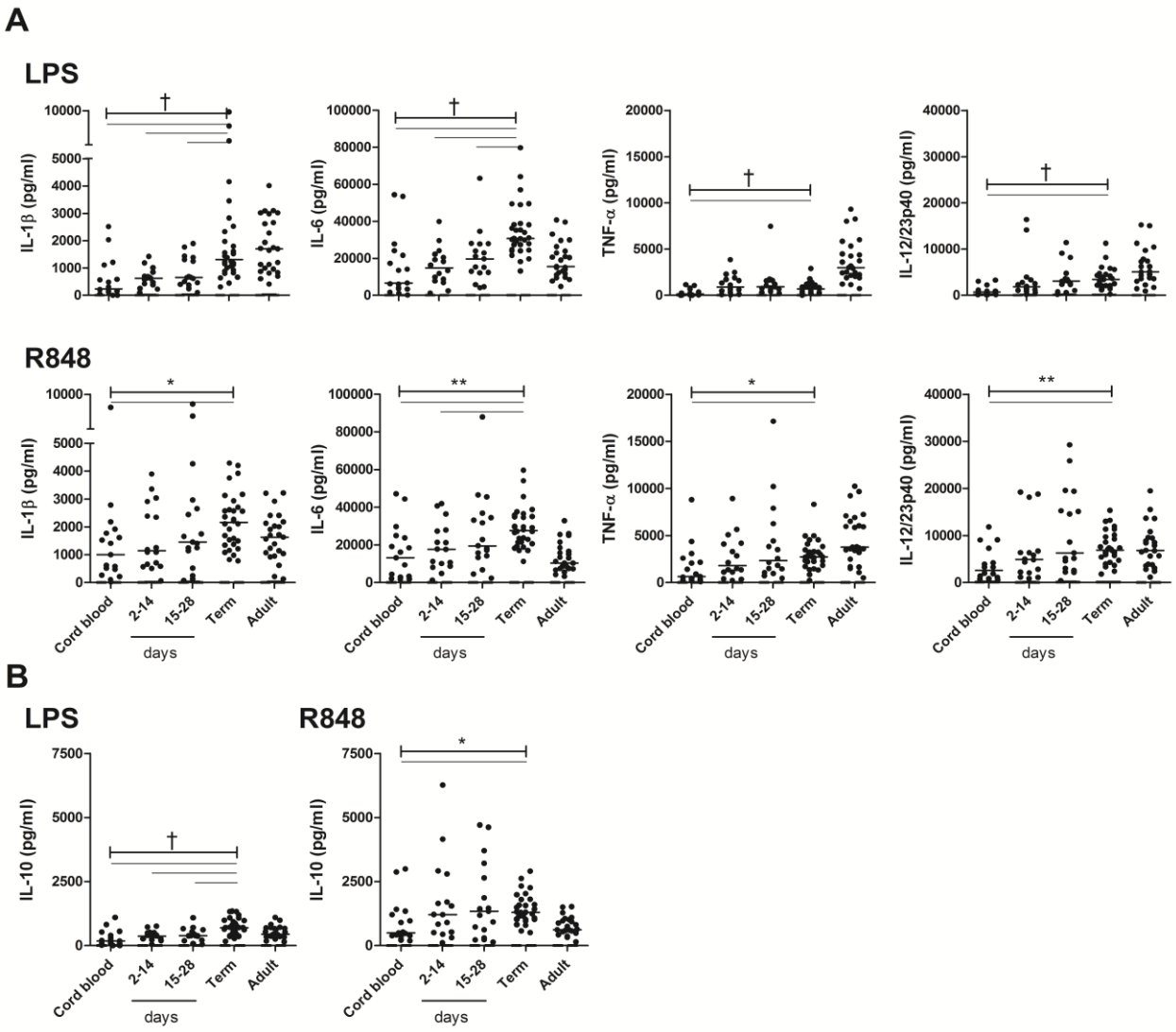


Figure 2.2 Toll-like receptor cytokine responses.

Whole blood **(A)** pro- and **(B)** anti-inflammatory cytokine responses in preterm neonates grouped by sampling period on cord blood, 2-14 and 15-28d of age, and in term cord blood and adults, following stimulation with lipopolysaccharide or R848 (bars represent median) Unstimulated cytokine levels (median) were undetectable (0 pg/ml) in all cases (not shown). Significant differences in cytokine responses across all three groups of preterm neonates and term neonates, as determined using an ANOVA, are shown as capped-end line (*$p < 0.05$; **$p < 0.01$; † $p < 0.001$). Each dot represents an individual subject. Statistical comparisons within each subgroup, as determined using a Dunn's post-test taking into account multiple comparisons, is shown as straight lines ($p < 0.05$).

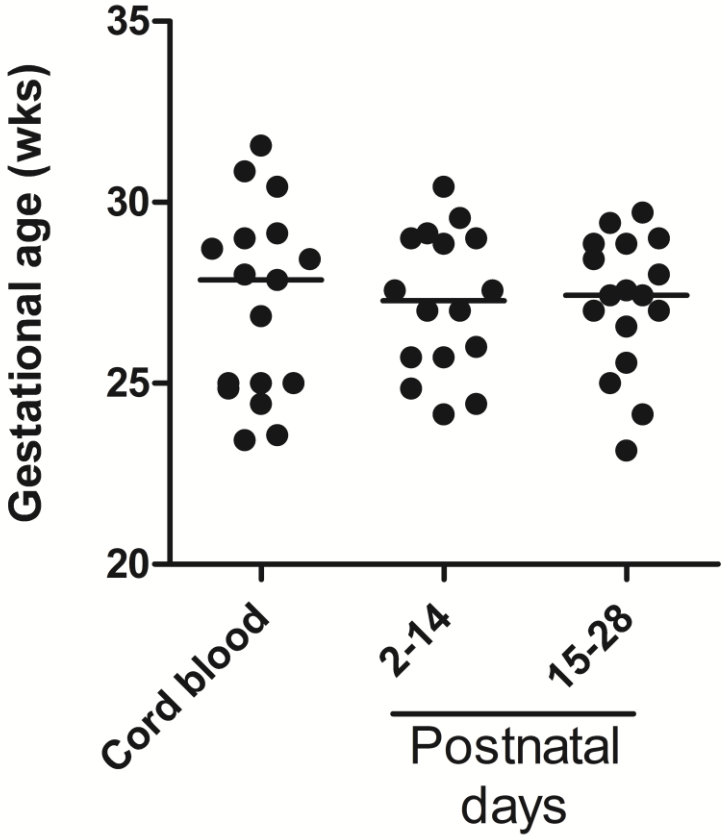


Figure 2.3 Gestational age distribution of preterm neonates by sampling period (days).

Cord blood was collected at delivery of the preterm. Postnatal preterm samples were collected between day 2-14 or day 15-28.

Table 2.3 Changes in TLR responses by postnatal age, adjusted for gestational age

TLR ligand	Cytokine	Estimate of effects (95%CI)	P value
LPS	IL-1β	742 (-140, 1631)	0.107
	IL-6	21301 (-1036, 43637)	0.068
	TNF-α	2450 (560, 4340)	0.014*
	IL-12/23p40	6076 (770, 11382)	0.029*
	IL-10	364 (-21, 756)	0.069
R848	IL-1β	2779 (-210, 5775)	0.075
	IL-6	36253 (10675, 61838)	0.008**
	TNF-α	76321 (490, 12663)	0.005**
	IL-12/23p40	17703 (6790, 28616)	0.003**
	IL-10	1939 (-308, 4186)	0.098

*p<0.05; **p<0.01

2.3.2 TLR reactivity at the single-cell level in premature neonates

To confirm that the attenuated TLR reactivity was not due to a lack of circulating immune cells in whole blood, cell compositions were enumerated using flow cytometry. Monocyte proportions were substantially higher in preterm neonates during the neonatal period, compared to term neonates, adults, or even compared to preterm cord blood samples, whereas proportions of granulocytes, lymphocytes and dendritic cells were comparable (**Figure 2.4**).

To further exclude a lack of cytokine-producing cells, TLR-induced production of IL-6, TNF-α and IL-12/23p40 were also analyzed at the single cell level by intracellular cytokine flow cytometry detection in two main cytokine-producing innate immune blood cell types: CD14-expressing monocytes and CD11c-expressing dendritic cells. A representative flow cytometry diagram of intracellular cytokine responses from a representative preterm postnatal blood sample is shown in (**Figure 2.5A**). As shown in **Figure 2.5B**, production of IL-6 and TNF-α was substantially reduced in a subgroup of preterm postnatal monocytes, compared to term cord blood or adults. Dendritic cells are by and large the main source of IL-12/23p40 and therefore these cells were primarily examined for production of this specific cytokine involved in the differentiation of T helper 17 responses (Lavoie et al. 2010). Similar to the reduced IL-6 and TNF-α responses observed in preterm postnatal monocytes, production of IL-12/23p40 was also markedly reduced in dendritic cells from both term cord blood and preterm postnatal blood (**Figure 2.5B**). The inability of DCs to produce IL-12/23p40 could influence the reduced differentiation into Th1 cells seen in neonates. Collectively, these data confirm that innate immune cells are present in preterm neonates but that these cells are hypo-reactive to TLR stimulation *in vitro*.

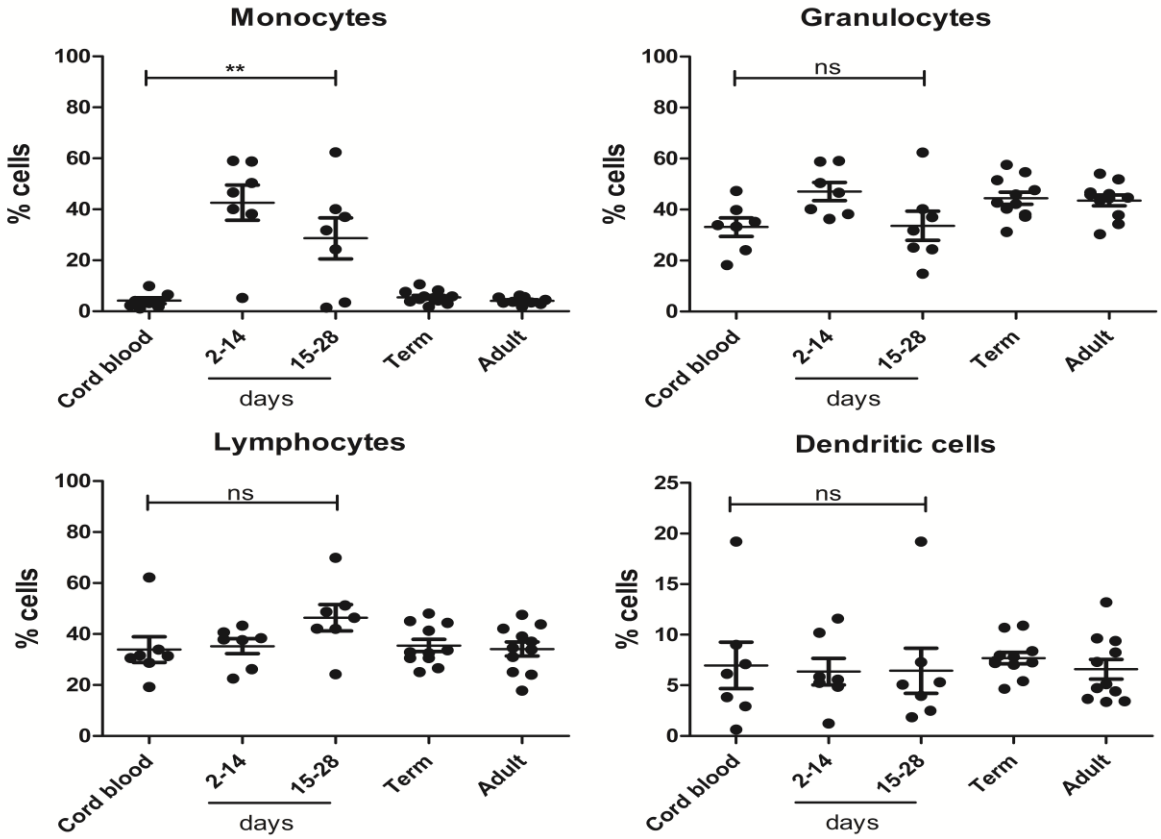


Figure 2.4 Flow cytometry enumeration of blood immune cells.

Ex vivo whole blood was frozen in FACSLyse solution and stained for major immune cell types. Live cells were gated by forward scatter and side scatter and subsequently examined for specific cell types. Percentage of cells is the total of the live (FSC/SSC) gate. Monocyte (CD14+/HLA-DR+ cells), lymphocyte (CD3+ or CD19+ cells), dendritic cell (CD14-/HLA-DR+/CD11c+ cells) and granulocyte counts (determined by FSC/SSC) were determined using multi-parameter flow cytometry; bars represent medians; **$p<0.01$ by ANOVA.

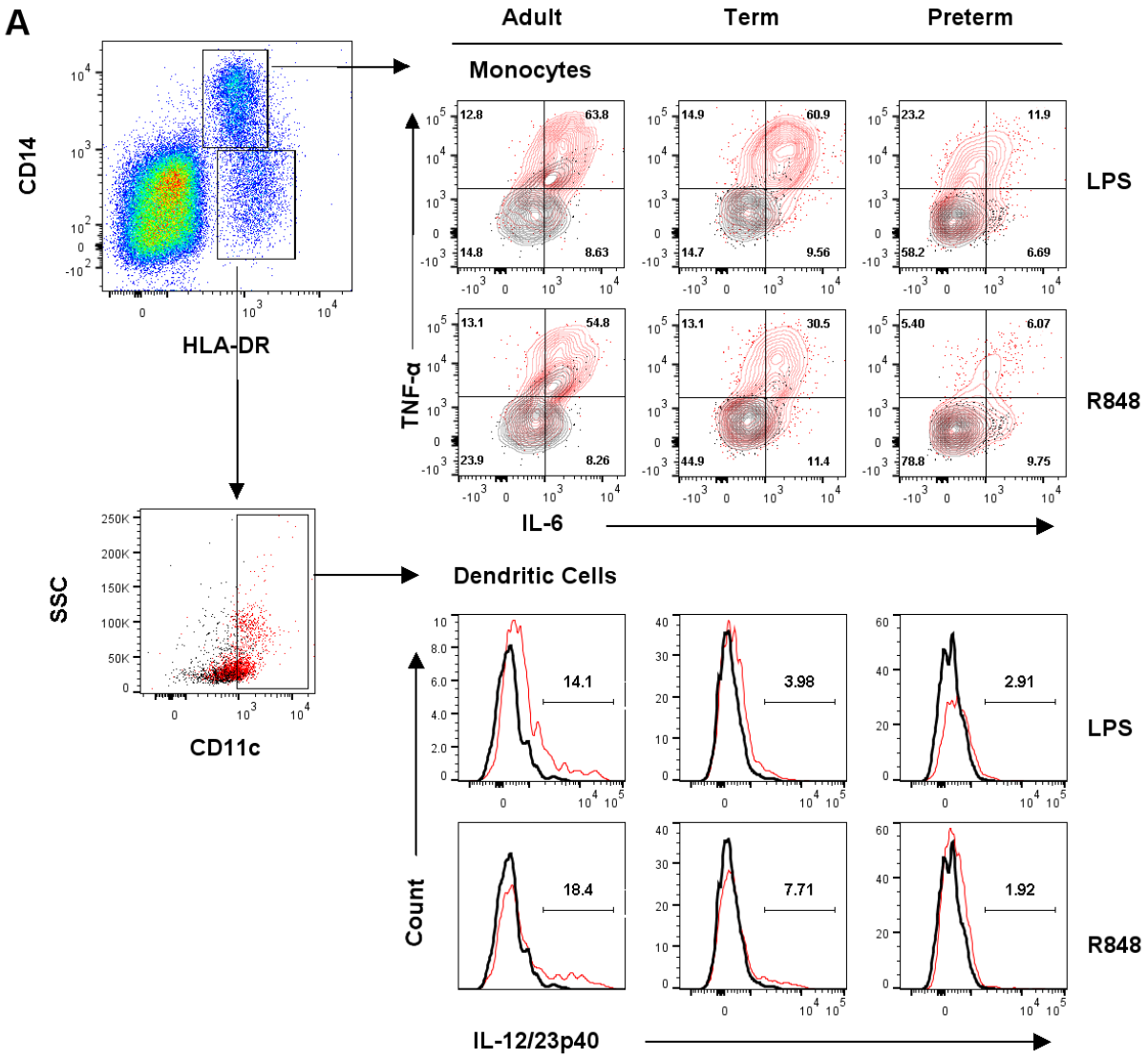


Figure 2.5 Single-cell cytokine production in innate immune cells.

A 6 hr stimulation of whole blood with the TLR ligand, in the presence of brefeldin A, followed by freezing in FACSLyse buffer and intracellular staining. **(A)** Representative flow cytometry gating strategy of each group, adult, term cord blood and preterm postnatal blood (mean gestational age and birth weight: 27.1wk and 1,063g, respectively; median postnatal age: 13days, range 7-23 days). Gating on DCs shows stimulated DCs in red and the fluorescence minus one (FMO) in black

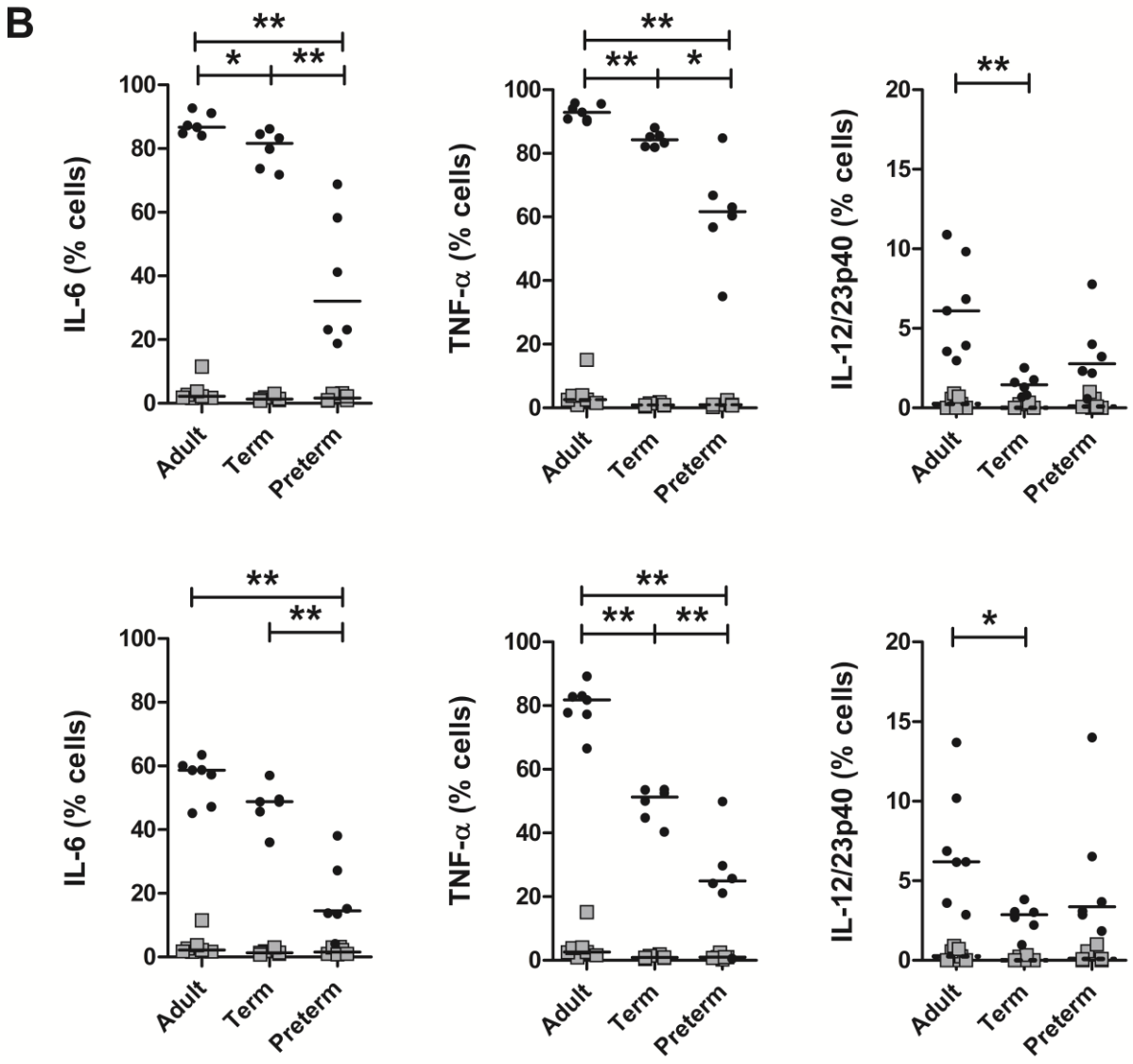


Figure 2.5 Single-cell cytokine production in innate immune cells (continued)

A 6 hr stimulation of whole blood with the TLR ligand, in the presence of brefeldin A, followed by freezing in FACSLyse buffer and intracellular staining. **(B)** Aggregated data for lipopolysaccharide- and R848-induced cytokine responses (black circles) in monocytes (for IL-6 and TNF-α) and in dendritic cells (for IL-12/23p40) compared to unstimulated responses (gray squares). *p<0.05; **p<0.01. (6 subjects per group)

2.3.3 Contribution of perinatal factors to the maturation of postnatal immune responses

To assess whether responses were independently influenced by common perinatal factors linked to prematurity (including chorioamnionitis; antenatal corticosteroids; presence of labour; mode of delivery of the infant; and use of magnesium sulfate), the contribution of these factors was examined in the group of preterm neonates using partial correlations, adjusting for gestational age. As shown in **Figure 2.6**, none of the factors had significant effects on the production of cytokines in the whole blood postnatal samples, indicating a developmental maturation rather than attenuation due to exogenous perinatal factors.

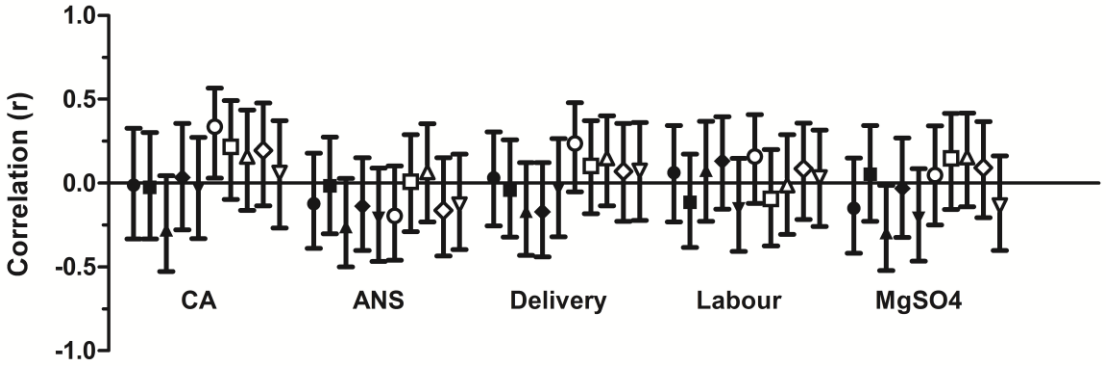


Figure 2.6 Effect of perinatal factors on the postnatal maturation of whole blood TLR responses.

Data show correlations (y-axis) adjusted for gestational age, with 95% confidence intervals (bars). LPS (black symbols) and R848 (white symbols) responses by cytokine; IL-1β (circle), IL-6 (square), TNF-α (upward triangle), IL-12/23p40 (diamond), IL-10 (downward triangle); ANS: antenatal corticosteroids; CA: chorioamnionitis. MgSO4: magnesium sulfate.

2.4 Discussion

To our knowledge, this is the first study that has measured TLR activity of preterm immune cells during the neonatal period. We confirm profoundly attenuated postnatal responses, consistent with our previous responses measured on cord blood (Lavoie et al. 2010; Sharma et al. 2014). The use of single-cell flow cytometry allows confirmation that the functional attenuation in TLR responsiveness is not due to a lack of innate immune cell composition. The measure of responses on whole blood provides a more global determination of anti-microbial responses. Indeed, previous studies have demonstrated that neonatal plasma suppresses TLR-mediated cytokine production, indicating that both cellular and soluble humoral factors are important in the functional determination of immune responsiveness (Belderbos et al. 2012; Levy et al. 2004). Overall, these data suggest a maturation of TLR function during the neonatal period from low levels at birth to higher levels at later postnatal age. These observations provide essential support to the premise that the increased risk of infection in preterm neonates is due to a developmental immaturity of innate immune anti-microbial responses.

Our data are consistent with previous studies that have measured a low surface expression of TLRs on blood mononuclear cells of preterm neonates, with TLR2 and TLR4 expression increasing during the first 2 months of age (Shen et al. 2013). However, because TLR-dependent cytokine responses generally correlate poorly with their corresponding receptor gene or protein expression, it is important to validate the impact of a reduced expression of PRR at the functional level by examining relevant cytokine responses (Strunk et al. 2012). Others reported that endotoxin (TLR4) responses in monocytes from preterm neonates born below 32 weeks of gestation are already mature (i.e. comparable to responses measured at the full term of gestation) by the second week of age, although it remained unclear whether these differences

were due to developmental differences in cell composition independent of gestational age (Shen et al. 2013).

Our data help address an important paradox in neonatal research. High circulating levels of inflammatory cytokines have been repeatedly linked to worse outcomes such as bronchopulmonary dysplasia (Paananen et al. 2009), white matter injury, and neurodevelopmental impairments in preterm infants (Strunk et al. 2014). These results have led to speculations that the innate immune system of these infants is hyper- rather than hypo-responsive during the neonatal period (Wright & Kirpalani 2011). However, this hypothesis was based mainly on random measures of inflammatory mediators in serum, and had, to the best of our knowledge, never been confirmed by functional data measuring the 'reactivity' of the postnatal innate immune system. In fact, studies using cord blood have consistently shown that the innate immune defenses of the preterm infants are hypo-reactive to PRR stimulation, compared to term-born infants or adults. Our data extend these observations, namely that preterm neonates continue to display decreased TLR-agonist induced production of both pro-inflammatory (IL-1β, IL-6, TNF-α, IL-12/23p40) and regulatory (IL-10) cytokines over the entire first month of life. Also, previous cord blood studies have suggested that immune cells from preterm neonates may in fact be biased toward an anti-inflammatory state characterized by higher IL-10 production compared to other pro-inflammatory cytokine responses (Lavoie et al. 2010). However, this was not observed in our current study. This then raises the question: why are basal levels of inflammation elevated in the subgroup of preterm neonates evolving towards worse outcomes? A possibility is that the high basal inflammation reflects an early maturation of immune functions in affected infants compared to unaffected peers either due to exogenous or genetic factors. In support of this hypothesis, we observed considerable heterogeneity in immune

responses over the first 28 days of age. The possibility that worse outcomes may result from an early immune maturation of antimicrobial responses does raise potential clinical concerns of using experimental therapies aiming to “boost” immune reactivity at an earlier stage, in order to prevent neonatal infections in this age group. Additionally, the high circulating levels of systemic inflammation in these infants despite observing an attenuated innate immune responsiveness (Chang et al. 2011) may reflect impaired mechanisms of resolution of the inflammation in the context of a sustained exposure to pro-inflammatory factors including oxidative stress or repeated septic episodes.

TLRs play a critical role in protecting against microbes in early life, as indicated by an increased vulnerability to specific bacterial and viral infections in humans with loss-of-function mutations along these molecular pathways (Picard et al. 2010). The risk of infections in these patients is greatest during the neonatal/early childhood period, underlining the importance of these receptors for immune protection during infancy (Currie et al. 2004; Picard et al. 2010). As shown from previous epidemiological studies, the incidence of sepsis sharply increases in these infants shortly after birth, peaking during the first week of postnatal age, then slowly declining thereafter (Lavoie 2009; Isaacs 2003). Although additional reasons may underlie the increased vulnerability of these infants to sepsis, including a high need for invasive life-saving interventions early on, and the prompt microbial colonization of their skin and mucosal membranes after birth, our data mirror this period of vulnerability. This window of vulnerability temporally delineates a high-risk period during which exposure to skin- or mucosal-breaking invasive interventions should be insistently minimized, in order to limit the risk of sepsis.

2.4.1 Limitations

Some limitations to our study should be acknowledged. We were unable to detect a major effect of perinatal factors, (i.e. chorioamnionitis, antenatal corticosteroids, exposure to magnesium sulfate, presence of labour, etc.) on the maturation of innate immune responses in the group of preterm infants. Although our analysis was not specifically powered to detect subtle effects due to a limited sample size, we can exclude large effects related to an exposure to antenatal corticosteroids or chorioamnionitis that could be implied from animal models (Kramer et al. 2005; Newnham et al. 2003). Preterm neonates were sampled at a single time point with no repeated samples for any preterms. A longitudinal sampling of preterms would provide more information about the immune response changes over the postnatal period. Similarly, we lacked postnatal samples from term neonates, which was a choice we made to limit the invasiveness of the study. Our study also highlights the importance of corroborating observation in populations of human infants, even if ethically challenging, in order to validate animal observations. Moreover, our study certainly informs as to the considerably larger sample size that would be required in order to detect more subtle effects from perinatal factors on the maturation of innate immune functions.

2.4.2 Conclusions

In conclusion, we show that the ability of preterm neonates to respond to PAMP is profoundly attenuated over the first month after birth in a preterm neonate. We similarly observed reduction in both pro- and anti-inflammatory cytokine responses, yet with considerable heterogeneity in the degree and timing of postnatal maturation. Our data are directly relevant to our understanding of how and when immaturity of the developing innate immune system contributes to an increased risk of sepsis in these infants. Future studies are required to understand how variability in immune responses among individuals affects clinical outcomes.

Chapter 3: Enrichment of hematopoietic stem cells and immature progenitor cells in preterm postnatal blood predicts the risk of sepsis

3.1 Background

Neonates born prematurely are at risk of developing sepsis and it is the second leading cause of death in the neonatal age group (WHO, 2018, Fact sheet Newborns: reducing mortality**)**. The high frequency of very low gestational age (GA) babies experiencing infections has been previously documented (Lavoie et al. 2010), and it has been assumed that their susceptibility is due to immaturity of their immune cells. Studies into the immunological responses of premature neonates have demonstrated that they produce lower amounts of pro-inflammatory cytokines in response to stimulations than full term neonates (Strunk et al. 2012; Lavoie et al. 2010; Marchant et al. 2015); however, underlying causes for this immune attenuation are poorly characterized. The study of immune cells and their responses has traditionally been measured by the presence of proteins in the form of receptor expression, cytokines produced or the intracellular proteins required for the downstream signaling of cellular processes. This measures the final outcome of stimulation, but often requires extensive materials for an assay, for example a western blot, and requires *a priori* determination of the proteins to be assessed. The decreasing cost of RNA-sequencing and the increases in computing power have enabled unbiased analysis of small blood volumes from premature neonates, allowing non-targeted examination of immune maturation in the postnatal period.

To understand the susceptibility of preterms to infections researchers have utilized gene expression data to identify sepsis profiles in neonates (Cernada et al. 2014; Wynn et al. 2015;

Smith et al. 2014). These published data sets have examined different subsets of neonates, such as very low birth weight neonates (VLBW)(Cernada et al. 2014) and preterms (Wynn et al. 2015 and Smith et al. 2014) (**Table 3.1**). For example, Cernada et al (2014) were able to use only 0.5ml of blood from neonates, which is an advantage when dealing with the vulnerable population of preterm neonates. They examined VLBW and showed that Gram-positive and Gram-negative sepsis can have different transcriptomic profiles, but this could also be confounded by some of their controls having prior sepsis episodes and the small number of Gram-negative patients. Other groups have also attempted to define a whole blood transcriptomic "sepsis signature" in neonates (Sweeney et al. 2017; Raymond et al. 2017). Without doubt, defining such a signature has the potential to serve as a diagnostic or predictive marker in clinical settings. However, when inspecting results from published studies, it is apparent, that they cannot account for the influence of the patient's gestational and postnatal age. It is often the youngest GA babies that go on to develop sepsis and often the control group consists of older neonates. Indeed, Wynn et al. found that postnatal age is a more characteristic marker of the transcriptomic signature in preterms than infection status (Wynn et al. 2015). To better understand the effect of gestational and postnatal age on preterm transcriptomic profile we first need to define the baseline of these babies at various gestational ages to then be able to identify their profile during an infection. This would help us narrow down a diagnostic panel of genes that are most differentially expressed in the preterm and are able to identify sepsis and ideally the type of pathogen.

In chapter 2, we described the innate immune response of preterm babies in the postnatal period. This cohort comprises samples collected when babies were not infected or under suspicion of infection (this does not preclude possible infections *in utero* or in the postnatal

period prior to sampling). The whole-blood cell pellets associated with these stimulations were stored in a manner to protect and preserve the mRNA present. A subset of neonates in this cohort will be used to determine the transcriptomic profile of premature babies in an uninfected state. Gene expression data generated from our lab from unstimulated monocytes in preterm and term cord blood showed that age separated the two groups (data not shown), supporting our exploration of unstimulated samples in the three groups, which should have clearly different transcriptomic profiles.

In this chapter, we sought to determine the feasibility of RNA recovery from the cohort and identify the transcriptomic profile of the preterm postnatal samples. My hypothesis is that the preterm postnatal samples will have a different transcriptomic profile to the preterm cord blood and term cord blood, and subjects will cluster based on their postnatal age. A secondary hypothesis is that neonates who go on to later develop sepsis will have a distinct transcriptomic profile from their peers who do not have a culture positive sepsis event.

Table 3.1 Publicly available datasets of preterm whole blood gene expression

GEO accession number	Reference	Type of data	Platform	# samples	# individuals	Gestational age (sepsis) [mean and (range)]	Gestational age (control) [mean and (range)]
GSE69686	Wynn et al. 2015	Expression profiling by array	GPL20292	149	68	Early:33.3 (24-41) Early clinical: 33.2 (25-40) Late: 28.9 (24-40) Late clinical: 27.2 (24-30)	Early: 31.5 (27-36) Late: 26.8 (23-30)
GSE25504	Smith et al. 2015 and Dickinson et al. 2014	Expression profiling by array	GPL570; GPL6947; GPL13667; GPL15158	170		30.9 (23-41)	36.3 (24-42)
E-MTAB-4785	Cernada et al. 2014	Expression profiling by array	A-AFFY-Affymetrix GeneChip Human 1.0 ST GeneChip	36	36	27+/-2	28+/-2
GSE70461	Kalikstad et al. 2017	Expression profiling by array	GPL570	107	20	Not applicable	28.6 (23.5 - 37.1)

3.2 Materials and methods

3.2.1 Cohort and consent

Informed consent was obtained as described in chapter 2 for the postnatal preterm cohort. All preterm samples were prospectively acquired from NICU babies. Subjects were recruited across four postnatal age groups (1-14, 15-28, 29-43, >44 days) and all born under 30 weeks GA. The exclusion criteria were a current or suspected infection, antibiotic or corticosteroids (which may suppress immune responses) administration at the time of sampling. From our large cohort we performed a nested case-control analysis of babies that went on to develop sepsis after sampling. Term neonate controls were healthy babies >37 weeks GA delivered by cesarean section electively in the absence of labour. No other demographic or clinical data was collected on the term subjects. Clinical data for the premature neonates was collected (**Table 3.2**). Sepsis was defined as a blood culture positive event with suggestive clinical signs (as per the attending clinicians). Preterms with a sepsis event were matched by sex and closest Euclidian distance for gestational age, birth weight and postnatal day to preterms without a sepsis event (**Figure 3.1**).

Table 3.2 Clinical characteristics of the subjects

Clinical characteristics	Preterm cord blood	Preterm postnatal blood	Term cord blood
Gestational age (weeks; mean + range)	25.2 (23.4 - 26.9)	26.2 (24.6 - 29.1)	37-40
Postnatal age (days; mean + range)	0	18 (6 - 33)	0
Sex (% male)	100	77.8	N/A
Birth weight (grams, mean +range)	780 (650 - 1,045)	851 (690 - 960)	N/A
Chorioamnionitis (%)	33.3	22.2	N/A
CoNS sepsis (% of sepsis cases)	66.7	60	N/A
Other pathogen sepsis (% of sepsis cases)	33.3	40	N/A

CoNS: coagulase-negative *staphylococci*; N/A: not available

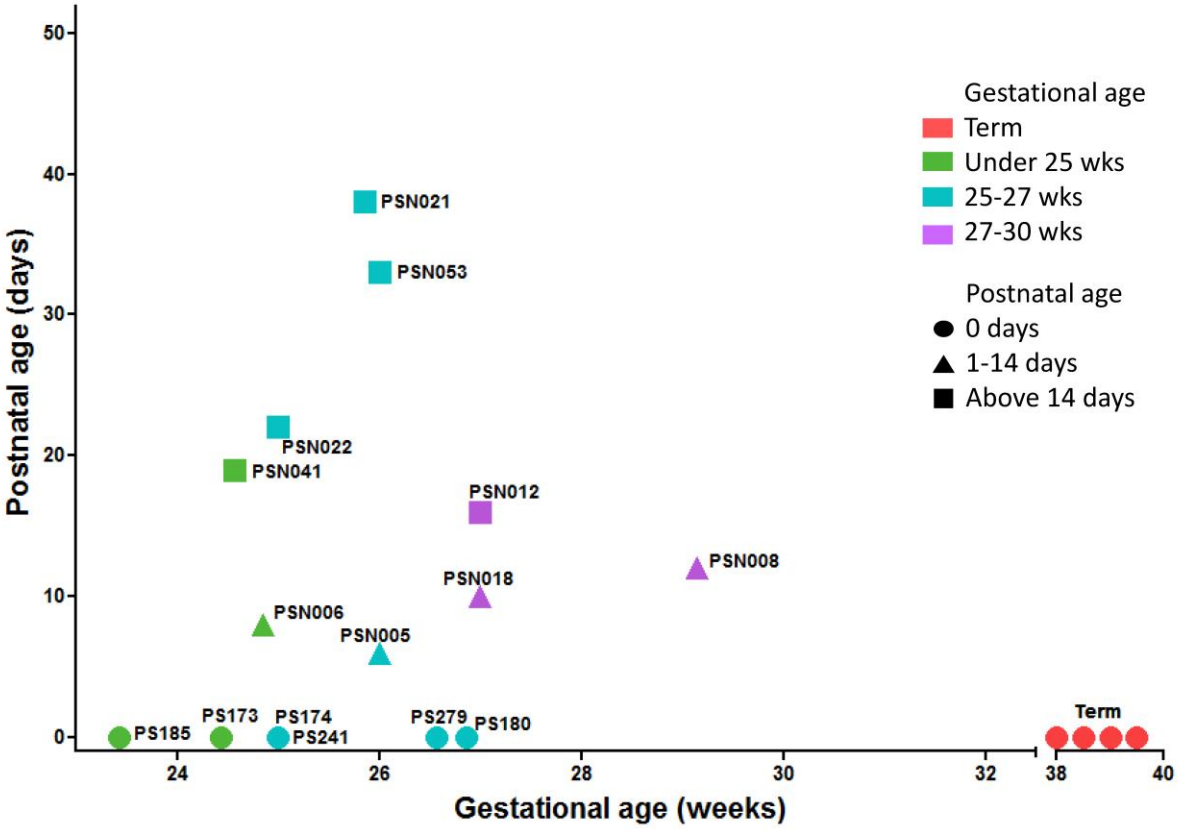


Figure 3.1 Selected preterm samples for whole blood RNA-sequencing

Gestational age and postnatal age of the preterm samples selected for sequencing. PS: preterm cord blood. PSN: preterm postnatal blood. Term: term cord blood.

3.2.2 Sample processing and RNA extraction

A detailed method of sample collection and processing can be found in chapter 2. Briefly, whole blood was collected and diluted 1:2 with RPMI1640 and incubated at 37°C for 24 hours. Blood was spun down and supernatant removed. Blood cell pellets were resuspended in 200 µl of RNAprotect (Qiagen) and stored at -80°C. RNA from the samples was extracted from the thawed whole blood cell pellets using the RNeasy Micro kit (Qiagen) as per the manufacturer's instructions. Immediately after RNA extraction total RNA quantity was determined using NanoDrop 2000 spectrophotometer (ThermoFisher) (**Figure 3.2**).

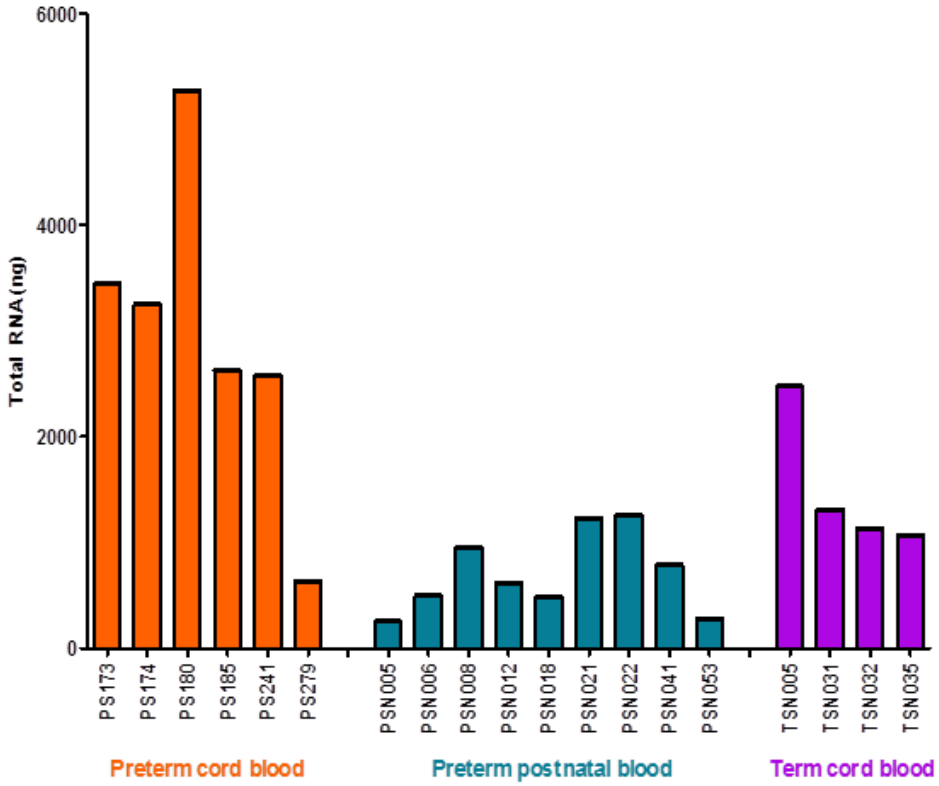


Figure 3.2 Total RNA for each sample.

RNA concentration determined by NanoDrop and multiplied by the total volume of RNase-free water for total RNA extracted. PS: preterm cord blood. PSN: preterm postnatal blood. TSN: term cord blood.

3.2.3 RNA cleanup, library preparation and RNA-sequencing

Samples with sufficient total RNA were processed with the Globin-Zero Gold rRNA removal kit (Illumina) by the Beijing Genomics Institute according to the manufacturer's instructions. Globin genes make up 70% of total RNA transcripts in whole blood (Wu et al., 2007) and their removal increases the detection of genes of interest (Shin et al., 2014; Field et al., 2007). With very limited starting material for our samples it was decided it was important to physically remove the globin and ribosomal RNA (rRNA). Post-globin/ribosomal depletion quality and quantity of RNA was measured with the Aligent 2100 Bioanalyzer (**Table 3.3**). Post-depletion samples had an average RNA integrity number (RIN) of 8.28 (range: 7.1-9.3) with good peaks at 18S and 28S, and an average concentration of 101.6 ng/ul (range: 12.33-264 ng/ul). All samples were deemed to be of sufficient quality for downstream library preparation and RNA sequencing.

The sample libraries were prepared using TruSeq Stranded Total RNA Library Prep Kit (Illumina). Samples were run on the HiSeq 2500 (Illumina) for 100bp, paired-ends and 30 million reads were collected per sample. Of the 30 million reads in each subject approximately 50% were exonic reads (**Figure 3.3A**) and each subject had 12-20 million exonic reads (**Figure 3.3B**). Raw sequencing data was broken into counted, unique mapped/not counted, trimmed, multimap and unaligned reads (**Figure 3.3**). We are interested in the counted reads as they represent the exonic genes. For each sample there are 10-20 million exonic reads. Sequences were aligned to GRCh37 (NCBI). One postnatal preterm sample, PSN015, was lost from further analysis and this leads to an un-matched postnatal preterm sample in later analyses.

Table 3.3 Post-globin and -ribosomal RNA depletion

Sample ID	Concentration (ng/µL)	Volume (µL)	Total Mass (µg)	RIN	28S/ 18S
PSN005	13.66	12	0.1639	8	1.6
PSN006	27	11	0.3	8.9	1.3
PSN008	128	10	1.28	8.6	2.5
PSN012	60	10	0.6	8.2	1.7
PSN015	12.33	10	0.123	9.5	2.1
PSN018	39	10	0.39	8	0.9
PSN021	100	8	0.8	7.6	1.7
PSN022	90	9	0.81	9.3	2
PSN041	62	10	0.62	9.2	1.2
PSN053	25.05	8	0.2004	7.1	1.2
PS185	132	11	1.45	8.1	2
PS173	147	27	3.97	8.2	1.8
PS174	138	22	3.04	7.7	1.1
PS241	236	9	2.12	8.9	2.5
PS279	53	9	0.48	7.8	1.7
PS180	264	24	6.34	7.4	1.5
TSN005	178	10	1.78	8.3	1.5
TSN031	125	10	1.25	8.9	1.8
TSN035	88	9	0.79	8.3	1.3
TSN032	114	10	1.14	7.6	1.4

PSN: preterm postnatal blood. PS: preterm cord blood. TSN: term cord blood.

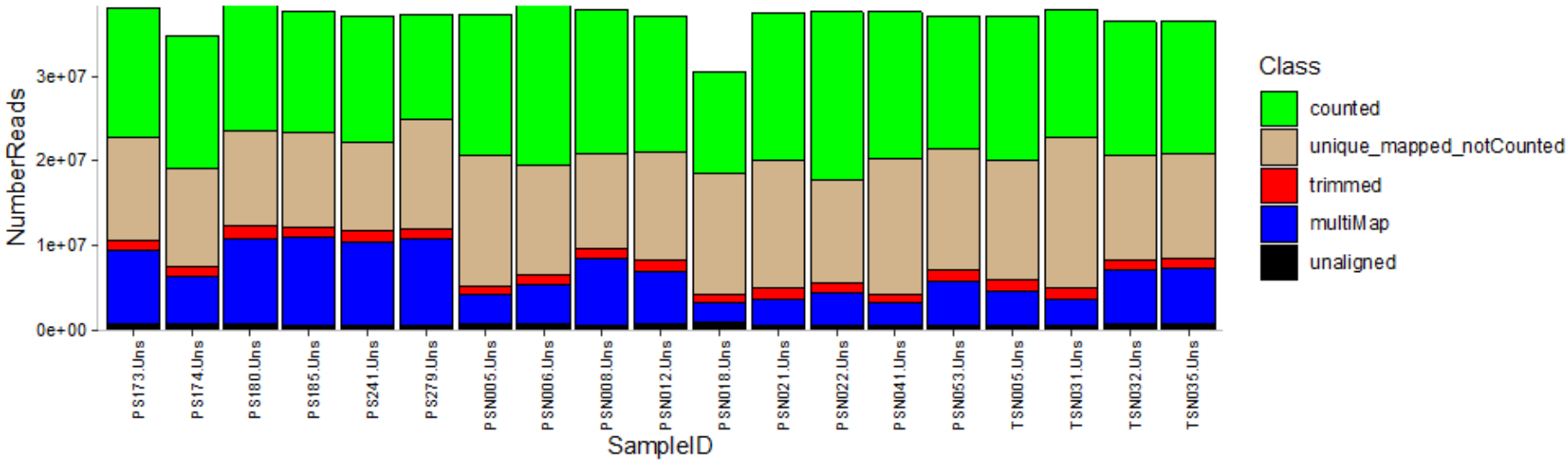


Figure 3.3 Quality control data on sequencing reads

All reads acquired from each sample. Counted reads are the exonic reads.
PS: preterm cord blood. PSN: preterm postnatal blood. TSN: term cord blood.

3.2.4 Data 'analysis'

Normalization and differential transcript analysis were conducted using the EdgeR framework with False Discovery Rate adjustment. Pathway enrichment among the genes differentially expressed was assessed using the Gene Set Enrichment Analysis (GSEA) approach and also by a network-based approach (http://genemania.org). The Benjamini-Hochberg procedure was used to control false discovery rate. The Hallmark gene sets (Liberzon et al. 2015) are composed of over 4,000 gene sets from the Molecular Signatures Database gene sets providing refined gene sets for preliminary analysis of the transcript data. Significantly differentially expressed genes were visualized by supervised hierarchical clustering based on group (preterm cord blood, preterm postnatal blood or term cord blood), adjusted GA (gestational age + postnatal days) and postnatal days.

3.3 Results

3.3.1 Available preterm sepsis data sets are limited

There are existing public datasets on whole blood studying preterm or very low birth weight infants with suspected or confirmed sepsis. After examining the clinical data on these babies, when it was included in the metadata available online, it appears that attempts to match the average GA of a sepsis-positive group with an uninfected control group may be skewed by the extremely high frequency of infections in the youngest gestational age group of preterms (**Table 3.1**). For example, Smith et al. (2014) contained samples from 30.9 (23-41) weeks GA neonates who experienced an infection alongside control subjects, no infection at sampling, that are an average of 6 weeks older at 36.3 weeks (range 24-42 weeks). It is widely accepted than preterms are <37 weeks GA and these public datasets include term neonates (38-42 weeks) in

their analysis. The gene expression signature described may be masked by gestational age, postnatal age or other factors unique to the premature neonate. Using control neonates of an older GA and different postnatal ages at the time of sepsis could complicate attempts to identify a signature transcriptomic profile of sepsis. The effect of postnatal age was demonstrated by Wynn at al. (2015) showing that the similarities between their postnatal samples, sampled for early-onset (< 3 days after birth) or late-onset (>3 days after birth) sepsis, were primarily associated with the postnatal age of the preterms and subsequently separated by their infection status (Wynn et al. 2015). This evidence suggested that the changes occurring in the postnatal period are a stronger influence on the transcriptomic profile than infection status or gestational age at birth. The issues with these datasets are that they cannot identify the underlying transcriptomic profile of the preterm during the postnatal period. Our cohort of uninfected preterm postnatal blood can fill this knowledge gap and provide a baseline for analysis of a sepsis profile.

3.3.2 Clinical information of subjects

Clinical descriptions of the subjects used, preterm cord blood vs. preterm postnatal blood are detailed in **Table 3.2**. For this subset, the infections were mostly coagulase-negative *staphylococci* (CoNS) (1/8 was *E. coli* sepsis at birth) and 26% of babies in the entire cohort (19/73) had a culture positive infection, of which 68% were CoNS. It should be noted that of our preterm cord blood subjects one had culture-positive sepsis at birth and another had concerns for sepsis at birth, the time of sampling. Two preterm cord blood subjects had chorioamnionitis as noted in the discharge summary.

3.3.3 Quality checks of the RNA extracted from whole blood pellets

We recovered RNA to perform globin and ribosomal RNA depletion. After depletion all samples were deemed to have sufficient quantity and quality to continue with library preparation and RNA-sequencing (**Table 3.3)**. Twenty samples were sequenced, six preterm cord blood, ten preterm peripheral postnatal blood and four term cord blood. The first objective was to prove that sufficient quantity and quality of RNA could be recovered from this cohort. We were able to successfully retrieve and sequence the RNA from these samples, enabling us to continue extracting and sequencing the entire cohort of 73 preterm babies in the future.

3.3.4 Preterm postnatal blood profile is more similar to term cord blood

A principal component analysis of the 3 groups (preterm cord blood, preterm postnatal blood and term cord blood) showed that preterm cord blood clustered distinctly from term cord blood. Interestingly, preterm postnatal samples were more similar to term cord blood than to preterm cord blood (**Figure 3.4**). PC1 represented 17.4% of the differences seen separating the preterm cord blood from the other two groups and PC2 made up 12.4% of the differences we see. To clarify the differences between the groups, we utilized gene set enrichment analysis (GSEA). The GSEA examined the expression of genes in a pathway and yielded a cumulative score comparing two groups of samples instead of inspecting each gene individually. Pathways curated by Hallmark were used and we identified 11 pathways that were upregulated in preterm cord blood (**Figure 3.5).** When comparing preterm cord blood to either group the most enriched gene set was for heme metabolism. This may be a characteristic of the nucleated red blood cells present in umbilical cord blood not related to the gestational age of the individual as it was also higher in term cord blood than preterm postnatal blood (**Figure 3.5**).

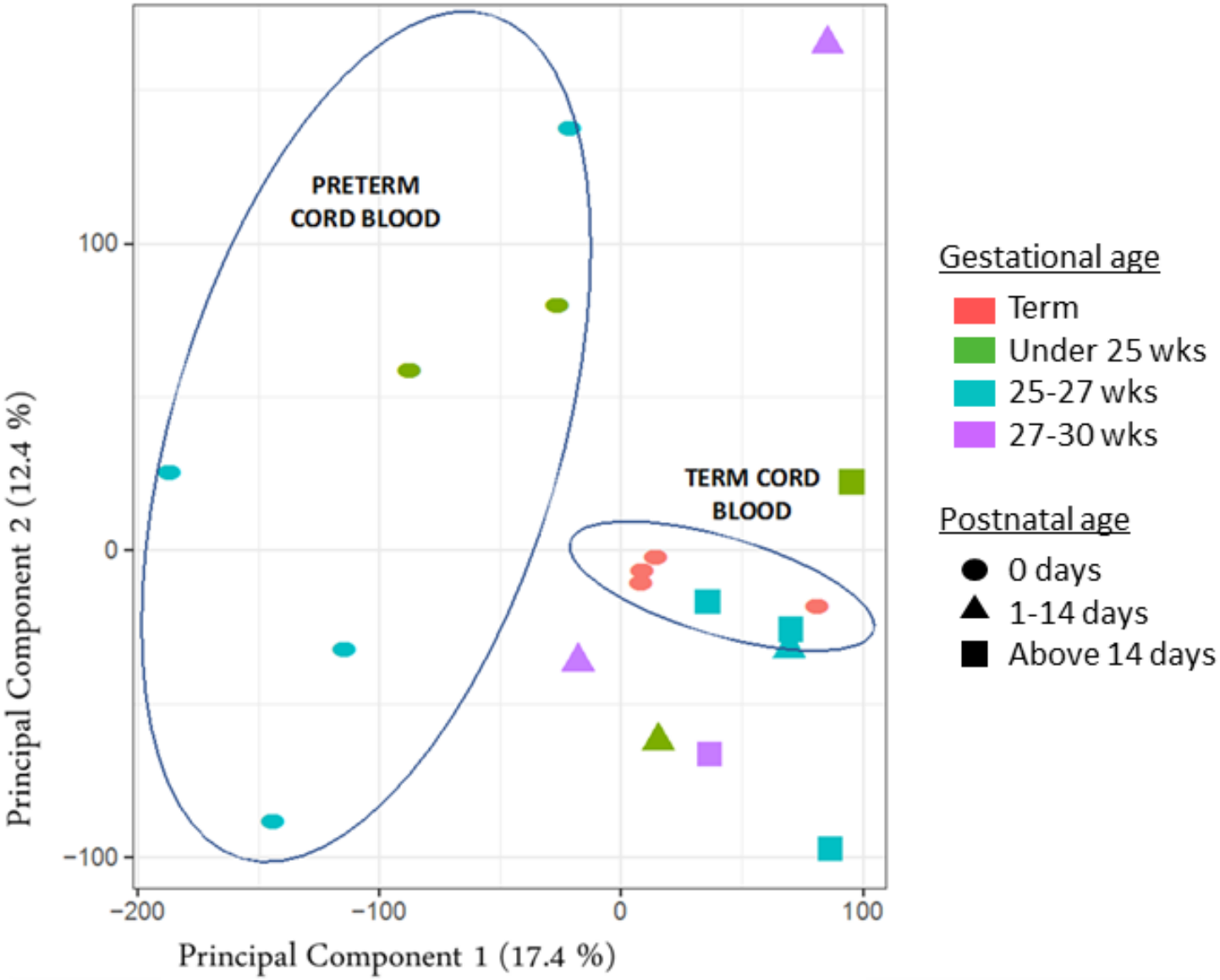


Figure 3.4 Principal component analysis of all samples

Distribution of all samples based on principal component 1 (17.4%) and 2 (12.4%).

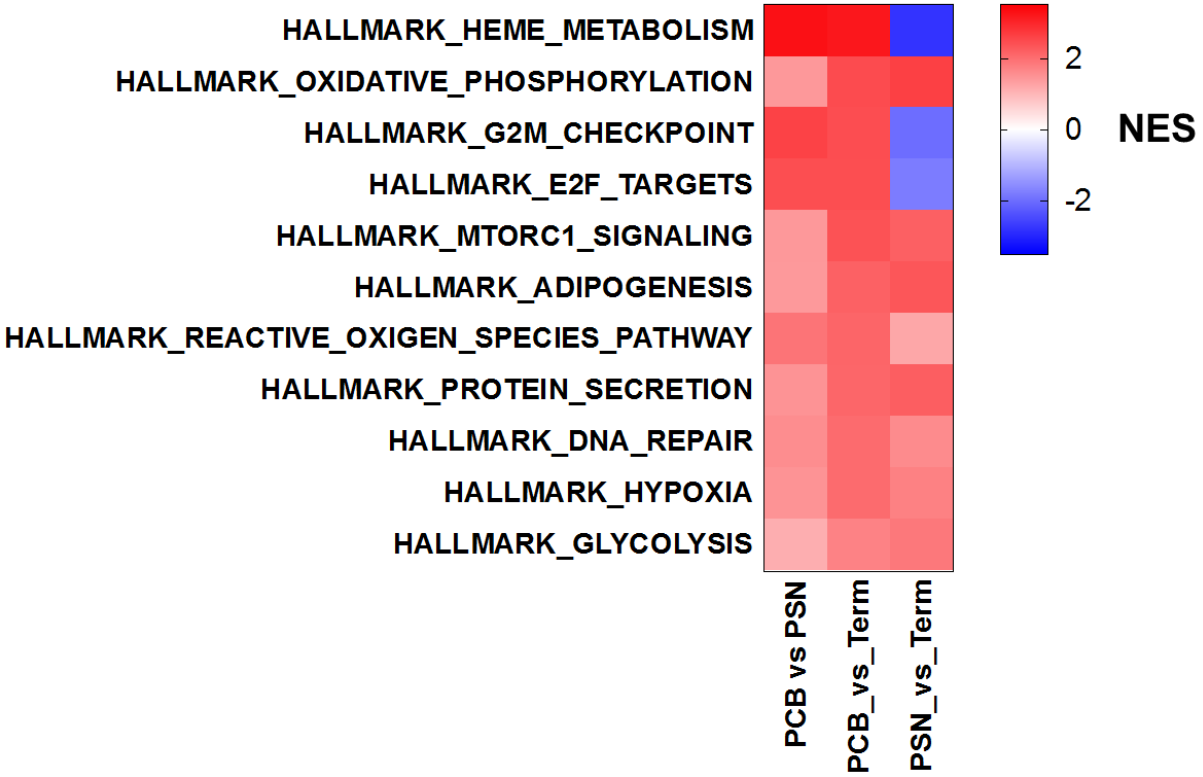


Figure 3.5 Enriched pathways in preterm cord blood and preterm postnatal blood.

Analysis (GSEA) between 3 groups of samples using the Hallmark database (FDR < 0.05). PCB: preterm cord blood. Scale is normalized enrichment score (NES). PCB: preterm cord blood. PSN: preterm postnatal blood. Term: term cord blood

3.3.5 Defining cell-types from whole blood

The strong overexpression of genes involved in heme metabolism suggested that differences in cell proportions could exist between the three age groups. Specifically, higher numbers of red blood cells in preterm cord blood might skew the transcriptomic signature. To address this hypothesis, we used a published data set that examined the transcriptomic profile of hematopoietic cells in a steady state (Novershtern et al. 2011) to define cell-type specific gene sets. mRNAs that were 2x higher in a certain cell-type than all the other cell subsets (FDR <0.05) were considered as signature mRNAs of that cell-type. Gene sets with at least 10 defining genes were extracted for 36 cell-types. Future reference to these gene sets will be as the Novershtern gene set or cell-types. A GSEA of the Novershtern gene set was performed to identify the proportions of cell-types enriched in preterm blood. The gene sets associated with erythroid progenitors and hematopoietic stem cells (HSCs) were enriched in preterm cord blood compared to term cord blood, while T cell subsets were enriched in term cord blood (**Figure 3.6**). Interestingly, the preterm postnatal samples demonstrated a shift in their transcriptomic profile away from the preterm cord blood and towards the term cord blood during the postnatal period, indicating that there were changes to the cell frequencies in the first month of life of a premature neonate, mimicking immune cell maturation. The enrichment of cell types in preterm cord blood compared to term cord blood is more easily visualized by **Figure 3.7**. Preterm cord blood samples are enriched in erythroid progenitor cell-types and granulocytes, while term cord blood is enriched in mature lymphoid cell-types. The analysis comparing preterm cord blood and preterm postnatal blood illustrates the transitional state of these postnatal samples with an enrichment of mature cell types (Figure 3.8). To integrate the Hallmark gene set information with the Novershtern cell-types, a sample level enrichment analysis (SLEA) was performed and

scores were compared between preterm and term cord blood (**Figure 3.9**). High correlation was seen between the metabolic and cell cycling processes and the HSC and erythroid progenitor cells, indicating that it is likely that these cells were providing the high cellular metabolic signal seen in the transcriptome signature.

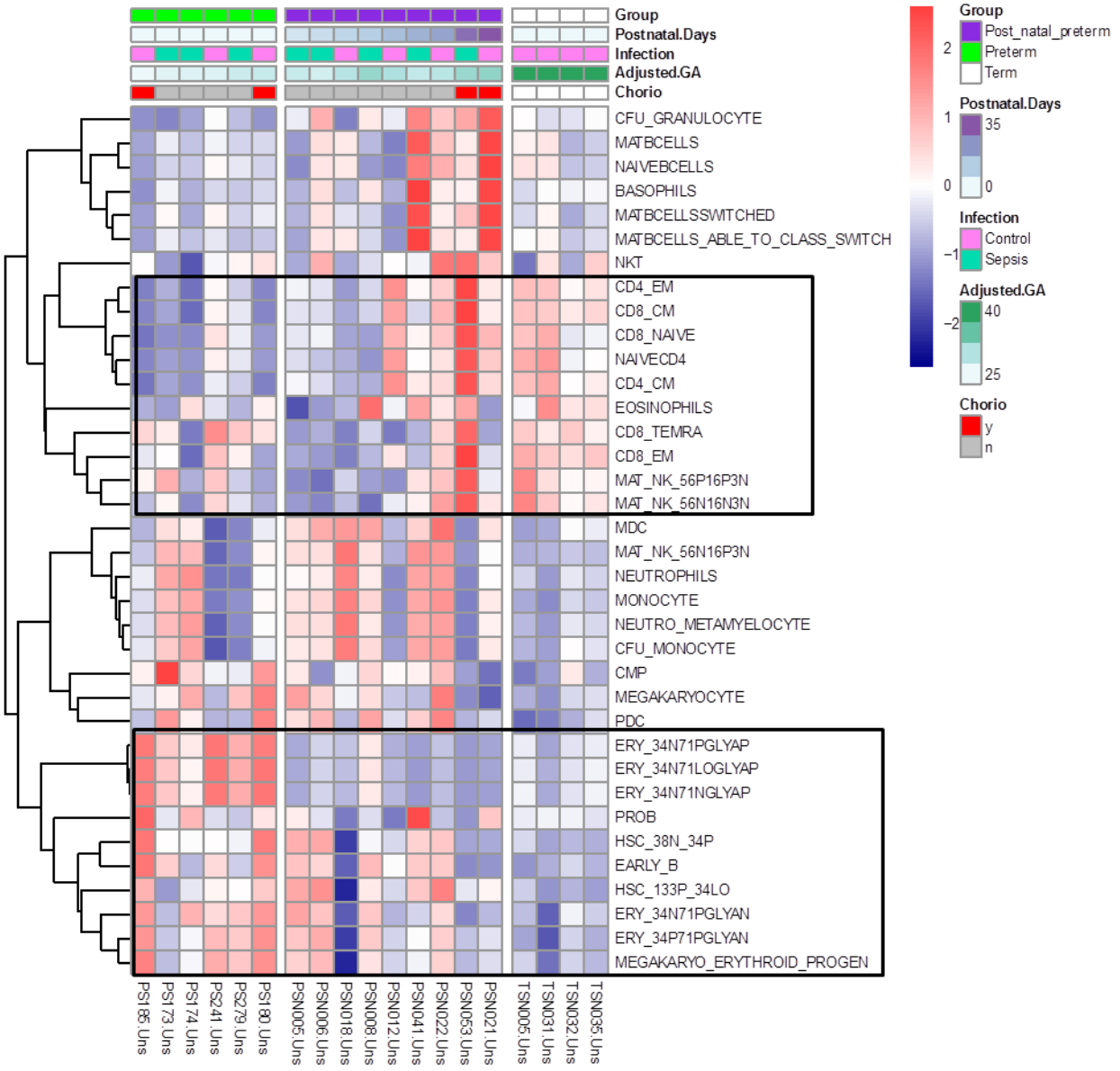


Figure 3.6. Preterm blood cell-type profiles mature during the postnatal period.

Supervised hierarchical clustering of the three groups for the cell-type gene sets (from the Novershtern et al. 2011 paper). Heatmap of cell-types represents the z-score (-2 to 2), the number of standard deviations an element is from the mean. Boxed groups indicate cell-types that undergo significant changes. Preterm: preterm cord blood. Post_natal_preterm: preterm postnatal. Term: term cord blood. Adjusted GA: gestational age + postnatal age. Chorio: presence of chorioamnionitis.

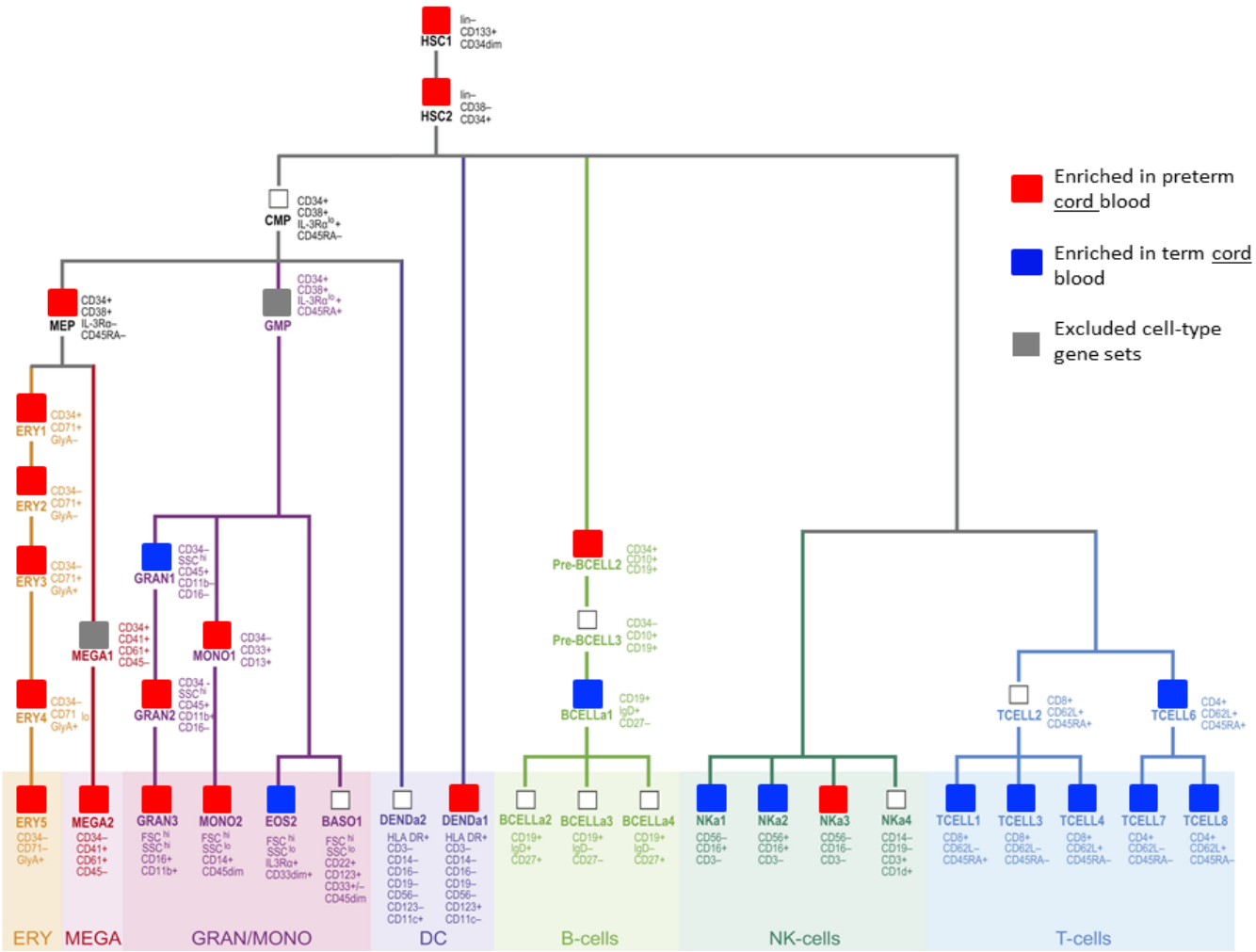


Figure 3.7 Enrichment of hematopoietic stem cell and erythroid progenitor cells in preterm cord blood

SLEA scores for cell-subset specific gene sets. Red: enriched in preterm cord blood. Blue: enriched in term cord blood. Gray: excluded cell-type gene sets. Figure adapted from Novershtern et al. 2011. Reproduced with permission.

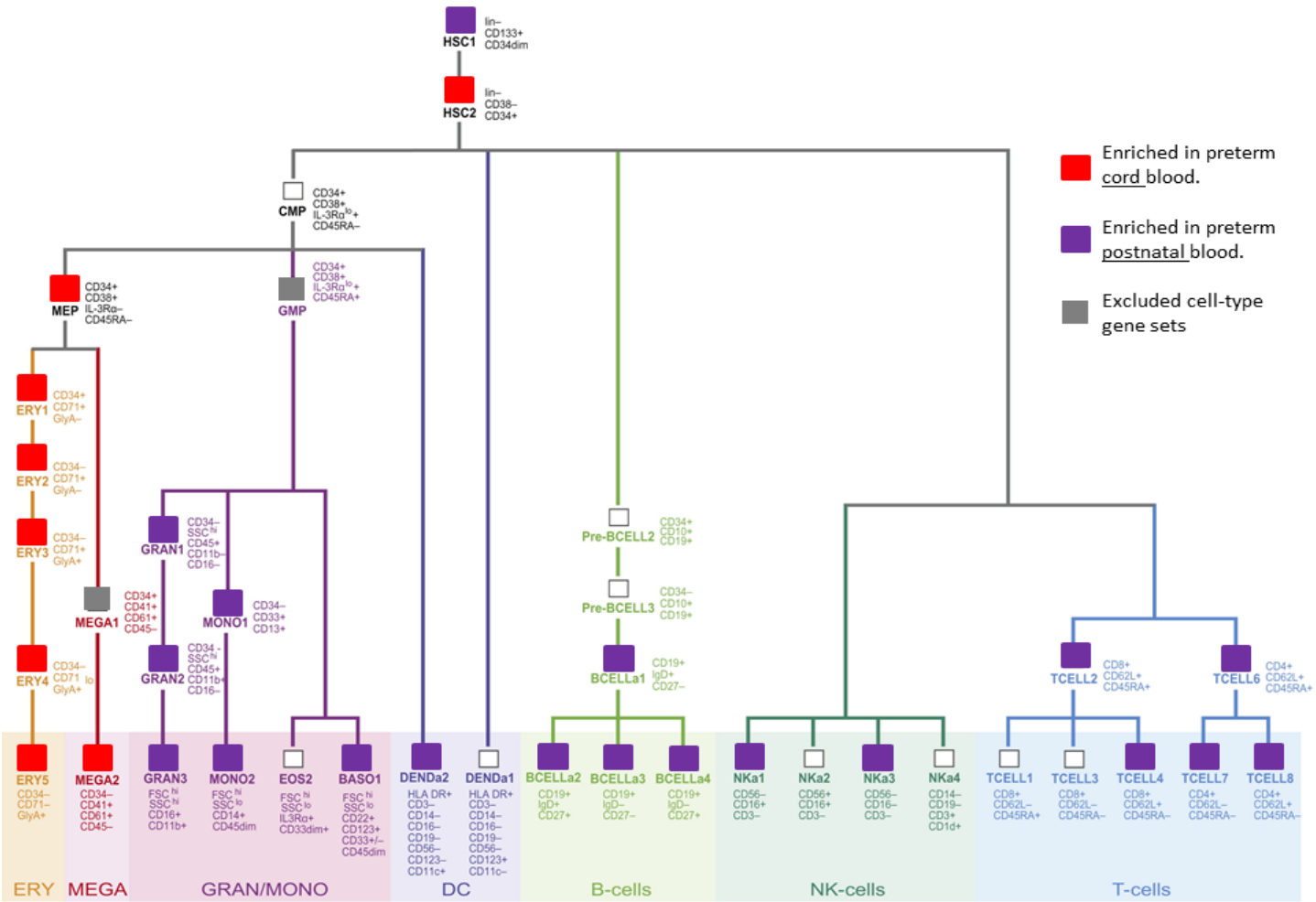


Figure 3.8 Enrichment of mature immune cell-types in preterm postnatal blood

SLEA scores for cell-subset specific gene sets. Red: enriched in preterm cord blood. Purple: enriched in preterm postnatal blood. Gray: excluded cell-type gene sets. Figure adapted from Novershtern et al. 2011. Reproduced with permission.

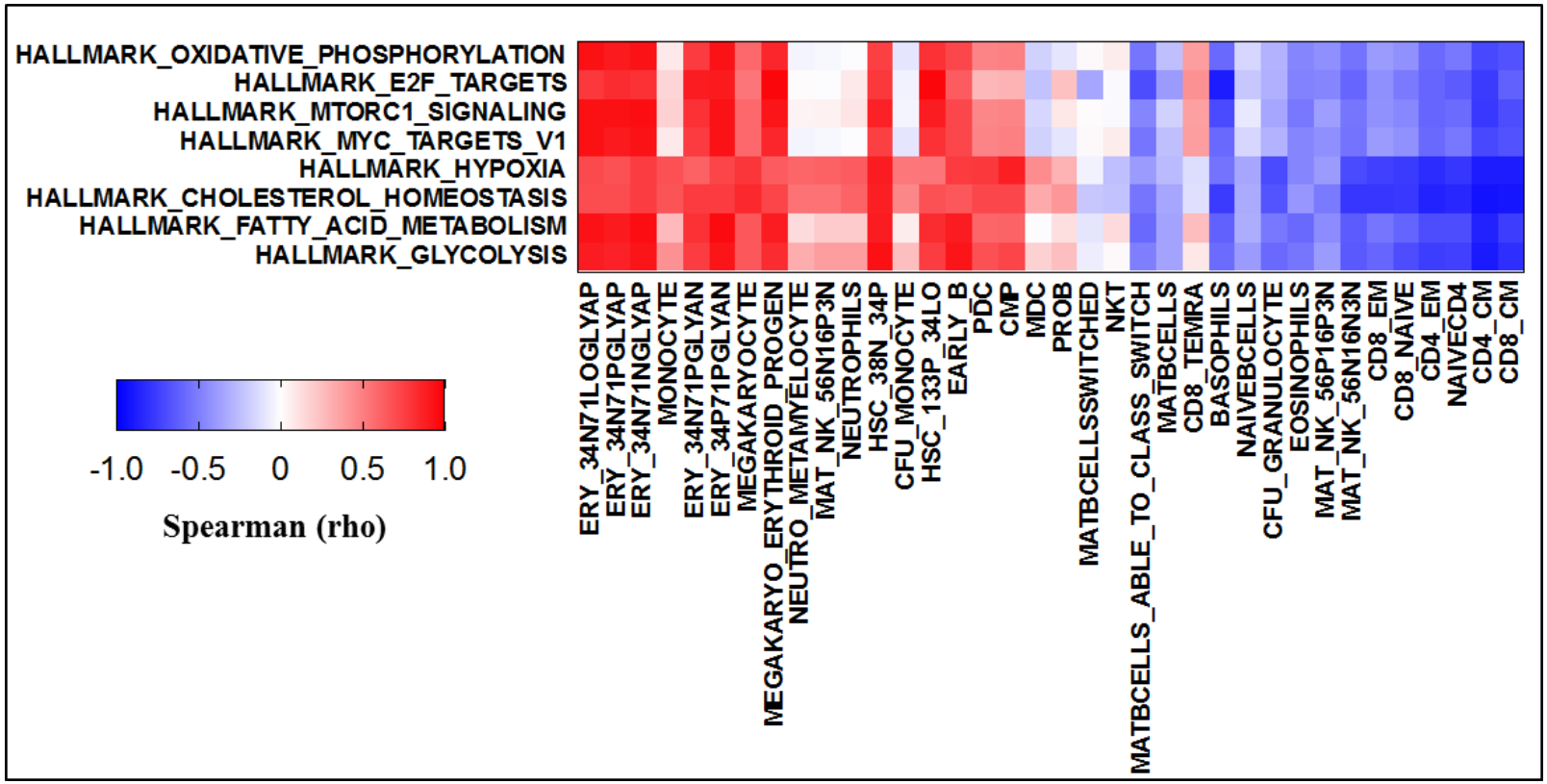


Figure 3.9 Cellular processes and metabolism gene sets are enriched in hematopoietic stem cells and erythroid progenitor cells

SLEA scores for cell-subset specific gene sets correlated with leading-edge SLEA scores of metabolic pathways. (Spearman correlation: abs(rho) > 0.6 is significant). Red: more enriched in preterm cord blood. Data from preterm cord blood vs term cord blood. Blue: less enriched in preterm cord blood. SLEA: sample-level enrichment analysis.

3.3.6 Validation of the changing cell-type signatures in a published data set

To validate our finding of the postnatal immune development in preterm babies from their transcriptomic profiles we can use existing datasets. As an example of a preterm postnatal dataset we have selected one examining the outcome of bronchopulmonary dysplasia, a chronic lung disease prevalent in premature babies (Kalikstad et al. 2017) since this dataset contains a large number of postnatal whole blood samples from premature neonates. These babies were sampled up to 8 times during their stay in the NICU and some clinical information is published (summarized in **Table 3.4**). From this published dataset we were able to extrapolate the gestational and postnatal age of these babies. To validate the changes of cell-type enrichment seen in our preterm postnatal subjects we applied the same analysis to the Kalikstad dataset (GSE70461). After applying the Novershtern transcriptomic cell-type gene sets to the data, supervised clustering for postnatal age confirms changes during the postnatal period from an immature, progenitor transcriptome towards a more mature blood profile with an increase in the abundance of T cell-associated transcripts during the postnatal period (**Figure 3.10**). However, this data set does not contain preterm or term cord blood that would capture the far ends of the immune cell development spectrum. Additionally, some preterm subjects had sepsis prior to or at the time of sampling further confounding the course of development. Preliminary analysis of the Kalikstad data set illustrates the need to continue sequencing our uninfected preterm cohort.

Table 3.4 Clinical characteristics of the Kalikstad dataset preterm postnatal samples

Clinical characteristics	N=107
Gestational age (weeks; mean + range)	28.6 (23.5 - 37.1)
Postnatal age (days; mean + range)	36 (1 - 226)
Sex (% male)	31
Birth weight (grams; mean + range)	1,113 (560 - 3,000)

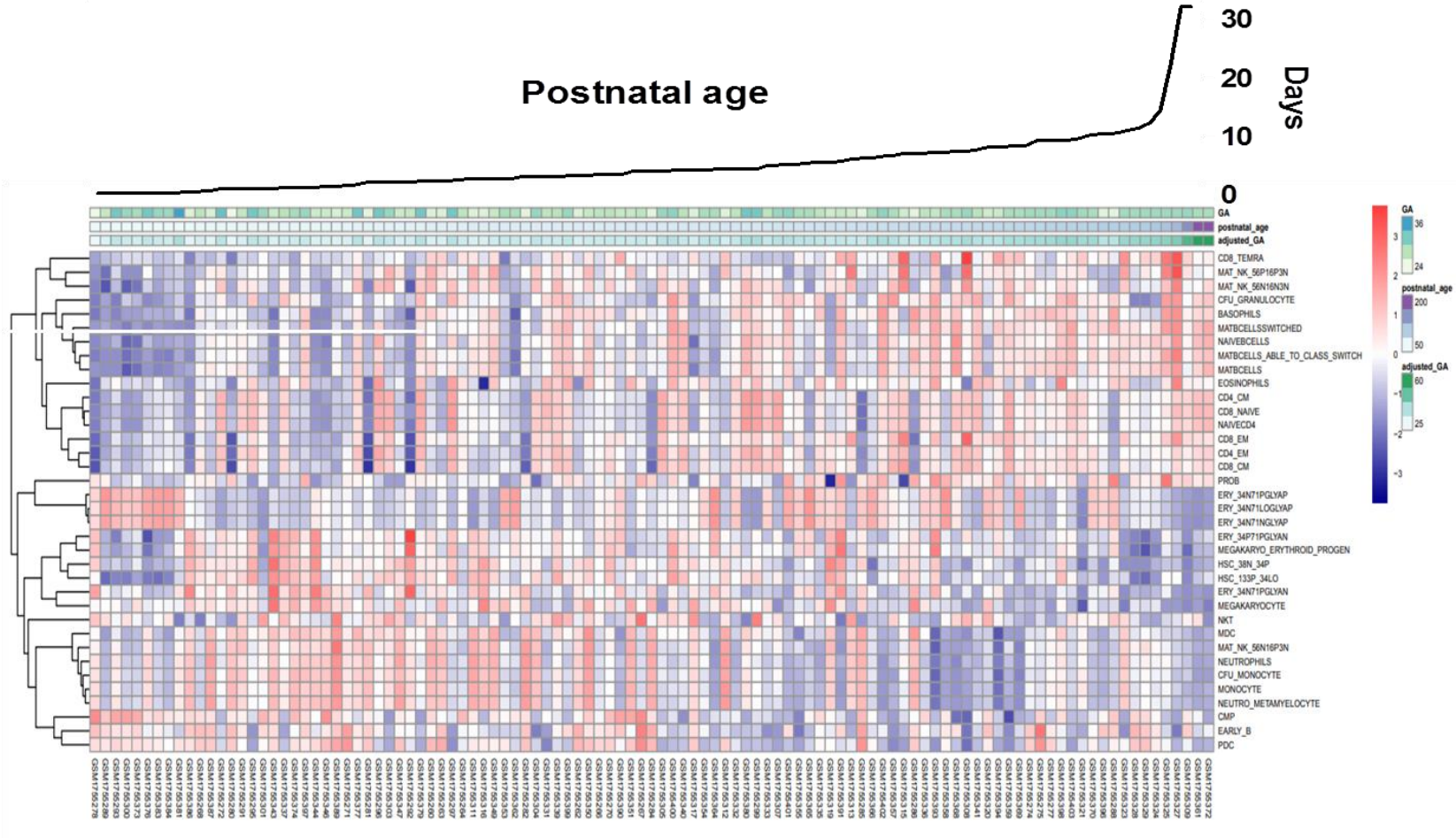


Figure 3.10 Postnatal preterm blood cell-type profile of Kalikstad data set.

Supervised hierarchical clustering of all postnatal preterm samples for the Novershtern cell-type gene sets by postnatal age. GA: gestational age. Kalikstad data from GSE70461. Adjusted GA: gestational age + postnatal age.

3.3.7 Predictability of sepsis from cord blood in preterm neonates

As postnatal preterms were not sampled during an infection (suspected or culture-positive) or when on antibiotics, to the best of our knowledge they were uninfected at the time of sampling. Each postnatally sampled preterm that later experienced a culture-positive sepsis event was matched with a neonate that did not develop sepsis during their stay in the NICU. The sepsis event occurred on average 10 days (5-25 days) after sampling (**Table 3.2**), which is in line with epidemiological data. To identify a sepsis-prediction profile we matched our sepsis subjects for a few confounding factors (gestational age, postnatal age, birth weight, and sex). By matching for these factors, we hoped to detect a profile that is distinct in those preterms that went on to develop sepsis.

To confirm matching by gestational age, we show comparable gestational age between infants who developed sepsis and those infants without a sepsis event (**Figure 3.11**). Using the Novershtern gene sets, GSEA was performed on the preterm cord blood samples comparing sepsis to no-sepsis. The analysis identified an enrichment of monocyte and neutrophil gene sets in sepsis samples (**Table 3.5 and Figure 3.12**). The leading edge represents the core gene transcripts that make up the signal that is enriched in the gene set (Subramanian et al., 2005). The monocyte leading edge genes (from Novershtern, 2011) overlap with the inflammation gene set (from Hallmark) and associate with sepsis (FDR < 0.05) (**Figure 3.13**). Of this, 51 genes are annotated, and many are associated with inflammatory pathways (**Table 3.6** and **Figure 3.14**). These monocyte/granulocyte gene sets include members of TLR and inflammasome signaling pathways and suggest an elevated inflammatory state is predictive of sepsis in preterm cord blood.

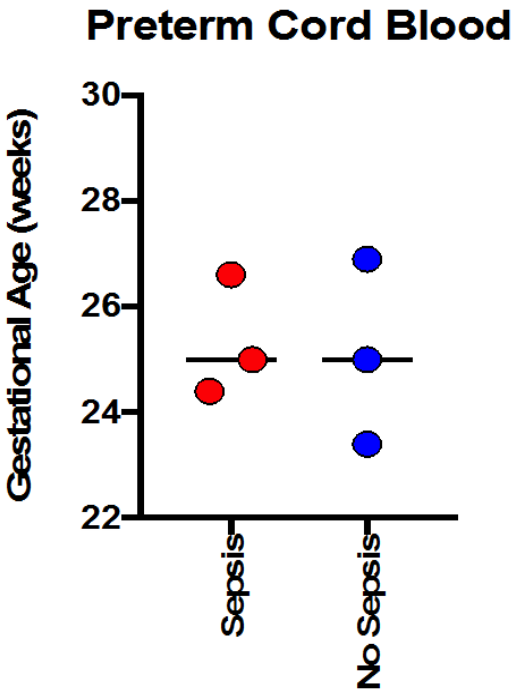


Figure 3.11 Matching by gestational age for preterm cord blood.

Sepsis was identified by a culture-positive sepsis event in the patients' clinical documentation. Three sepsis subjects were matched to no sepsis peers for gestational age, birth weight, sex and postnatal age.

Table 3.5 Cell-type gene sets associated with a future sepsis event in preterm cord blood

Cell-type gene set	Cell-type	SIZE	ES	NES	NOM p-val	FDR q-val
NEUTRO_METAMYELOCYTE	GRAN2	447	0.63	2.63	$<1x10^{-4}$	$<1x10^{-4}$
MONOCYTE	MONO2	758	0.57	2.46	$<1x10^{-4}$	$<1x10^{-4}$
CFU_MONOCYTE	MONO1	201	0.59	2.32	$<1x10^{-4}$	$<1x10^{-4}$
NEUTROPHILS	GRAN3	353	0.56	2.3	$<1x10^{-4}$	$<1x10^{-4}$
MAT_NK_56N16P3N	NKa1	252	0.57	2.29	$<1x10^{-4}$	$<1x10^{-4}$
MDC	DENDa2	151	0.5	1.89	$<1x10^{-4}$	$<1x10^{-4}$

Cell-type gene sets enriched in preterm cord blood samples with a future sepsis event. ES: enrichment score. NES: normalized enrichment score. NOM p-val: nominal p value estimates the significance of enrichment score for a single gene set. FDR q-val: p value after adjusting for false discovery rate (FDR).

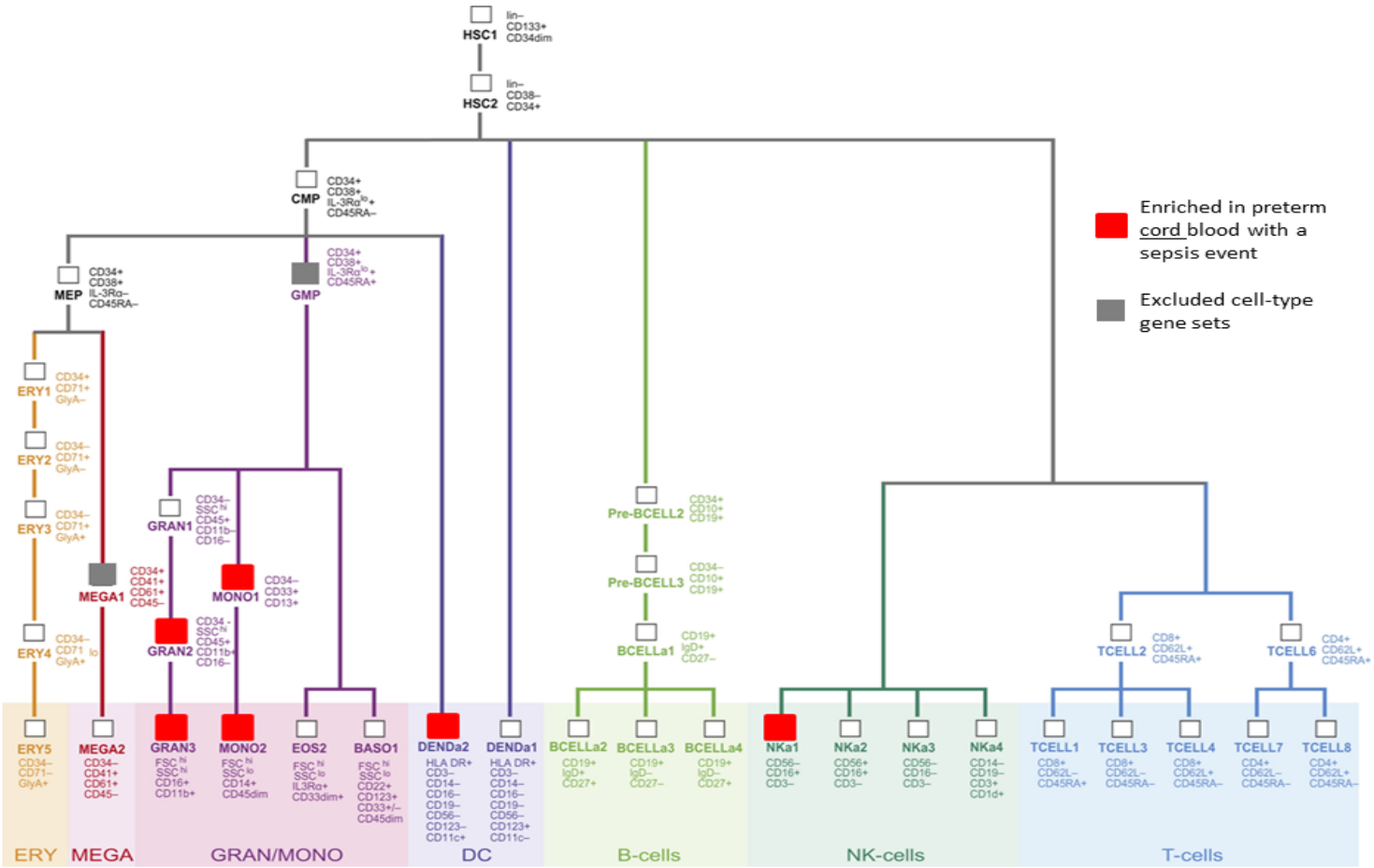
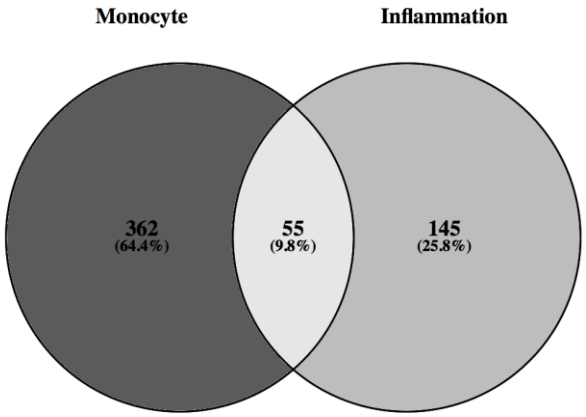


Figure 3.12 Cell-types enriched in preterm cord blood with a sepsis event

SLEA scores for cell-subset specific gene sets comparing preterm cord blood samples with a future sepsis event with controls (not blood culture positive event). Gran2, Gran3, MONO1, DENDa2 and NKa1 cell types are enriched in the cord blood of preterms with a sepsis event compared to those who did not. Red: enriched in preterm cord blood with a sepsis event. Gray: excluded cell-type gene sets. Figure adapted from Novershtern et al. 2011.Reproduced with permission



Hallmark Gene set	SIZE	ES	NES	NOM p-val	FDR q-val
TNFA_SIGNALING_VIA_NFKB	199	0.4491154	1.746554	$<1x10^{-4}$	0.00662586
P53_PATHWAY	198	0.4246946	1.636658	$<1x10^{-4}$	0.01081233
ALLOGRAFT_REJECTION	198	0.3908934	1.525422	$<1x10^{-4}$	0.02638716
WNT_BETA_CATENIN_SIGNALING	41	0.4804327	1.459695	0.03036053	0.04188498

ES: enrichment score. NES: normalized enrichment score. NOM p-val: nominal p value estimates the significance of enrichment score for a single gene set. FDR q-val: p value after adjusting for false discovery rate (FDR).

Figure 3.13 Preterm cord blood sepsis predicting gene sets.

Monocyte and inflammation leading edge genes are involved in inflammatory pathways (e.g. TNF-α, p53 and allograft rejection) associated with sepsis (FDR <0.05). Table represents Hallmark gene sets enriched in the leading edge genes overlapping between monocyte and inflammation.

Table 3.6 Genes shared by monocyte (Novershtern) and inflammation (Hallmark) gene sets

Gene	Gene name		Gene	Gene name
CDKN1A	cyclin dependent kinase inhibitor 1A		RHOB	ras homolog family member B
MCL1	BCL2 family apoptosis regulator		IFNGR2	interferon gamma receptor 2
NFIL3	nuclear factor, interleukin 3 regulated		CCR2	C-C motif chemokine receptor 2
APAF1	apoptotic peptidase activating factor 1		CEBPD	CCAAT enhancer binding protein delta
FRAT1	WNT signaling pathway regulator		GADD45B	growth arrest and DNA damage inducible beta
TLR2	toll like receptor 2		NUMB	NUMB, endocytic adaptor protein
SGK1	serum/glucocorticoid regulated kinase 1		MCL1	MCL1, BCL2 family apoptosis regulator
MAPKAPK3	mitogen-activated protein kinase-activated protein kinase 3		CEBPA	CCAAT enhancer binding protein alpha
PLK2	polo like kinase 2		DUSP1	dual specificity phosphatase 1 [*Homo sapiens*
IFI30	lysosomal thiol reductase		ZFP36L1	ZFP36 ring finger protein like 1 [*Homo sapiens*
SLC16A6	solute carrier family 16 member 6		NLRP3	NLR family pyrin domain containing 3 [*Homo sapiens*
SERPINB8	serpin family B member 8		KLF4	Kruppel like factor 4
TLR1	toll like receptor 1		KLF10	Kruppel like factor 10
TLR6	toll like receptor 6		CASP1	caspase 1
IFNGR1	interferon gamma receptor 1		TNFAIP2	TNF alpha induced protein 2
SAT1	spermidine/spermine N1-acetyltransferase 1		KDM6B	lysine demethylase 6B
PLXNB2	plexin B2		CD1D	CD1d molecule
CD86	CD86 molecule		SRGN	serglycin
CD4	CD4 molecule		NCF4	neutrophil cytosolic factor 4
HLA-DMA	major histocompatibility complex, class II, DM alpha		IER3	immediate early response 3
PTPRE	protein tyrosine phosphatase, receptor type E		VDR	vitamin D receptor
LY86	lymphocyte antigen 86		HBEGF	heparin binding EGF like growth factor
CTSS	cathepsin S		FOS	Fos proto-oncogene, AP-1 transcription factor subunit
ELF4	E74 like ETS transcription factor 4		MXD1	MAX dimerization protein 1
TNFAIP6	TNF alpha induced protein 6		SPI1	Spi-1 proto-oncogene
VEGFA	vascular endothelial growth factor A			

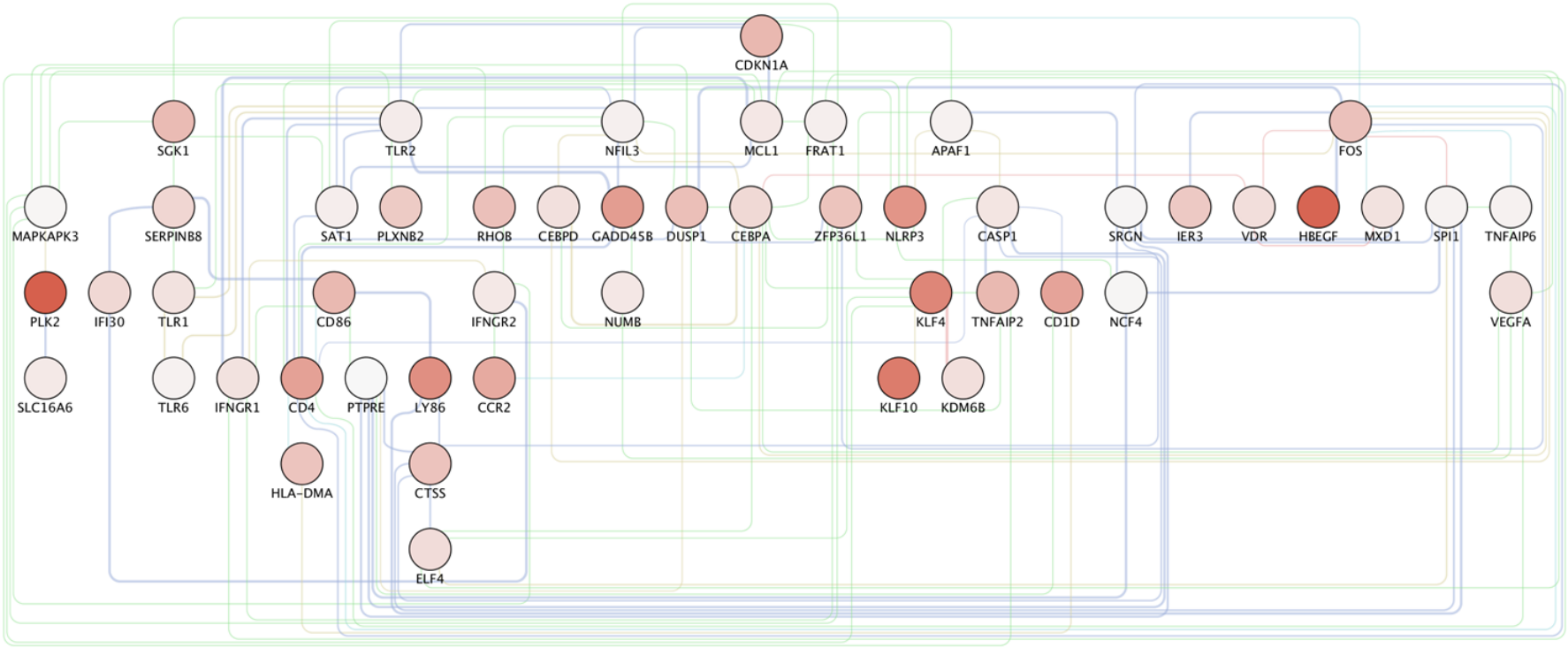


Figure 3.14 Monocyte related genes predictive of sepsis include members of TLR and inflammasome signaling

Overlapping genes between monocyte leading edge and inflammatory pathway leading edge. Gradient of the nodes shows fold upregulation in preterm cord blood. Edges are based on network inference (GeneMania).

3.3.8 Ability to identify a transcript profile in the postnatal preterms with a sepsis event

Samples were paired for gestational age, however one no sepsis sample was lost to analysis as mentioned in the methods section, unbalancing the matching for postnatal age (**Figure 3.15**). The GSEA performed on the 5 preterm postnatal sepsis samples versus the four no sepsis samples indicated an enrichment of gene sets associated with HSCs and erythroid progenitors (**Table 3.7 and Figure 3.16**). This was a profile similarly enriched in the preterm cord blood when compared to term cord blood (**Figure 3.6**). The upregulation of cellular processes and cellular metabolism in these same samples (**Table 3.8**) is likely associated with these progenitor cells. The preliminary predictive sepsis profile for preterm postnatal blood is an immature immune phenotype with an enrichment of HSC/erythroid gene sets as the best predictors for sepsis.

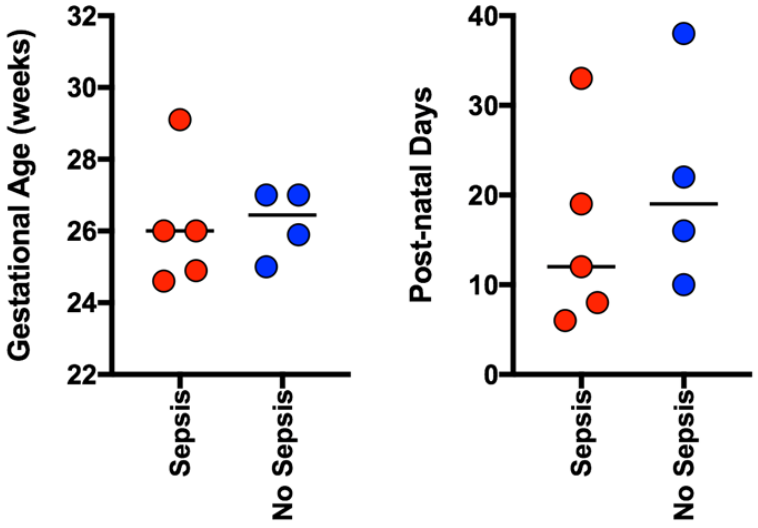


Figure 3.15 Matching of preterm postnatal subjects.

Sepsis was identified by a culture-positive sepsis event in the patients' clinical documentation. Three sepsis subjects were matched to no sepsis peers (i.e. stable infants with no clinical signs of sepsis) for gestational age, birth weight, sex and postnatal age (days). One subject in the no sepsis group was lost to analysis during sequencing.

Table 3.7 Transcriptomic profile predicting sepsis in preterm postnatal subjects (Novershtern cell-types)

Cell-type gene set	Cell-type	SIZE	ES	NES	NOM p-val	FDR q-val
ERY_34P71PGLYAN	ERY1	519	0.51	2.29	$<1x10^{-4}$	$<1x10^{-4}$
ERY_34N71PGLYAN	ERY2	463	0.51	2.28	$<1x10^{-4}$	$<1x10^{-4}$
MEGAKARYO_ERYTHROID_PROGEN	MEP	644	0.47	2.15	$<1x10^{-4}$	$<1x10^{-4}$
ERY_34N71LOGLYAP	ERY4	879	0.43	1.96	$<1x10^{-4}$	$<1x10^{-4}$
HSC_38N_34P	HSC2	467	0.43	1.92	$<1x10^{-4}$	$<1x10^{-4}$
ERY_34N71PGLYAP	ERY3	1252	0.41	1.91	$<1x10^{-4}$	$<1x10^{-4}$
ERY_34N71NGLYAP	ERY5	852	0.4	1.84	$<1x10^{-4}$	$<1x10^{-4}$
HSC_133P_34LO	HSC1	980	0.38	1.77	$<1x10^{-4}$	$<1x10^{-4}$
PROB	Pre-BCELL3	176	0.38	1.54	$<1x10^{-4}$	0.006

ES: enrichment score. NES: normalized enrichment score. NOM p-val: nominal p value estimates the significance of enrichment score for a single gene set. FDR q-val: p value after adjusting for false discovery rate (FDR).

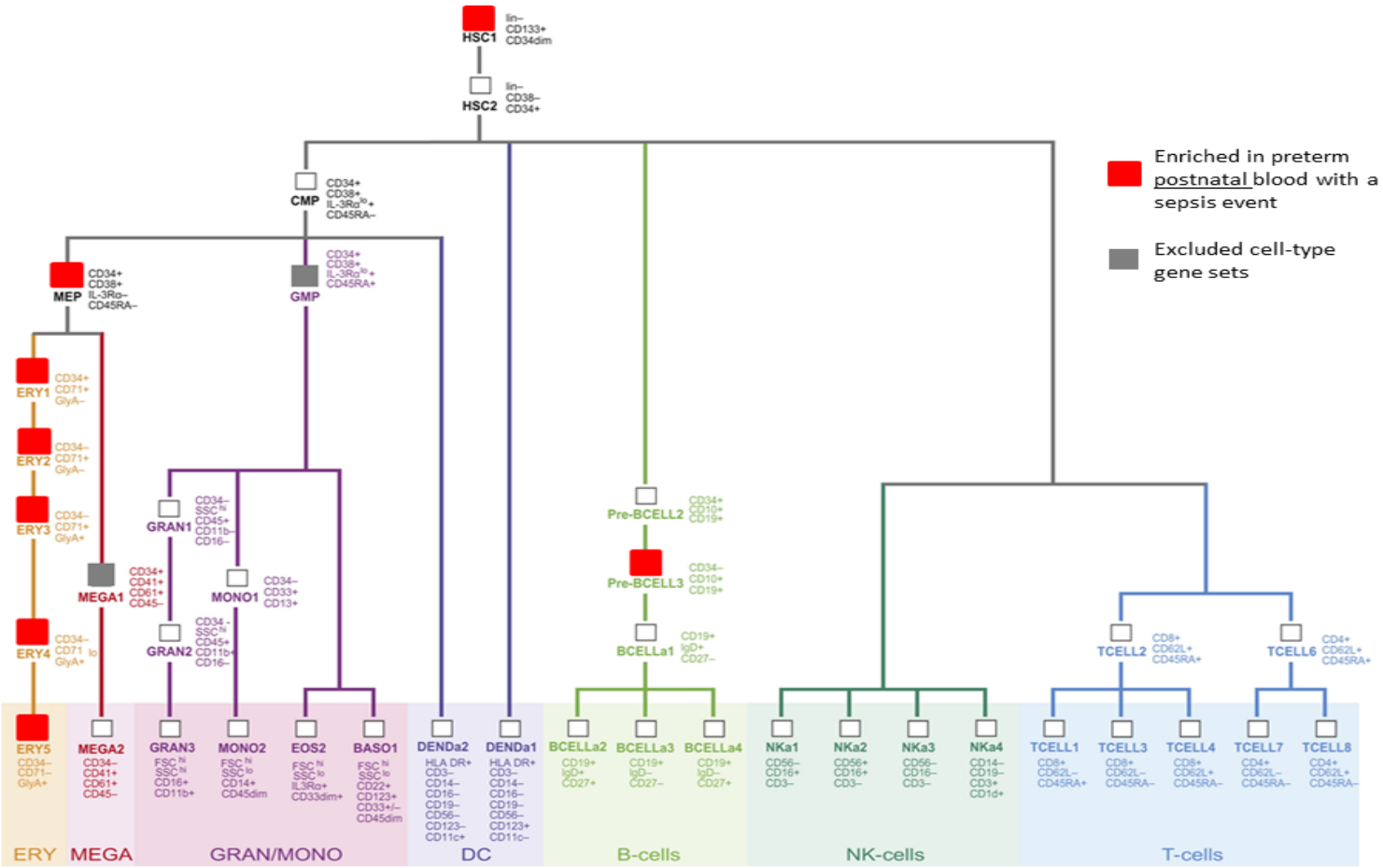


Figure 3.16 Cell-types enriched in preterm postnatal blood with a sepsis event

SLEA scores for cell-subset specific gene sets comparing preterm postnatal blood samples with a future sepsis event with controls (not blood culture positive event). HSC, progenitors (MEP) and erythroid cells (ERY1 to 5) are enriched cell-types (red) in preterm postnatal samples that had a subsequent sepsis event. Red: enriched in preterm postnatal blood with a sepsis event. Gray: excluded cell-type gene sets. Figure adapted from Novershtern et al. 2011.Reproduced with permission.

Table 3.8 Hallmark gene sets upregulated in postnatal preterms prior to a sepsis event

Hallmark Gene set	SIZE	ES	NES	NOM p-val	FDR q-val
OXIDATIVE_PHOSPHORYLATION	199	0.6480665	2.6335275	$<1x10^{-4}$	$<1x10^{-4}$
MYC_TARGETS_V1	199	0.5345765	2.2032447	$<1x10^{-4}$	$<1x10^{-4}$
MYC_TARGETS_V2	58	0.593127	2.0271602	$<1x10^{-4}$	$<1x10^{-4}$
FATTY_ACID_METABOLISM	151	0.4842905	1.918757	$<1x10^{-4}$	$<1x10^{-4}$
DNA_REPAIR	142	0.475454	1.8596214	$<1x10^{-4}$	$6.41\ x10^{-4}$
MTORC1_SIGNALING	199	0.4543665	1.8595903	$<1x10^{-4}$	$5.49\ x10^{-4}$
E2F_TARGETS	199	0.451368	1.8571514	$<1x10^{-4}$	$4.81\ x10^{-4}$
UNFOLDED_PROTEIN_RESPONSE	111	0.4506599	1.7065523	0.0032626	0.0031075
G2M_CHECKPOINT	198	0.4132883	1.684985	$<1x10^{-4}$	0.0034257
IL2_STAT5_SIGNALING	199	0.4082355	1.6793956	$<1x10^{-4}$	0.0031143
HEME_METABOLISM	194	0.3991696	1.6309947	$<1x10^{-4}$	0.005125
PI3K_AKT_MTOR_SIGNALING	104	0.4019245	1.5210906	0.0049261	0.0115383
REACTIVE_OXIGEN_SPECIES_PATHWAY	47	0.4581284	1.4872519	0.0237226	0.0164327
CHOLESTEROL_HOMEOSTASIS	72	0.4121621	1.4753515	0.0177305	0.0172729

ES: enrichment score. NES: normalized enrichment score. NOM p-val: nominal p value estimates the significance of enrichment score for a single gene set. FDR q-val: p value after adjusting for false discovery rate (FDR).

3.4 Discussion

There are published datasets examining differences between preterms of varying ages over the postnatal period, but often, they examined the RNA derived from purified mononuclear cells (Kwinta et al. 2017; Zasada et al. 2014). The limitation of this is the exclusion of granulocytes and potentially other cells that might influence the immune response. Previous work on whole blood in preterms has shown that postnatal age has a greater influence on the transcriptomic profile than GA or infection status (Wynn et al. 2015). They found that with unsupervised hierarchical clustering, clustering occurred depending on the preterm's postnatal age at sampling (<3 days or > 3 days) and only after that on their infection status (Wynn et al. 2015). Hence, we expected samples to segregate based on the origin of the sample (preterm cord blood vs. preterm postnatal blood vs. term cord blood), and by the postnatal age of the neonates. We did see that the preterm postnatal samples were different from preterm cord blood, but it was surprising that preterm postnatal samples were more similar to the term cord blood. This could be due to advanced maturation of the preterm immune system with postnatal age. Alternatively, we cannot exclude that there is a unique transcriptional program associated with a preterm delivery that is reversed after birth in preterm infants, making them more similar to term infants.

When examining the cell types present in the whole blood using the Novershtern gene sets there was an increased presence of hematopoietic stem cell (HSC) signature, megakaryocyte-erythroid progenitor (MEP), early B, pDC and common myeloid progenitors (CMP) in preterm cord blood compared to term cord blood. The Spearman correlation shows that it is the progenitor cell-types are the likely source of the elevated cellular processes, metabolism and activity from the Hallmark gene sets. The next question raised was whether this is due to the function, activity, or metabolism of these cells specifically in preterm cord blood, or just their

abundance within preterm cord blood. We have not yet been able to answer this question, but we are currently pursuing single-cell RNA-sequencing studies to address this.

The changes in the transcriptomic profile of the postnatal preterms suggest that there are changes in the proportion of different cell types, indicating continued development of the immune repertoire. Petter Brodin's group (Olin et al. 2018) has serially sampled a large cohort of babies, acquiring extensive plasma proteomics, cell composition and some RNA sequencing on a very limited volume of blood. They describe a decrease in the proportion of neutrophils after birth and an increase in the T cell populations (Olin et al. 2018). This is similar to the cell-types we see in the postnatal samples, but they do not examine the erythroid progenitors and HSC as they only assessed well characterized, mature immune cell-types. They note that the phenotype (frequencies of cell populations and plasma protein concentrations) of cord blood from term and preterm neonates were highly diverse, but the preterms converges with the term cord blood by 3 months after birth. Our babies represent a snapshot along this path to convergence and we see that regardless of gestational age or postnatal age the preterm postnatal samples are more similar by principal component analysis to term cord blood. However, the Olin et al. (2018) data do not account for immunological function, nor do they characterize the immature and progenitor cell types, which we have found to be enriched in preterm cord blood and in those babies who are at risk of developing sepsis.

Our cohort did not sample infected premature infants as many others have but identifies a baseline transcriptomic signature of these babies in the postnatal period. Can we correct for gestational age and postnatal days in the transcriptomic profile of preterm babies? Ideally, this would enable us to predict which babies are more susceptible to infections early in the neonatal period to encourage changes in care or better monitoring of at-risk infants. The strength of our

cohort is the clinical data collected on these babies and the ability to identify if they experienced sepsis events or other serious outcomes, such as necrotizing enterocolitis and bronchopulmonary dysplasia. To study the *ex vivo* changes in transcriptome of neonatal immune cells to TLR4 stimulation some have utilized purified proteins such as LPS to examine the transcriptomic changes of neutrophils in term cord blood and adults (Mathias et al. 2017). Another group utilized whole *E. coli* and *S. epidermidis* to examine the changes in the transcriptome of purified monocytes, identifying clear separation in the profile of the two pathogen responses, but were unable to differentiate term from preterm subjects (de Jong et al. 2017). A potential explanation for their lack of detection of a difference is that we have shown transcriptome differences between preterm and term cord blood monocytes are localized within metabolic and ribosomal pathways, with the majority of genes being similarly expressed (Kan et al. 2018). Our cohort provides an examination of many circulating cells and has identified hematopoietic stem cells (HSCs) and erythroid progenitors as upregulated in preterm cord blood; these cells are missed in studies of purified cell populations. Our RNA-sequence dataset, once completed, will illustrate the development of cell-types in the postnatal period and in parallel provide paired samples to examine each subject's response to bacterial and viral infections. The data will provide insight into how preterm neonates respond to infections and if an enrichment of immature, progenitor cells corresponds to the low pro-inflammatory cytokine production in preterms we observed in chapter 2.

Our third aim was to determine if the transcriptome could identify those infants who developed sepsis after sampling. By matching sepsis subjects with infants of similar gestational age that did not develop sepsis, we control for the effect of gestational age and the remaining transcriptomic profile could indicate a potential signature of increased susceptibility to infection.

In preterm cord blood subjects that developed sepsis there was an enrichment of a mature and inflammatory cell-types. However, the small number of preterm cord blood subjects with sepsis requires sequencing of more samples to strengthen this conclusion. Additionally, one preterm cord blood subject had confirmed sepsis at the time of sampling, which could overpower the profile of the other two subjects in the sepsis group. The preterm postnatal subjects with a future sepsis event had an enrichment of immature, HSC and erythroid progenitor cell-types. This progenitor profile may provide a new risk factor for sepsis that could be identified and monitored in premature neonates after birth. The caveat to these conclusions is that we cannot rule out a culture negative sepsis event in the no-sepsis control subjects, which could confound these predictive profiles.

Researching gene signatures of preterm infants during the neonatal period may inform the development of sepsis-gene signatures, which could enable a more rapid detection of a blood stream infection than traditional blood culture. Blood cultures are notoriously difficult for diagnosis of sepsis in preterm neonates. There is a high incidence of false positives due to contamination and false negatives can occur when insufficient blood is collected. As a result, clinicians often rely on a clinical diagnosis of sepsis in the absence of a positive blood culture. This study defined sepsis as a culture positive event, but there is the possibility that some of our no-sepsis preterms may have experienced a clinical sepsis event. In the NICU, if we could screen a preterm early after birth to determine if they are more prone to developing a sepsis episode, we could limit their exposure to activities that increase the chance of introducing a pathogen into the bloodstream. These babies could be monitored more closely for non-descriptive signs of a bloodstream infection, such as tachycardia, bradycardia, hypothermia, hyperthermia, lethargy, feeding intolerance, low blood sugar, and breathing problems (Camacho-Gonzalez et al. 2013),

in the hope of identifying an infection early and being able to treat with the appropriate antibiotics. Our cohort and profiles of uninfected preterms could be linked to other data sets that identified separate clusters from Gram-positive and Gram-negative sepsis (Cernada et al. 2014). Our work will help to clarify the baseline preterm postnatal transcriptomic profile and complement the efforts to identify preterm neonates with sepsis. In the future we could validate which differentially expressed genes can quantify the frequency of HSC and erythroid progenitor cell types in preterm cord blood and identify a baby's individual risk of developing sepsis.

In addition, this cohort can inform about mechanisms underlying immune maturation and attenuation in premature neonates. Even though transcript levels do not correlate perfectly with their functional protein levels due to posttranscriptional regulatory mechanisms, (Vogel & Marcotte 2012), transcriptomic signatures can be provide limited information on cell function and status. In this cohort, we not only have RNA samples from unstimulated but also *ex vivo* stimulated whole blood (LPS, R848). When we finish sequencing the cohort we will be able to compare each baby's basal transcriptome to examine how their gene expression profile changes with exposure to TLR4 and TLR7/8 stimulation. Since we have functional readouts (cytokine secretion by ELISA and a subset with flow cytometry), we can correlate the transcriptomic signature with the capacity to produce a pro-inflammatory immune response. Ideally, we would identify if the cytokines' mRNAs are upregulated with PAMP stimulation compared to terms or if the reduced cytokine secretion is due to regulation mechanisms downstream of transcription as described in preterm monocytes (Kan et al. 2018). Identifying genes and pathways that are predictive of high or low cytokine production could further our knowledge of preterm neonates' unique immune status. A more mechanistic understanding can open novel therapeutic avenues that are needed to advance treatment in neonatal intensive care.

3.4.1 Limitations

This is a selected subset of sixteen preterm samples and four term cord blood samples from Chapter 2. The primary objective of this study was to determine if we could recover sufficient mRNA from these samples, and to establish feasibility of our approach. In line with this objective, we have successfully demonstrated that major maturation differences can be detected across the neonatal age, supporting further analyses on the entire cohort of samples. The main caveat of the analysis in this chapter is the small sample size, limiting power and type II error to support the conclusions we have drawn. The preliminary sample size also limits our ability to explore the relationships with clinical co-variables, which we will attempt once we complete the RNA-sequencing of the entire cohort. Also, the use of 24h cultured whole blood sample is an ideal comparison against stimulated sample (not yet sequenced), though the period of culture may have altered the blood cell composition and transcriptomic profile.

Whole blood provides practical information about multiple immune cell types in an unbiased manner. However, a whole blood model does not easily provide information on the transcriptional profile of each immune cell type. In the future it would be helpful to analyze the LPS and R848-stiumulated samples and to further de-convolute the data to identify the immune response signature of the different cell types. Alternately single-cell RNA sequencing could help further refine the types of immature and progenitor cells present in preterms. Single-cell RNA sequencing could help us to characterize the development of these cells when standard immune cell markers may be insufficient.

3.4.2 Conclusions

The primary objective of this nested, case-control study was to confirm that we could acquire sufficient mRNA from our samples. The secondary goal was to provide preliminary data about the maturation changes that occur in preterm infants during the postnatal period and how these changes may be used to identify a profile predictive of a future sepsis event. The preliminary data generated have demonstrated the value of this cohort and support its complete sequencing. Using the uninfected and *ex vivo* stimulated samples we will not only be able to identify the developmental changes during the postnatal period, but also ask how these transcriptomes change with a bacterial (TLR4 – LPS) or viral infection (TLR7/8 – R848). Of note, immune status is only one of many factors that could lead to a baby developing sepsis, there also needs to be the exposure and the opportunity for the pathogen to breach the physical barriers and enter the bloodstream. Medical interventions that most premature neonates receive during their stay in the NICU are each a chance for a pathogen to enter the host and develop into sepsis.

Chapter 4: Innate-like production of IL-8 by naïve neonatal CD4 T cells

4.1 Background

The neonatal adaptive immune system is largely naïve, antigen-inexperienced, except for a small proportion of cells whose specificity remains unclear (Zhang et al. 2014). To compensate for the lack of recall antigen responses necessary for a rapid, successful eradication of micro-organisms by the adaptive immune system, neonatal naïve T cells may possess innate-like functions. Earlier studies identified that up to 50% of neonatal naïve CD4+ T cells in preterm and term neonates can immediately produce abundant IL-8 without prior need for activation, proliferation and differentiation (Gibbons et al. 2014). IL-8 is an inflammatory chemokine that is a powerful chemoattractant for neutrophils (Remick 2005). Though adult T cells can produce IL-8 after differentiation (Gasch et al. 2014; Himmel et al. 2011), only a small minority (<10%) of adult T cells produce IL-8 in an innate-like manner compared to term neonates (30-50%). A high frequency of IL-8 producing naïve CD4 T cells was also evident in peripheral blood from neonates born earlier in gestation (Gibbons et al. 2014). In addition to myeloid cells, IL-8 is produced in large quantities by endothelial cells where it serves to recruit and activate neutrophils as professional phagocytes. IL-8 also results in upregulation of MHCII and co-stimulatory molecules and thus, may function to bolster the capacity of neutrophils to act as APCs (Kambayashi & Laufer 2014).

As many T cells emerge from the thymus, Van den Broek and colleagues examined patients who underwent a thymectomy within the first month after birth, in order to better understand the dynamics and origin of innate-like naïve CD4 T cells (van den Broek et al. 2016). The proportion of IL-8-producing T cells was greatly reduced in thymectomized patients (van den Broek et al. 2016). Naïve IL-8-producing CD4 T cells were also enriched for recent thymic

emigrant (RTE) markers, and preferentially expressed the central naïve T cell marker CD31. These findings suggest that the neonate's increased proportion of IL-8-producing naïve CD4 T cells is dependent on sustained thymic output. These authors also linked increased calcium flux to IL-8 production in these cells, suggesting that they are more prone to antigen activation (van den Broek et al. 2016). Altogether, these data fundamentally change our perspective of naïve T cells in humans to a heterogeneous spectrum of cells including innate-like naïve cells recently emerged from the thymus to more peripherally "differentiated" naïve T cells (van den Broek et al. 2018).

In trying to understand the physiological relevance of these *in vitro* observations it is important to mention that evidence of innate-like IL-8 production in these cells was shown following PMA-ionomycin stimulation, which is not physiologic. This raises important questions: what are the circumstances that might trigger or utilize naïve CD4 T cell innate-like (IL-8-producing) immune functions *in vivo*? How do innate-like functions help neonates compensate for the lack of immunological memory at birth? In mice, a skin infection has been shown to trigger rapid homing of neutrophils to draining lymph nodes where most naïve T cells reside (Bogoslowski et al. 2018), implying the existence of a direct talking mechanism between T cells and neutrophils. However, addressing these questions mechanistically has been challenging as IL-8 is a human cytokine that has no direct counterpart in mice, complicating murine *in vivo* studies. Despite reports of IL-8 production by TCR-stimulated T cells (Gibbons et al. 2014) the proportion of IL-8+ cells and secreted levels were not compared levels produced in myeloid cells, a major source of IL-8. Also, how IL-8 production compares between neonatal and adult cells was not rigorously examined in earlier studies (Gibbons et al. 2014; van den Broek et al. 2016). Co-engagement of Toll-like receptors, in addition to the T cell receptor has

been shown to augment IL-8 production, but this increase was relatively modest (Gibbons et al. 2014). These observations led us to develop experiments to understand what makes T cells produce IL-8 in an innate-like manner *ex vivo*. The production of IL-8 by naïve CD4 T cells has been documented in preterm cord blood and during the postnatal period, in addition to term cord blood (Gibbons et al. 2014). To overcome blood volume restrictions of preterm samples and properly investigate these IL-8-producing cells we utilized term cord blood as the representative neonatal samples. In this chapter, we sought to more specifically investigate the phenotype and TCR-driven production of IL-8 by human naïve T cells and determine whether co-receptor engagement can help trigger a strong IL-8 response in these cells. We specifically test the hypothesis that IL-8 production by naïve neonatal T cells can be enhanced by antigen-presenting cells (APCs).

4.2 Materials and methods

4.2.1 Consent and blood collection

Blood was collected from healthy adult volunteers at the BC Children's Hospital and from the placenta of term (36-42 weeks) newborns delivered by cesarean section at the BC Women's Hospital. All blood samples were obtained by venous puncture into (sodium) heparinized vacutainers (BD Biosciences), or into citrate bags (for cord blood) (Macopharma) and processed within 8 hours of collection. In some cases, refrigerated cord blood samples were used, except for samples where APCs were obtained. Our studies were approved by the Children's & Women's Research Ethics Board (#H07-02681).

4.2.2 Cell purification methods

Mononuclear blood cells were extracted from cord or peripheral blood according to a previously published protocol (Lavoie et al. 2010). Briefly, whole blood diluted in PBS was placed on a Ficoll-paque density gradient solution to separate the non-nucleated red cell/granulocyte fraction from the mononuclear cell fraction. Cells were counted and, if required, further purified by magnetic isolation. The purification of naïve CD4+ T cells was performed using a negative selection kit (EasySep™ Naïve CD4+ T cell Isolation Kit I/II) (StemCell Technologies, Vancouver, Canada). Briefly, mononuclear cells are coated with a cocktail of antibodies to bind all non-naïve CD4+ T cells (e.g. monocytes, B cells, CD8 T cells, and CD45RO+ T cells). EasySep™ Dextran RapidSpheres™ are added, attaching to all antibodies. Magnetically labelled cells are separated, and remaining cells are untouched CD3+ CD4+ CD45RA+ CD45RO-. For isolation of APCs a positive selection kit for CD3+ T cells (StemCell Technologies), was utilized according to the manufacturer's instructions for CD3-depletion. FACS isolation of naïve CD4 T cells was performed gating for CD3+ CD4+ CD25- CD45RO- and CCR7+ (Razzaghian et al, manuscript in preparation), using the following antibodies: anti-CD3 – PE (HIT3a) and anti-CCR7 – AlexaFluor647 (3D12), from BioLegend anti-CD4 – BV605 (OKT4) and anti-CD45RO – AlexaFluor700 (UCHL1), from eBioscience/ThermoFisher anti-CD25 – PECy7 (BC96), anti-Glycophorin A (MN) – FITC (10F7MN), on a BD FACS Aria. StemCell Kit magnet purified cells will be called purified naïve CD4+ T cells, while the use of FACS sorted cells will be explicitly stated. During the studies, purity checks were regularly performed for the magnet purified naïve CD4+ cells and the CD3-delpeted cells (APC crossover experiments) (**Table 4.1**).

Table 4.1 Purity of mononuclear cell subsets

(A) Purified naïve CD4 T cells (by negative-selection) and **(B)** CD3-depleted APCs for experiments in this chapter. Naïve CD4 T cells were assessed by staining for CD3+, CD4+ and CD45RA+.

A

Group	Cell type	Purity (% median, range)
Neonates	Naïve CD4 T cells	94.3 (86.0 – 98.7)
Adults	Naïve CD4 T cells	95.5 (59.0 – 98.8)

B

Group	Cell type	Cell proportion (% median, range)
Neonates	Monocytes (CD14+)	4.1 (0.01 - 8.3)
	B cells (CD19+)	3.5 (1.1 - 5.6)
	T cells	< 0.02
Adults	Monocytes (CD14+)	9.9 (9.4 - 18.2)
	B cells (CD19+)	11.5
	T cells	< 0.03

4.2.3 Stimulations of T cells

Stimulations with phorbol 12-myrisate 13-acetate (PMA) (100ng/ml) (Sigma-Aldrich) and ionomycin (1ug/ml) (ThermoFisher Scientific/Invitrogen,) were done in the presence or absence of brefeldin A (Sigma-Aldrich). For T cell activation, DynaBeads™ Human T-activator CD3/28 for T cell expansion and activation (ThermoFisher Scientific) were used at 1:1 (cells to beads), unless otherwise stated. For TCR activation anti-CD3 (clone: OKT3) was used at 1ug/ml (Mike Williams – UBC antibody core, Vancouver, Canada). To inhibit the function of protein kinase C (PKC), cells were pre-incubated for 1hr with the PKC inhibitor Sotrastaurin (α, βI, δ, ε, η, and θ forms) (Selleck Chemicals) at various concentrations and subsequently stimulated with PMA and ionomycin at the above concentrations and incubated at 37°C for 4 hours.

4.2.4 Surface and intracellular flow cytometry

Cells were cultured for the required amount of time and subsequently stained for surface receptor expression and intracellular cytokines/chemokines as previously described (Lavoie et al. 2010). Briefly, surface antibodies in 1x PBS were added to cells after supernatant was removed and incubated for 20 minutes at room temperature in the dark. For intracellular staining, the FoxP3 kit (ThermoFischer/eBioscience) was used according to manufacturer's instructions to fix and permeabilize the cells on ice. If fixable viability dye was used, it was added for 20 minutes at room temperature prior to surface antibody staining. Phenotype panels included the markers in **Table 4.2**.

Table 4.2 Phenotyping antibodies used to characterize IL-8 production in naïve CD4 T cells

Receptor	Clone	Isotype	Colour	Manufacturer
CD3	UCHT1	Mouse IgG1, k	eF450	eBioscience/ThermoFisher Scientific
CD4	OKT4	Mouse IgG2b, k	BV605	BioLegend
CD31	WM59	Mouse IgG1, k	APC-Cy7	BD Biosciences
CD38	HIT2	Mouse IgG1, k	BV711	BioLegend
CD45RA	HI100	Mouse IgG2b, k	APC-eF780	eBioscience/ThermoFisher Scientific
CD45RA	HI100	Mouse IgG2b, k	PerCP-Cy5.5	eBioscience/ThermoFisher Scientific
CD45RO	UCHL1	Mouse IgG2a, k	AF700	BioLegend
CD14	MP4-25D	Rat IgG1 k	BV421	BioLegend
CD93	R139	Mouse IgG2b, k	FITC	BD Biosciences
CD96 (TACTILE)	NK92.39	Mouse IgG1	PerCP-eFluor710	eBioscience/ThermoFisher Scientific
CD97	VIM3b	Mouse IgG1, k	FITC	BD Biosciences
CD127 (IL-7Rα)	HIL-7R-M21	Mouse IgG1, k	APC-R700	BD Biosciences
CD127 (IL-7Rα)	A019D5	Mouse IgG1, k	APC	BioLegend
CD52	4C8	Mouse IgG3, k	BV510	BD Biosciences
IL-27Rα	191106	Mouse IgG2b	PE	R&D Systems
IL-8	BH0814	Mouse IgG2b, lambda	FITC	BioLegend
IL-8	E8N1	Mouse IgG1, k	PECy-7	BioLegend

4.2.5 APC-T cell cross-over experiment

For pre-incubation of APCs, CD3-depleted MNCs were cultured with LPS (10ng/ml) or curdlan (10ng/ml) in complete RPMI1640 (cRPMI) supplemented with 10% human serum (Gemini Bio Products), 2mM L-glutamine (Gibco/ThermoFisher) and penicillin/streptomycin (ThermoFisher) for 24 hours. Allogenic naïve T cells were purified with negative selection kit as described above and added to the APCs at a ratio of 1:1. To stimulate the T cells, anti-CD3 (OKT3) at 1ug/ml was added to the culture and incubated at 37°C for a further 6 and 24 hours in the presence of brefeldin A. Subsequently, the plate was spun down and the supernatant was removed and stored at -80°C. The cells were then stained for surface markers CD4 (BV605, clone OKT4), CD45RA (APC–eF780, clone HI100), CD14 (BV421, clone MP4-25D), CD19 (PE, clone HIB19), and intracellular IL-8 (FITC, clone BH0814) and run on a BD LSRII. MNCs at naturally occurring proportions (day 1) were stimulated for 24hrs with the PAMP followed by the addition of anti-CD3 (OKT3) for FMO controls and anti-CD3/28 beads for maximum stimulation and stained with antibodies and run as above.

4.2.6 Transwell neutrophil migration assay

The neutrophil isolation protocol was used as previously published (de Goede et al. 2017). Briefly, density centrifugation of diluted whole blood, as described for MNC extraction, followed by a 30-minute incubation with dextran-saline solution for sedimentation of the granulocytes. Subsequently, RBCs were lysed with isotonic addition of NaCl. The migration assay was performed using a transwell system (HTS 96-well plate, 3uM – polycarbonate membrane) (Corning) and adapted from (Himmel et al. 2011). Migration was induced by a 2-fold serial dilution of recombinant human IL-8 (rhIL-8) (R&D Systems) (maximum

100,000pg/ml). To block the IL-8 induced migration of neutrophils, the recombinant IL-8 was pre-incubated with anti-IL-8 (clone #6217, R&D Systems) for 1hr prior. The medium used is CST OpTmizer T cell Expansion SFM (Gibco/ThermoFisher), 2mM L-glutamine (Gibco/ThermoFisher), penicillin/streptomycin (ThermoFisher), and CTS supplement (Gibco/ThermoFisher). Neutrophils (100,000 cells/well) were incubated for 30 minutes at 37°C in the transwell apparatus, with a consistent temperature during the migration. The upper wells were removed and placed into a new 96-well plate and the contents of the upper wells were discarded. The lower well's contents were removed and replaced with 25mM EDTA in 1x PBS and incubated at 37°C for 10 minutes. The plate was gently tapped to loosen partially migrated cells attached to the bottom of the membrane. The PBS/EDTA was removed and added to the tube with contents of the bottom well. All conditions were performed in duplicate. Tubes were centrifuged at 500 x g for 5 minutes and the PBS/EDTA removed. Cells were re-suspended in 2% PFA. Samples were stored at 4°C and run the following day. Liquid counting beads (BD Biosciences) were added at 50,000 per tube and 5,000 beads were collected on a BD LSRII after gating on the beads. The total number of neutrophils collected per 5,000 beads was multiplied by 10 to estimate the total cell migration per well. The percentage of migration was graphed as the number of cells over the positive control (full migration – 100,000 cells directly into the bottom well and recovered by the same method).

4.2.7 Data analysis

Flow cytometry data was analyzed using FlowJo software version 10. Statistical analysis was performed using GraphPad Prism version 5.03. An unpaired two-tailed t-test was used to compare the intracellular production or secretion of IL-8 between neonate and adult groups, a p-value <0.05 was deemed to be significant. The published data sets were analyzed by GEO2R (Gene Expression Omnibus by NCBI) for differential expression between adult and cord blood CD38+ cells (Gibbons et al. 2014) and for 0h and 72h for the Th0 condition (Tuomela et al. 2012). Differential gene expression was performed in GenomeStudio (Illumina) and further processed and normalized using the lumi package in R (Razzaghian et al. manuscript in preparation).

4.3 Results

4.3.1 APC-independent production of IL-8 by naïve neonatal CD 4 T cells

The main function of IL-8 is to attract phagocytes to the site of infection and to understand the role of APCs we tested whether they are required for production of this chemokine by naïve T cells. Naïve CD4 T cells from cord blood or peripheral adult blood were isolated by FACS, or negative selection by magnetic column (to obtain “untouched” cells). Both neonatal and adult naïve CD4 T cells produced IL-8 upon stimulation with PMA/ionomycin as detected by intracellular flow cytometry or within culture supernatants by ELISA (**Figure 4.1A** and **Figure 4.1B**). However, neonatal CD4 T cells secreted more IL-8 when measuring cytokine present in cultures (**Figure 4.1B**). In addition, the frequency of IL-8-production was considerably higher among neonatal naïve CD4 T cells relative to adult naïve CD4 T cells (**Figure 4.1C**). However, on a per cell-basis, the amount of IL-8 produced by naïve CD4 T cells

remained substantially lower than monocytes which are another major source of IL-8 (**Figure 4.1D**). In experiments, secreted IL-8 levels were >20-50-fold greater in monocytes than in naïve T cell cultures regardless of the age of the naïve CD4 T cell donor (data not shown). These observations indicate that increased production of IL-8 is inherent to neonatal naïve CD4 T cells.

Next, we determined whether naïve CD4 T cells produce IL-8 upon stimulation through the T-cell receptor complex rather than pharmacologic treatment with PMA and ionomycin. Intracellular production of IL-8 was detectable at 4hrs and increased at 24hrs regardless of age (**Figure 2A**). However, we detected no statistically significant differences in secreted or intracellular IL-8 levels between neonates and adults (**Figure 4.2A**). According to previous data, neonatal T cells produce an un-glycosylated form of IL-4 that is not secreted (Hebel et al. 2014). Therefore, we wanted to confirm that most of the IL-8 detected intracellularly is secreted. Comparing cells assayed in the presence or absence of brefeldin A, our data confirmed that most of this IL-8 is secreted rather than only produced intracellularly (**Figure 4.2A)**

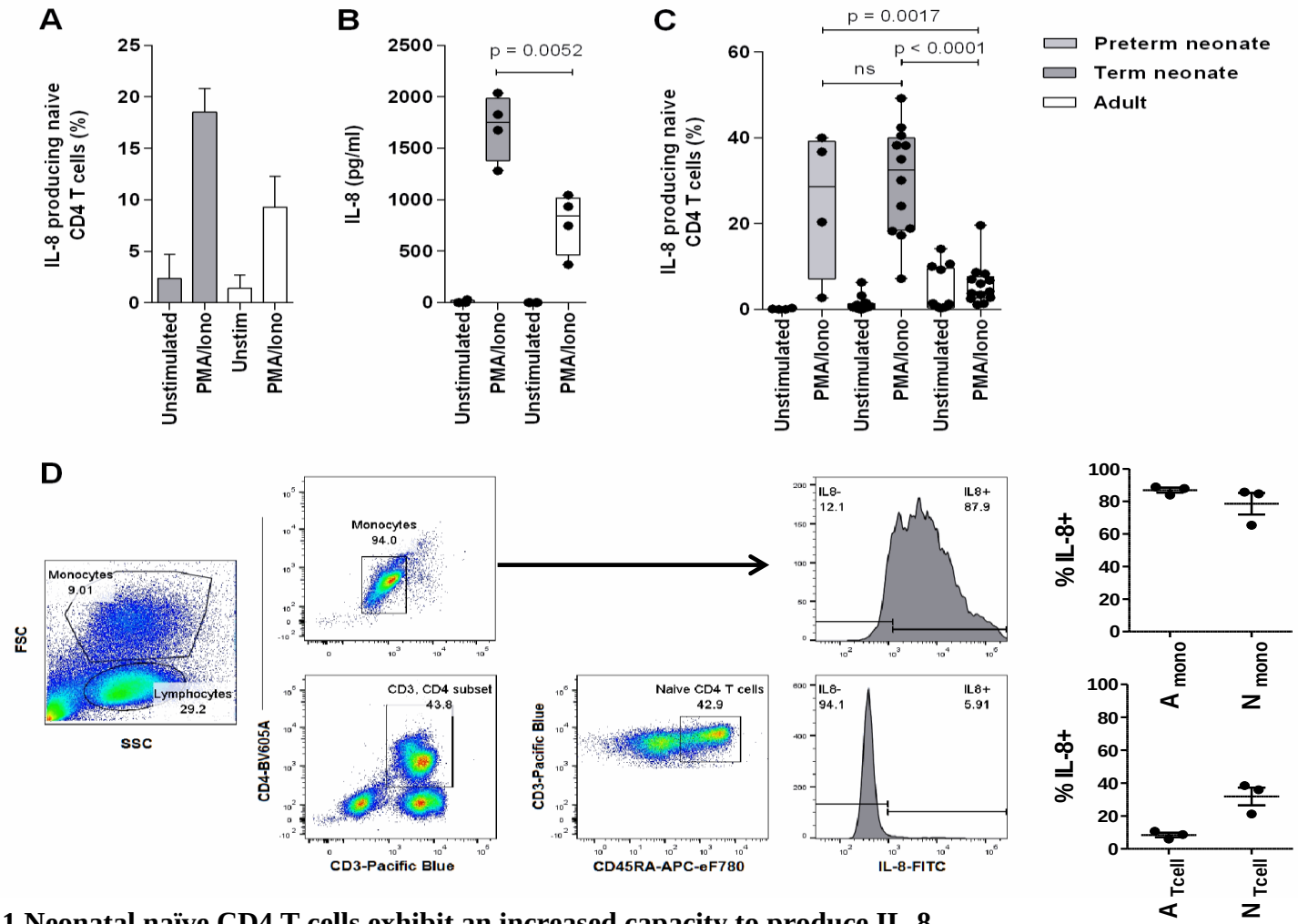


Figure 4.1 Neonatal naïve CD4 T cells exhibit an increased capacity to produce IL-8.

(A) Intracellular IL-8 production (flow cytometry) by magnet-purified samples (n=2 neonatal and 2 adult, mean ± SEM), **(B)** IL-8 secretion (ELISA) by FACS-purified naive CD4 T cells (n = 4 neonatal and 4 adult samples), or **(C)** Intracellular IL-8 production in CD3+/CD4+/CD45RA+ T cells from mononuclear cells stimulated with PMA/I (4hr) (n = 4 preterm, 6 term neonatal and 8 adult samples). Box and whiskers, mean + min to max. P values by unpaired 2-tailed t-tests. **(D)** Comparison of intracellular IL-8 production between naïve CD4 T cells and monocytes, among mononuclear cells stimulated with PMA/I (4h), representative figures are an adult. A Mann-Whitney U two-tailed test revealed no statistically significant difference. Mean ± SEM. SEM: standard error of the mean. Mono: monocyte. T cell: naïve CD4 T cells. A: adult. N: neonatal.

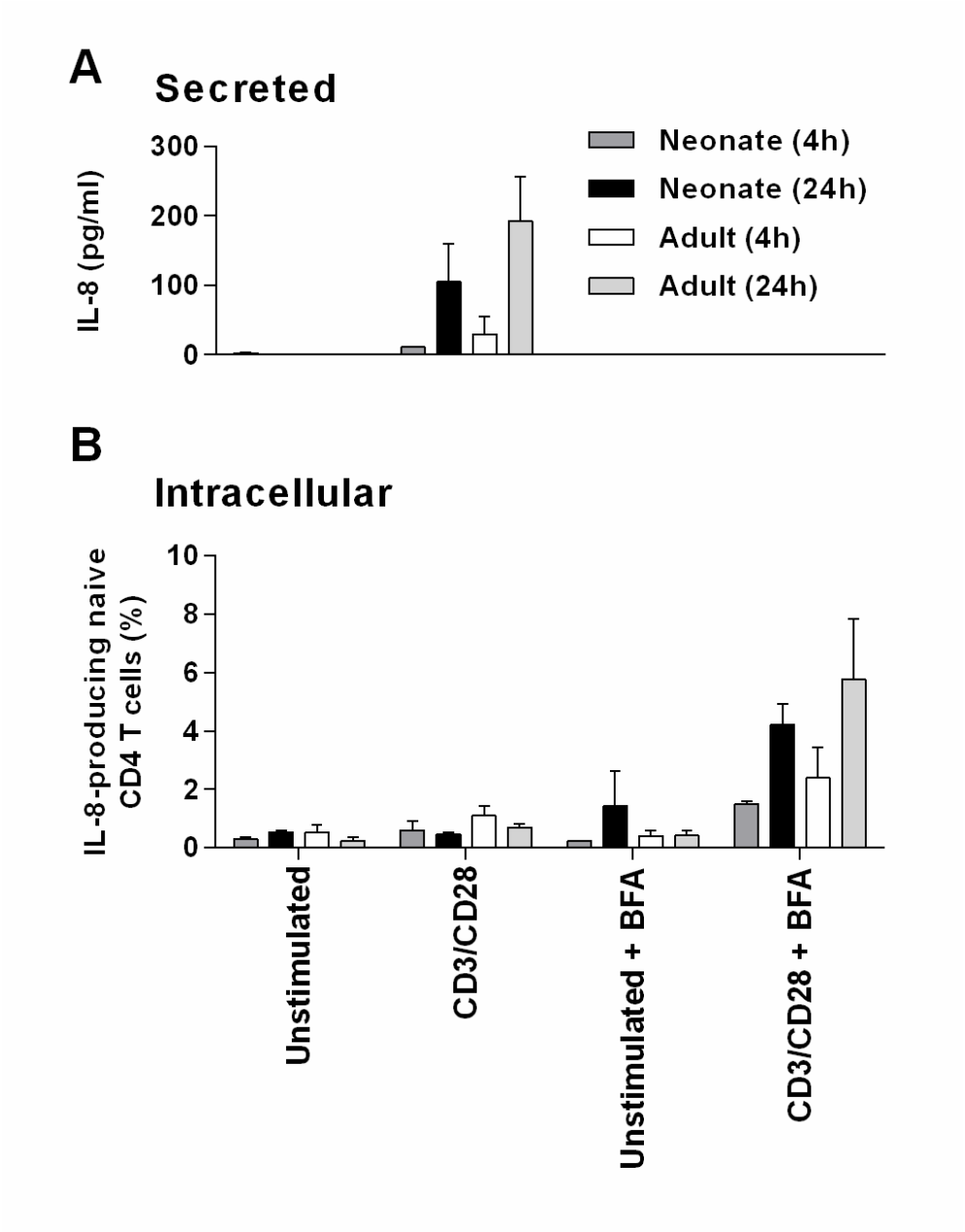


Figure 4.2 TCR stimulation of IL-8 production in naïve CD4 T cells.

(A) Intracellular and corresponding **(B)** secreted IL-8 were measured in magnet-purified naïve CD4 T cells (n = 2-3 neonatal and 3-4 adult samples) stimulated with anti-CD3/CD28 beads (CD3/CD28) for 4h or 24hr, in the presence or absence of brefeldin A (BFA). Mean +/- SEM. Mann-Whitney U, two-tailed test was used to compare neonate and adult samples and showed no significant difference. Some analyses could not be performed due to the low number of samples in the group. SEM: standard error of the mean.

4.3.2 Role of APCs in the production of IL-8 in naïve CD4 T cells

Our experiments above suggested increased IL-8 production in naïve T cells in mononuclear cell cultures compared to isolated T cell cultures (**Figure 4.1A** vs **4.1C**). This indicated that APC interactions may be required to enhance innate-like responses in these T cells. To further examine this question, we conducted cross-over experiments where either adult or neonatal T cells were stimulated in presence of neonatal or adult APC co-cultures (illustrated in **Figure 4.3A**). As shown in **Figure 4.3B**, the proportion of IL-8-producing cells was greater at 24h than at 6h, but we did not observe differences in T cell IL-8 production between neonatal or adult APCs.

Next, we tested whether pre-activating APCs using LPS (a TLR4 agonist) or curdlan (a dectin-1 agonist) to stimulate T cell IL-8 production. To this end, APCs were pre-activated with either of these PAMPs for 24h and incubated with allogeneic T cells in presence of anti-CD3 antibody stimulation (OKT3; 1ug/ml) (illustrated in **Figure 4.4A**). These experiments were highly complex due to the number of donors, especially with the need for two neonatal donors back-to-back for each experiment. Therefore, we were only able to repeat these experiments five times (necessitating 10 neonatal donors and an equal number of adult donors). IL-8 production was increased in neonatal and adult T cells in presence of pre-activated APCs, compared to non-pre-activated APC (**Figure 4.4B**). Again, the proportion of IL-8-producing T cells was greater at 24h than 6h. Though my experiments will need replication in a larger number of subjects, we were unable to detect specific effects due to the age of the APC donors, possibly due to sample size.

The effect of APC pre-activation on IL-8 production by naïve CD4 T cells was also confirmed in autologous co-cultures using MNCs from each donor, unseparated MNCs at naturally occurring ratios (**Figure 4.4C**). With no PAMP the production of IL-8 by anti-CD3 stimulation was significantly reduced in both neonates and adults, compared to LPS. Finally, production of IL-8 was maximized with anti-CD3/CD28 stimulation, instead of only anti-CD3, regardless of a pre-activation with PAMP, implying that IL-8 production in the naïve CD4 T cell requires T cell antigen activation and co-stimulation (**Figure 4.4D**). Note that in autologous culture experiments, the proportion of IL-8-producing naïve T cells was generally higher than in allogeneic experiments, which may have been due to differences in APC/T cell ratios in the co-cultures. Nonetheless, this further supports an important role of APCs in augmenting innate-like production of IL-8 production in T cells.

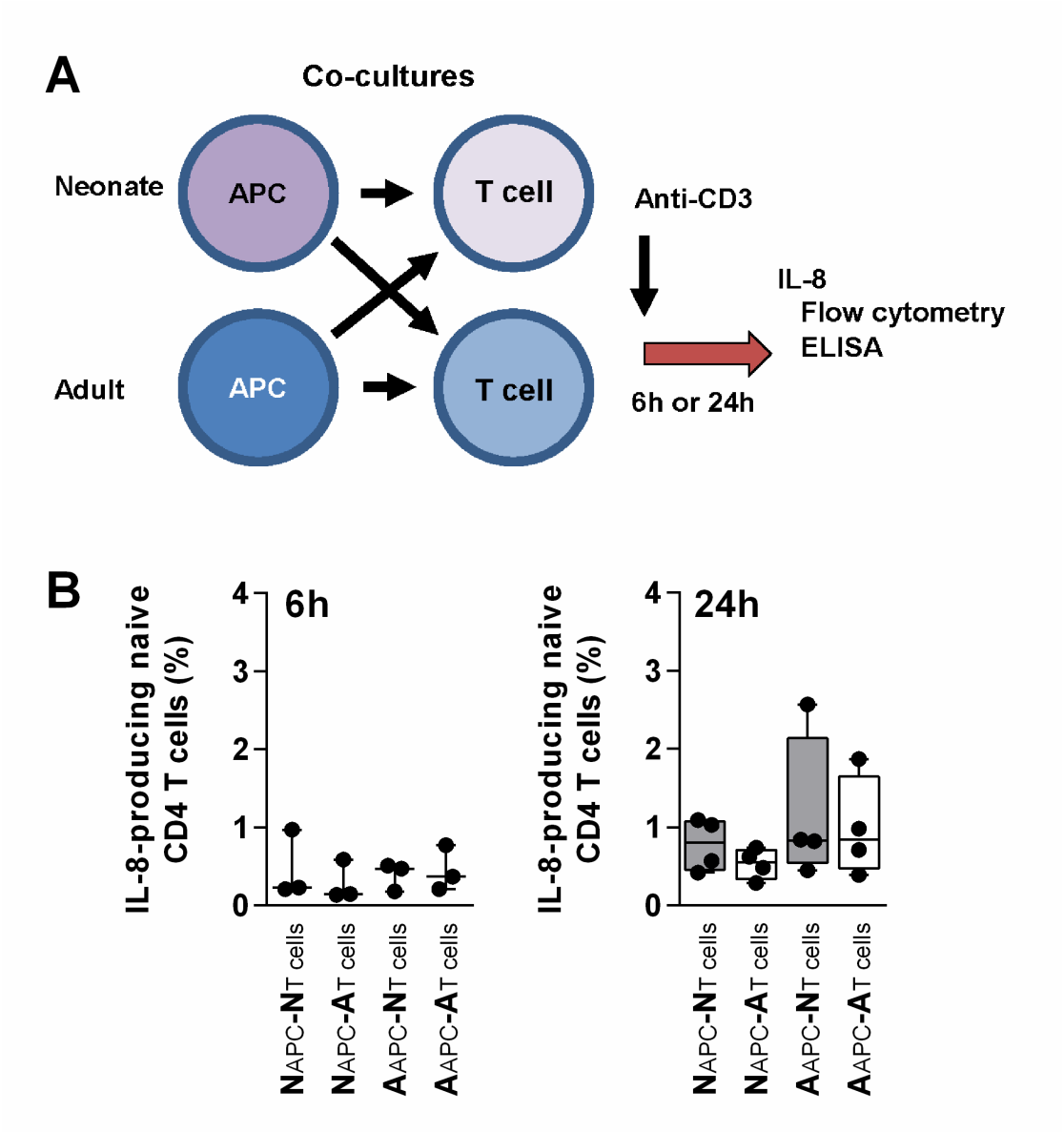


Figure 4.3 Effect of APC on IL-8 production by naïve CD4 T cells

(A) Experimental method APC-T cell co-cultures. All co-cultures were allogeneic with a new T cell donor on day 2. **(B)** Purified APCs (CD3-depleted MNCs) and naïve CD4 T cells stimulated with anti-CD3 measured for intracellular IL-8 production by ICC after 6h and 24h. 4-5 independent experiments per group. Box and whiskers, mean + min-max. A Mann-Whitney U, two-tailed test was used to compare samples, and all showed no significant difference. Supernatants were collected, but the ELISA to examine the cytokine milieu created by the APC pre-activation has not yet been performed.

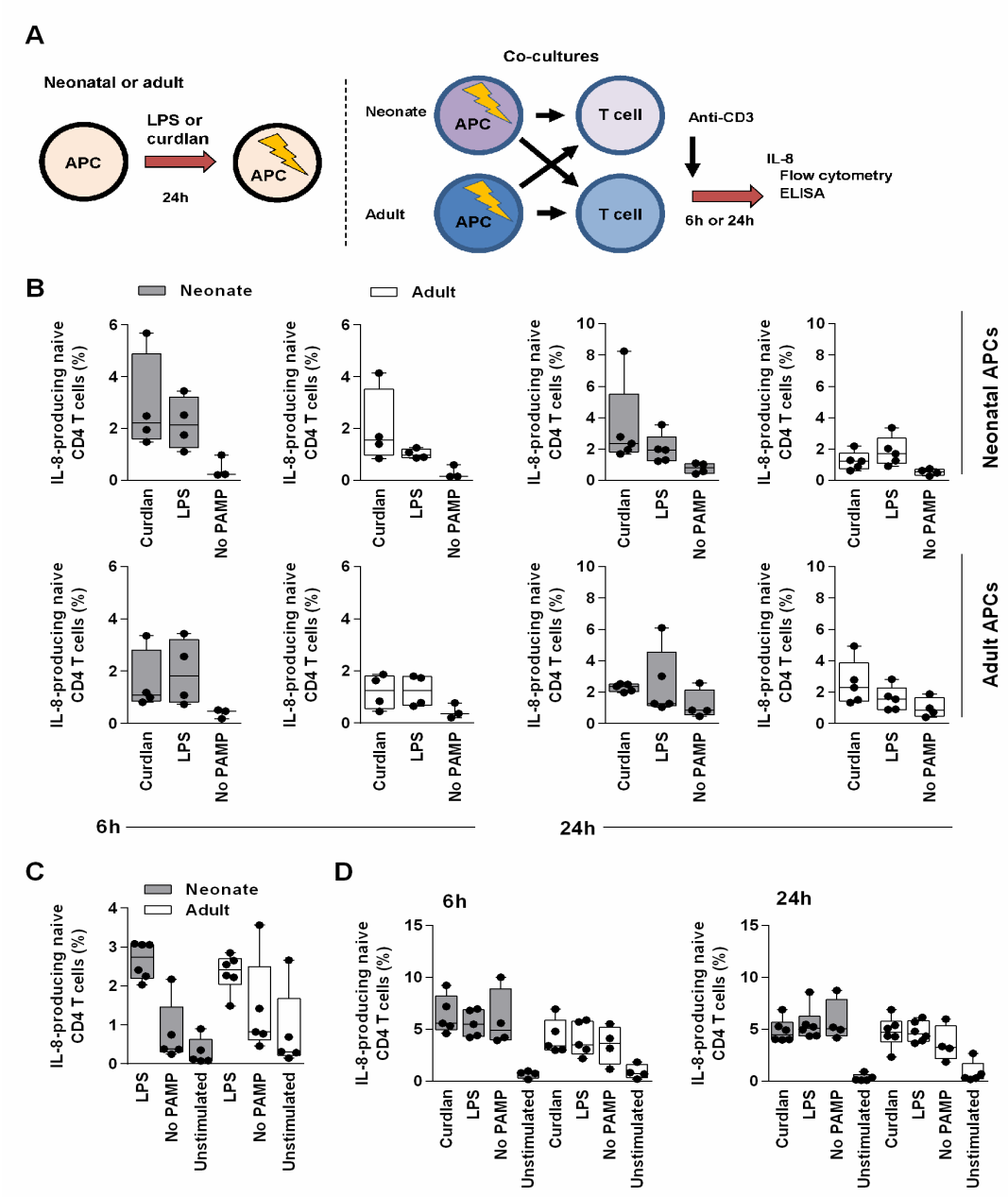


Figure 4.4 Effect of APC pre-activation on IL-8 production by naïve CD4 T cells

(A) Experimental method APC-T cell co-cultures with pre-activation of allogeneic APCs. **(B)** Purified APCs pre-activated and cultured with allogeneic naïve CD4 T cells stimulated with anti-CD3 only, **(C)** Pre-activated MNCs stimulated with anti-CD3 only (24h) and **(D)** Pre-activated APCs and autologous naïve CD4 T cells stimulated with anti-CD3/28 measured for intracellular IL-8 production by ICC after 6h and 24h. 4-5 independent experiments per group. Box and whiskers, mean + min-max. A Mann-Whitney U test to compare curdlan and LPS stimulations or LPS and no PAMP showed no statistical significance ($p>0.01$). MNC: mononuclear cell. APC: CD3-depleted MNCs

4.3.3 Role of protein kinase C in naïve T cell production of IL-8

The major difference between the ability of PMA/ionomycin to trigger innate-like IL-8 production in naïve T cells compared to antigenic stimulation led us to investigate the signaling molecules involved in this response. Ionomycin increases calcium flux into the cell and phorbol 12-myristate 13-acetate (PMA) is a well-known activator of protein kinase C (PKC).
There are 15 isoforms of PKC in humans, divided in subfamilies based on second messenger requirements. The family of conventional (or classical) PKCs includes isoforms α, β1, β2 and γ that all require calcium, diacylglycerol (DAG) and a phospholipids for activation. PKC δ, ε, η and θ require DAG, but do not require calcium for activation (Lim et al. 2015). T cells express multiple isoforms of PKC that can modulate activation in these cells (Keenan et al. 1997). PKC-beta activation is required for production of IL-2 in naïve CD4 T cells and is found at comparable levels in cord blood and adult cells (Hassan & Reen 1997). PKC θ is specifically expressed in T cells (Isakov & Altman 2002), is crucial for TCR signaling (Isakov & Altman 2012), and is critical for the development of *in vivo* Th2 responses in mice (Marsland et al. 2004).

In attempting to dissect the role of each of these pathways on the production of IL-8 in naïve CD4 T cells, we found that ionomycin, PMA or even phytohemaglutinin (PHA, a lectin known to trigger T cell differentiation by cross-linking surface glycoproteins) alone resulted in lower IL-8 production compared to PMA/ionomycin (**Figure 4.5A**). Combining ionomycin with PHA or CD3/CD28 stimulation did not increase the production of IL-8 by neonates or adults. However, the combination of anti-CD3/28 with PMA induced the most potent IL-8 production in neonatal naïve CD4 T cells, both as a percentage of IL-8-producing cells (**Figure 4.5A**), and by mean fluorescent intensity (MFI) (**Figure 4.5B**) compared to anti-CD3/28 alone. These data

strongly suggest that PKC activation is required for production of IL-8 independent of the effect of TCR activation on calcium flux. The fact that the pan-PKC inhibitor (Sotrastaurin), which is known to inhibit PKCs α,β,δ,ε, η and θ, completely blocked PMA/ionomycin-induced IL-8 production, in a dose-dependent manner in these cells confirmed the involvement of PKCs (**Figure 4.5C**).

4.3.4 Phenotypic characterization of IL-8 producing naïve CD4 T cells

Here, we sought to better define the phenotype of IL-8-producing naïve CD4 T cells, using an unbiased exhaustive approach. Earlier reports (Gibbons et al. 2014) showed that these cells preferentially express CD38, a cyclic ADP ribose hydrolase glycoprotein and adhesion molecule. CD38 is an interesting molecule whose expression is tightly regulated in a subset of T cells and has been linked to disease progression in chronic lymphocytic leukemia (Deaglio et al. 2010; Zucchetto et al. 2009). CD38 is recruited to the immunological synapse in T cells with antigen stimulation (Muñoz et al. 2008) and regulates cytoplasmic Ca2+ in peripheral blood but not tissue resident T cells (Deaglio et al. 2001). CD38 is also present on B cells, NK cells, monocytes and DCs (Zilber et al. 2000; Lund et al. 1996; Frasca et al. 2006). A high level of CD38 expression on neonatal naïve CD4 T cells was first described by Clement et al. (Clement et al. 1990).

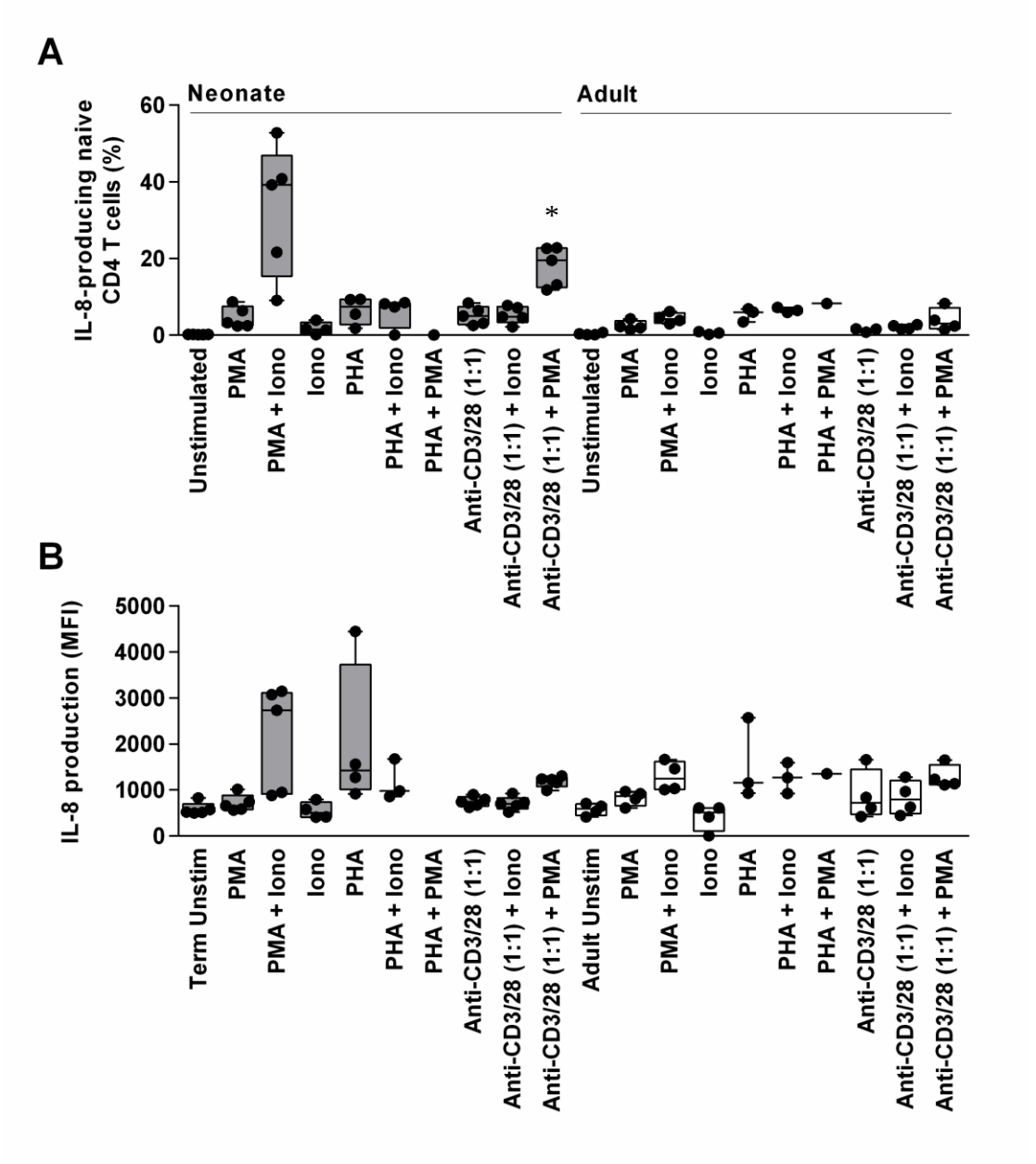


Figure 4.5 Production of IL-8 is PKC-dependent.

MNCs stimulated for 4 hours with different combinations of treatments. % of IL-8 produced **(A)** and MFI of IL-8 **(B)**. Represent mean + SEM of 4-5 independent experiments per treatment. Box and whiskers, mean and min to max. Manu-Whitney U two-tailed test showed statistical difference (* $p<0.01$) compared to anti-CD3/28 alone in the neonate (A). MNC: mononuclear cell. PMA: phorbol 12-myrisate 13-acetate. PHA: phytohemagglutinin. PKC: protein kinase C.

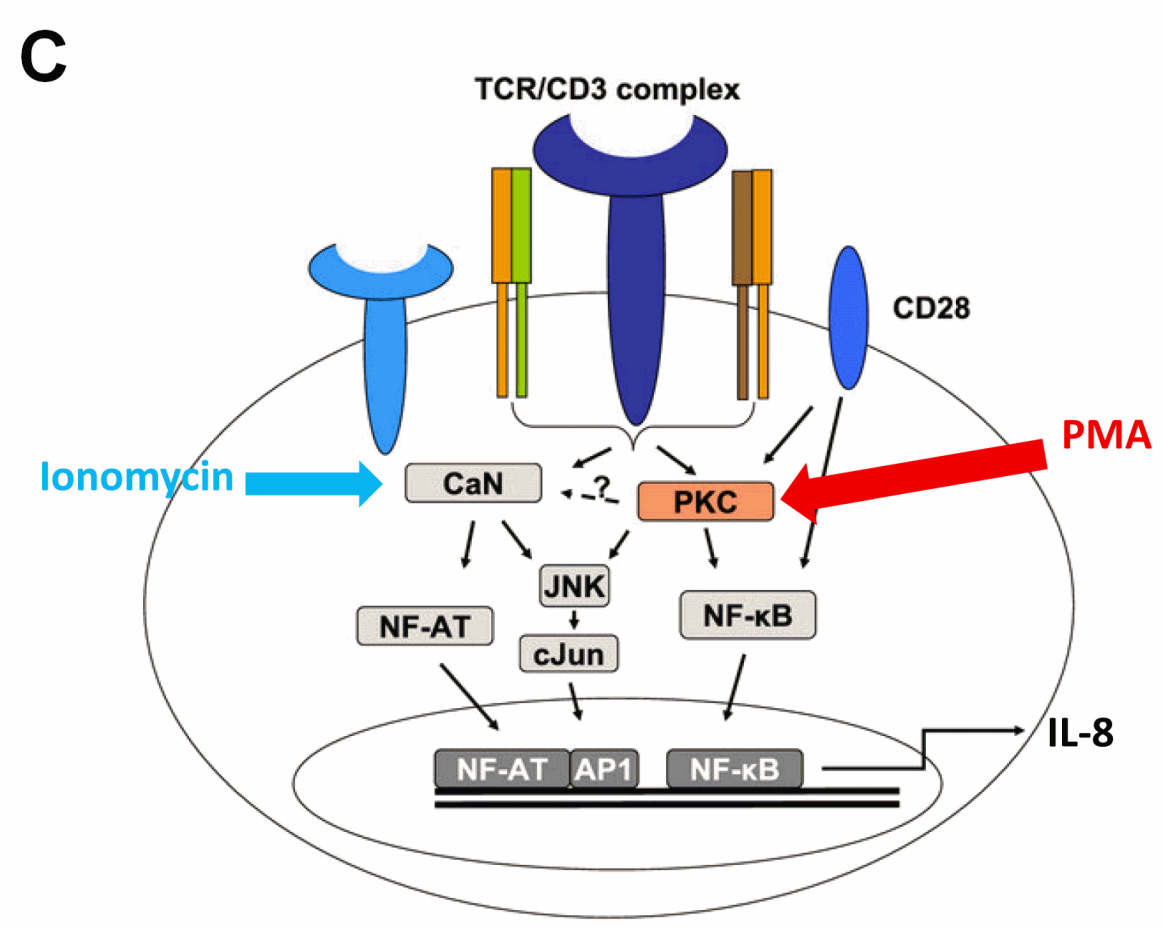


Figure 4.5 Production of IL-8 is PKC-dependent (continued)

(C) Mechanism of activation by PMA and Ionomycin on T cell signaling (adapted from Baan et al, 2012). CaN: calcinurin. PKC: protein kinase C.

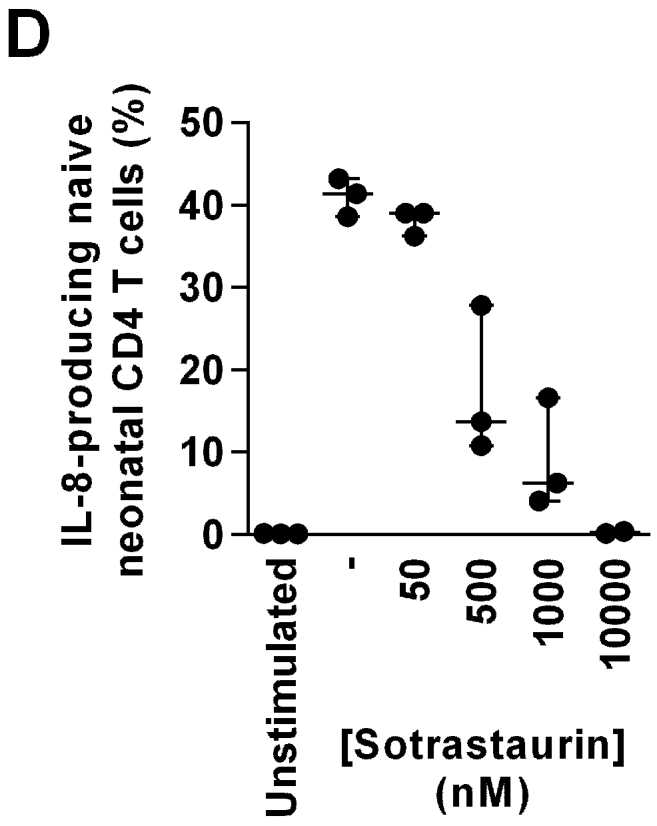


Figure 4.5 Production of IL-8 is PKC-dependent (continued)

(D) Pre-incubated with PKC inhibitor (Sotrastaurin) IL-8 production was measured by ICC after 4h PMA/Ionomycin. Represents 3 independent term cord blood samples. Box and whiskers, mean and min to max. MNC: mononuclear cell. PMA: phorbol 12-myrisate 13-acetate. PKC: protein kinase C.

We mined three genome-wide gene expression datasets, including two published, publicly available ones (Gibbons et al. 2014 and Tuomela et al. 2012) plus our own (Razzaghian et al, manuscript in preparation) to examine differential gene expression between naïve CD4 neonatal and adult T cells. These datasets were examined for differences in surface marker expression, hypothesizing that differential expression in these genes could correspond to the surface marker expression of IL-8 producing naïve CD4 T cells. Ideally, we would have sequences and analyzed IL-8+ and IL-8- naïve CD4 T cells in neonates and adults, but this was not possible at the time.

In the first dataset, Gibbons et al. (Gibbons et al. 2014) examined naïve CD4 T cells sorted based on the presence or absence of CD38 expression (CD38+ neonate, CD38+ adult and CD38- adult) and subsequently stimulated with PMA/ionomycin for 1.5 hours. In the second dataset, Tuomela et al. (Tuomela et al. 2012) isolated CD4 T cells by magnetic bead, positive-selection from cord blood, and stimulated these cells for 72 hours in either Th0- or Th17-polarizing conditions. In our own dataset, we sorted neonatal and adult naïve CD4 T cells (phenotype as described in methods section), which also included genome-wide methylation data using the 450K gene array (Razzaghian et al, manuscript in preparation). The characteristics of each of these datasets are summarized in **Table 4.3**.

Table 4.3 Gene expression data sets used to identify markers for neonatal naïve CD4 T cells

Dataset	Subject groups	Cell type and stimulation	Platform
Gibbons	Cord blood Adult blood	CD38+/- naïve CD4 T cells stimulated with PMA/I (1.5h)	Affimetrix GeneChip Human Genome 1.0 ST array
Tuomela	Cord blood	Magnet purified CD4 T cells in Th0 or Th17 polarizing conditions for 0-72h	Illumina Human HT-12 V3.0 expression beadchip
Razzaghian	Cord blood Adult blood	FACS purified naïve CD4 T cells *ex vivo*	Illumina Human HT-12 V4.0 expression beadchip

Integrating data from these three datasets, we selected eight differentially expressed (or differentially methylated) genes among surface markers, that may be enriched in neonatal compared to adult naïve CD4 T cells (**Table 4.4**). However, differential expression of these genes does not necessarily mean that the corresponding proteins or surface receptors are specific to the IL-8-producing T cells. An ideal approach would be to directly sort IL-8-producing cells versus non-IL-8 producing cells and sequence the two cell types.

To examine the presence of the receptors of interest on the cells we stained *ex vivo* MNCs from adults and neonates (**Figure 4.6A**). A high number of naïve CD4 T cells expressed CD31, CD38, CD52 and CD127 in both adults and neonates (**Figure 4.6B**). The other markers were not detected or only at very low levels. CD93 was found on the CD3-negative population using t-SNE analysis (**Figure 4.6C**).

To determine if the receptors are present on IL-8-producing cells we stimulated MNCs for 4 hours with PMA/ionomycin and gated on IL-8+ naïve CD4 T cells. Representative gating for two receptors is shown in **Figure 4.6D** (CD31 and CD38) to confirm that IL-8 is produced by naïve (RA+/RO-) cells. Subsequent analysis for IL-8+ cells was done on CD4+ CD3+ cells gating on all the CD45RA+/RO- (naïve) or CD45RA-/RO+ (memory) and subsequently gated with quadrants for the production of IL-8 in CD31+ or CD38+ cells. Cumulative data for the expression of selected markers show a similar profile of receptor presence on neonate and adult samples (**Figure 4.6E**). CD31 and CD38 are present on 50-80% of the IL-8+ cells, but CD52 and CD127 are absent or are downregulated with stimulation.

Table 4.4 List of genes of interest with cell tropism and function (from UniProt.org)

Surface markers	Cell type	Function	Data set
CD31 (PECAM-1)	Recent thymic emigrants (RTEs) and endothelial cells	Possibly involved in leukocyte migration, angiogenesis, and integrin activation.	Gibbons
CD38	Marker of cell activation of T/B/NK cells	Involved in cell adhesion, signal transduction, and regulates intracellular Ca^{2+} levels	Gibbons, Razzaghian
CD52	Mature lymphocytes. Cell bound and soluble forms	Highly negative molecule, possibly anti-adhesion	Razzaghian
CD93	Expressed on neutrophils, activated macrophages, a subset of dendritic cells and of natural killer cells.	Initially thought to be a receptor for C1q, but now is thought to instead be involved in intercellular adhesion and in the clearance of apoptotic cells.	Tuomela; Razzaghian
CD96 (TACTILE)	NK and T cells in late phase activation	Adhesion of activated NK and T cells in late phase of immune response and may have a role in antigen presentation. Upregulated on T cells after activation, peaking 6-9 days after stimulation.	Gibbons, Razzaghian
CD97	Hematopoietic stem and progenitor cells, immune cells, epithelial cells, muscle cells as well as their malignant counterparts	Promotes adhesion and migration to sites of inflammation	Razzaghian
CD127 (IL-7Rα)	Various cells including naïve and memory T cells	Blocking apoptosis during differentiation and activation of T cells	Tuomela, Razzaghian
IL-27Rα	Highly expressed on lymphoid tissues (spleen, lymph nodes, peripheral leukocytes). Expression of CD4+ >CD8+ T cells. Weak expression on monocytes and B cells	Essential for transcription of STAT1 and augments induction of Tbet and Th1 differentiation	Gibbons

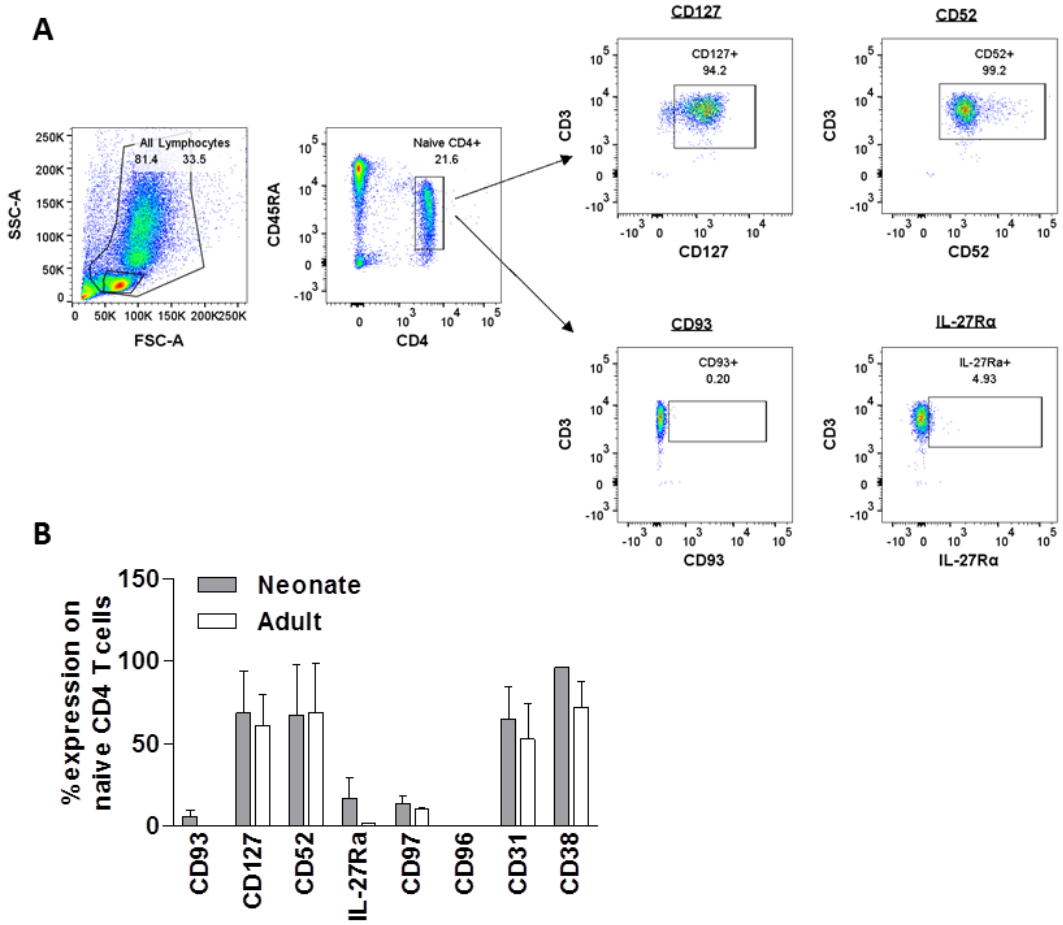


Figure 4.6 Receptors to identify IL-8 producing naïve CD4 T cells in neonates.

(A) Gating strategy for naïve CD4 T cells and receptors of interest. **(B)** *Ex vivo* expression of receptors of interest on naïve CD4 T cells from MNC culture. A Mann-Whitney U two-tailed test did not show any differences between neonates and adults. Represents mean +/- SEM. SEM: standard error of the mean.

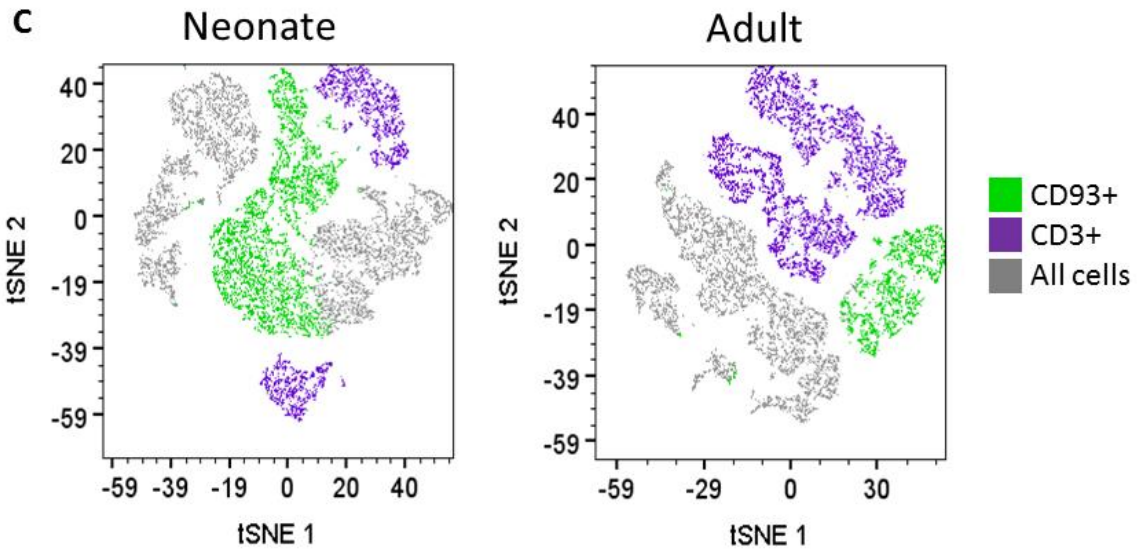


Figure 4.6 Receptors to identify IL-8 producing naïve CD4 T cells in neonates (continued)

(C) tSNE plots of term (left) and adult (right) MNCs show CD93 is no present on the CD3+ cells.

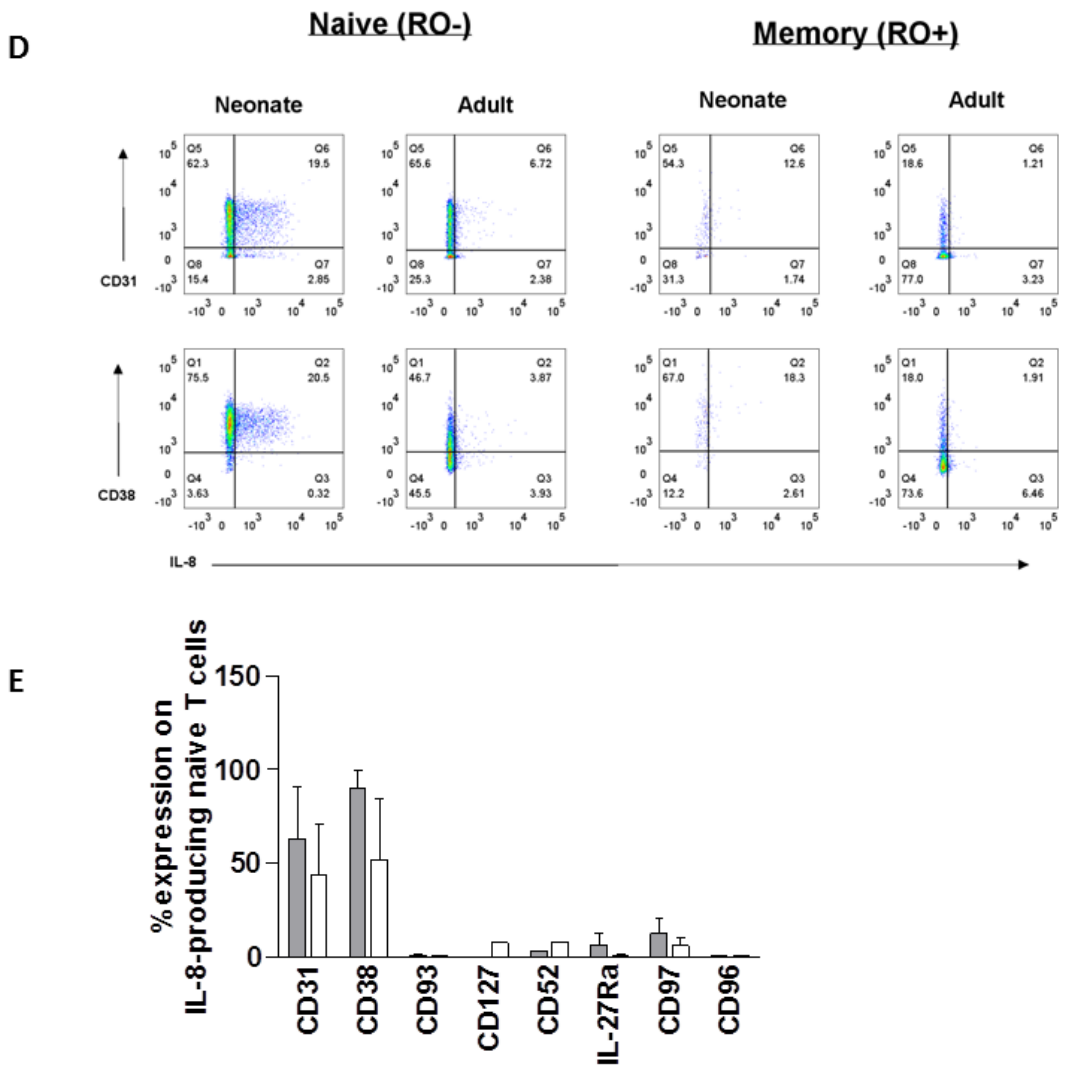


Figure 4.6 Receptors to identify IL-8 producing naïve CD4 T cells in neonates (continued).

(D) Representative markers (CD31 and CD38 showing production of IL-8 by naïve not memory CD4 T cells. **(E)** Expression of receptors on PMA/Iono (4h) stimulated IL-8 producing naive CD4 T cells. A Mann-Whitney U two-tailed test did not show any differences between neonates and adults. Represents mean +/- SEM. SEM: standard error of the mean.

During this work a publication examining the expression of CR1 (CD35) and CR2 (CD21), complement receptors, on RTEs suggested CR2 was a marker for the most naïve RTEs and these cells were producing IL-8 with stimulation (Pekalski et al., 2017). With this new information we decided to examine the expression of CR2 (CD21) on the IL-8-producing naïve CD4 T cells in cord blood as a possible marker for IL-8-produing cells. To examine the presence of CD21 on the IL-8+ T cells we first confirmed the presence of CD21 on the naïve T cell population ex vivo (**Figure 4.7A and B**). Thereafter, we stimulated cells with PMA/ionomycin and gated on the IL-8+ cells to determine the presence of CD31 and CD21 (**Figure 4.7C**). There were fewer IL-8-producing cells in the adult, but many expressed CD31+ (>68%) with less than half of the IL-8+ cells expressing CD21. For the neonates, 40% of the IL-8 producing cells expressed CD21, while approximately 50% of all IL-8+ cells were CD31+. This indicates that CD31 is a better marker for IL-8-producing cells, but it fails to capture the entire population in both adults and neonates. This leads me to conclude that CD21 is not a sufficient marker to identify the entire IL-8+ population of naïve CD4 T cells. In conclusion, according to our data only CD38-expressing, and to some extent CD31-expressing naïve CD4 T cells, were specifically enriched in IL-8-producing cells. Finally, as previous studies reported IL-8 expression in differentiated regulatory T cells (Himmel et al. 2011), we confirmed that IL-8 producing naïve T cells are also negative for CD25, found on activated and regulatory T cells. According to our data, none of the CD25-expressing T cells express IL-8 in an innate-like manner (**Figure 4.8**).

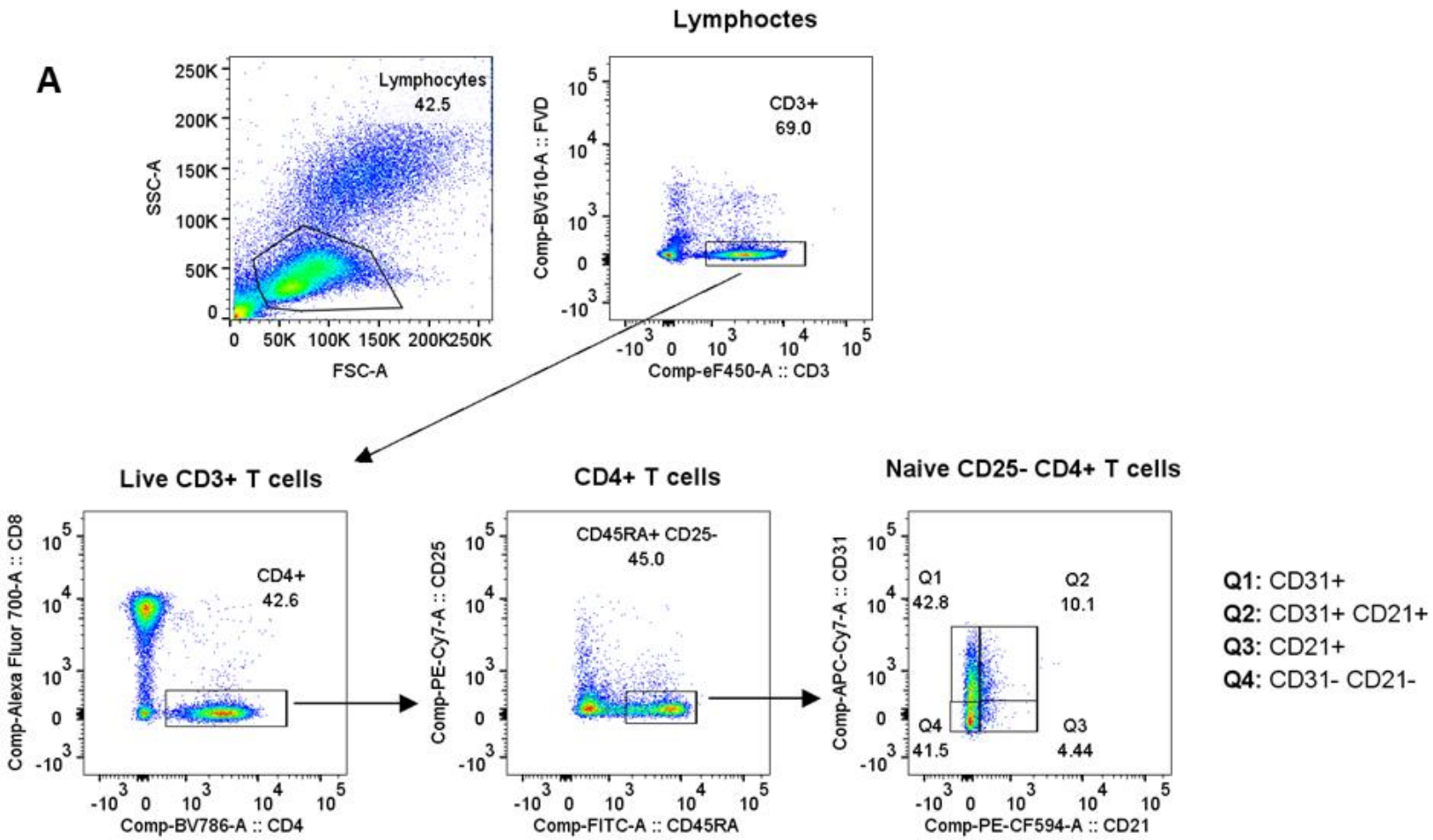


Figure 4.7 CR2 (CD21) is an insufficient receptor to identify IL-8 producing cells.

(A) Gating strategy to identify CD21+ and CD31+ naïve CD4 T cells.

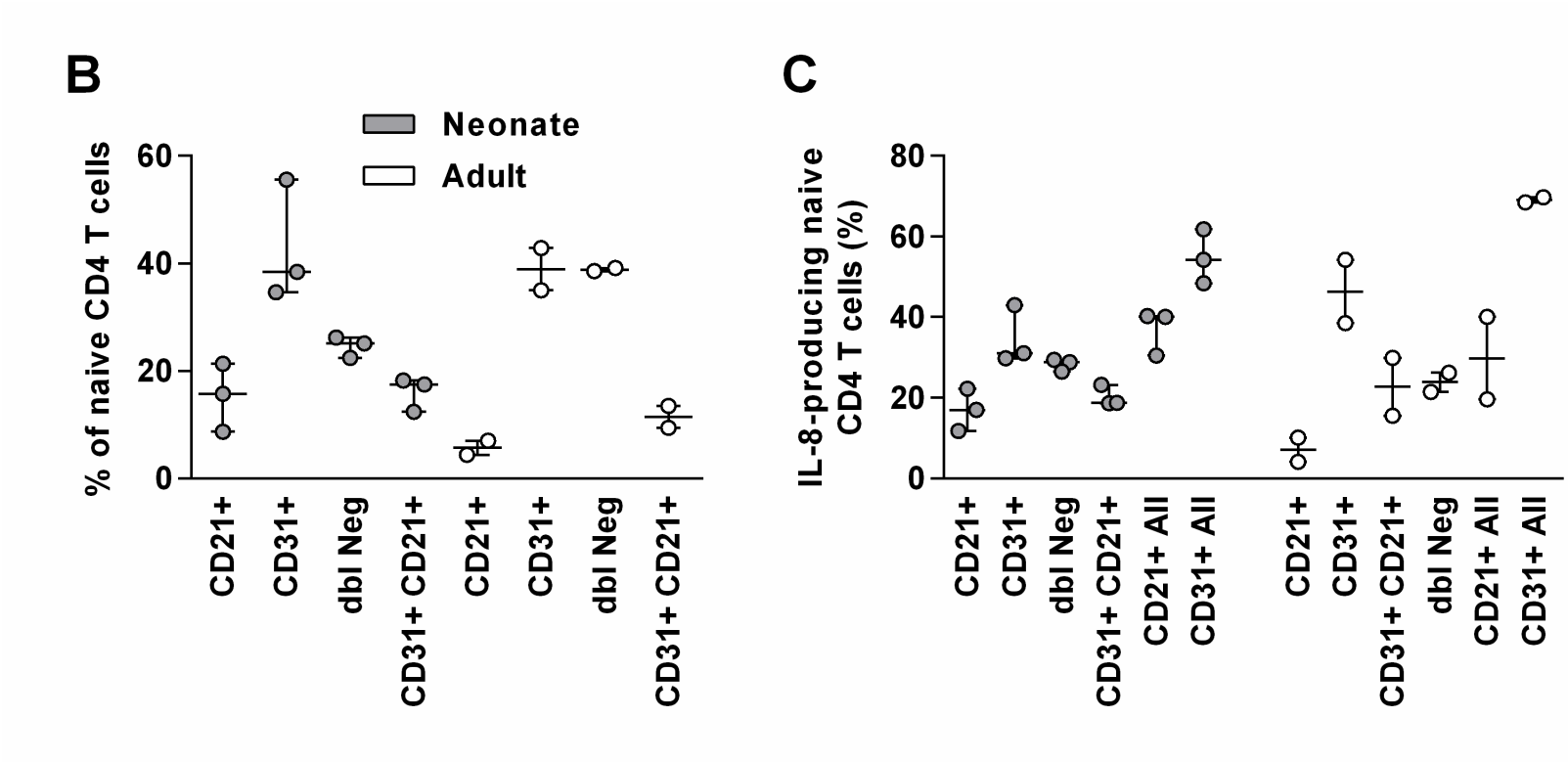


Figure 4.7 CR2 (CD21) is an insufficient receptor to identify IL-8 producing cells.

Presence of receptors on *ex vivo* **(B)** and PMA/ionomycin stimulated IL-8 producing **(C)** naïve CD4 T cells (3 neonates and 2 adults). Box and whiskers (mean + max to min).

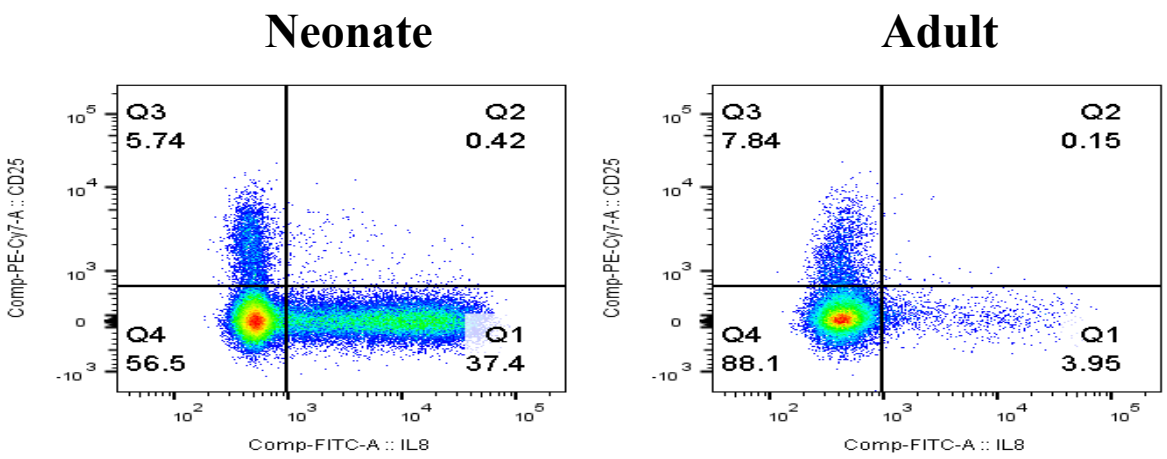


Figure 4.8 IL-8 producing naïve CD4 T cells lack CD25

MNCs stimulated with PMA/ionomycin (4hr) and examined for IL-8 production by ICC. Representative figures gated on naïve CD4 T cells for neonate and adult.

4.3.5 Chemotactic ability of neutrophils to IL-8

Finally, to begin investigating the putative effect of the IL-8 secreted by naïve CD4 T cells on neutrophils, we used an established neutrophil transmigration assay (Himmel et al. 2011). In this assay, neutrophils migrate toward human recombinant IL-8 (rhIL-8) and this migration can be blocked with an anti-IL-8 antibody (Himmel et al. 2011). We determined that 2,000-5,000 pg/ml of IL-8 would be required to detect migration of neutrophils. Several attempts were made to concentrate supernatants from TCR-activated naïve CD4 T cells, including up to $4x10^6$ cells. Consequently, the level of IL-8 secreted by these T cells was insufficient to detect neutrophil chemotaxis in this transmigration assay (**Figure 4.9A and data not shown**). Nonetheless, neonatal and adult neutrophils were found to migrate to rhIL-8 similarly in this assay (**Figure 4.9B**).

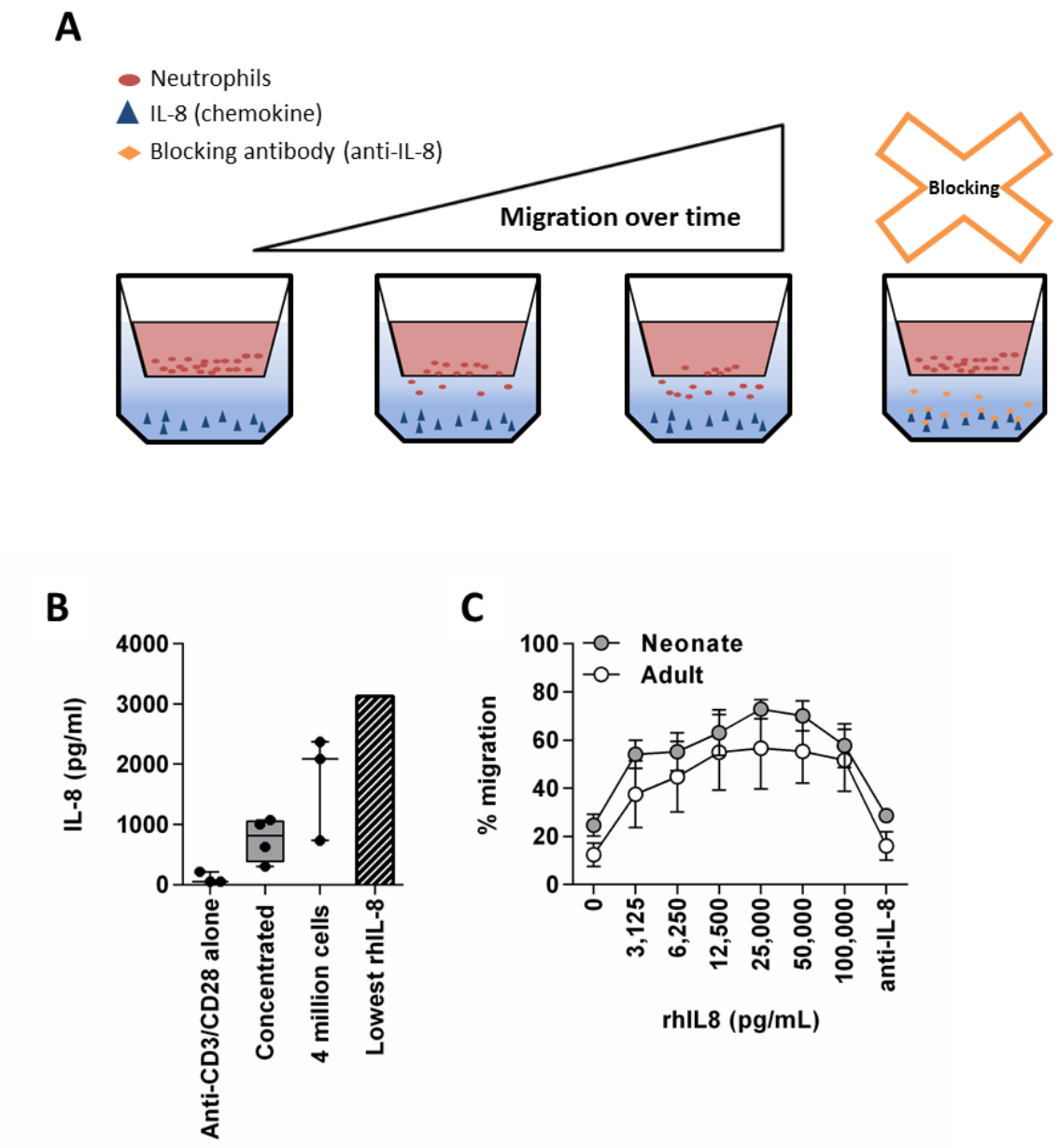


Figure 4.9 Neonatal neutrophil migration in response to IL-8.

(A) Experimental method for neutrophil migration assay. **(B)** Amount of IL-8 acquired from different stimulations from neonatal naïve CD4 T cells compared to lowest rhIL-8 used in migration assay's standard curve (3,125pg/ml). 100,000 purified naïve CD4 T cells were stimulated with anti-CD3/28 for 24 hours (anti-CD3/CD28). Concentrated sample represents 6+ wells concentrated (for proteins < 30kDa) and 4 x 10^6 cells naïve CD4 T cell. Represents mean + SEM of 3-4 independent experiments. **(C)** Percentage of migration for neonates and adults. Represents mean +/- SEM of 4 independent experiments. SEM: standard error of the mean.

4.4 Discussion

In this study we examined the production of IL-8 by naïve T cells in neonates and adults to better understand the physiological context and its purpose in the early immune response. The innate-like characteristic of these IL-8-producing cells is their production of a chemokine prior to differentiation. The rapid production of IL-8 is of interest for the immune response of neonates as it occurs by undifferentiated conventional T cells previously believed to be lacking effector functions. We speculate that the abundance of IL-8-producing naïve CD4 T cells in the neonate could serve to attract neutrophils at the site of infection at a critical developmental stage when more rapid recall immunological responses are largely absent.

To further investigate the physiological production of IL-8, we designed an assay to examine the effect of APCs on the production of IL-8 by naïve CD4 T cells. This experiment attempted to determine if there was an intrinsic effect of neonatal APCs and whether they could increase the amount of IL-8 produced by naïve CD4 T cells. In the APC-T cell crossover experiments we clarified that it was not the origin of the APCs, but the activation of the APCs that influenced the amount of IL-8 produced. Though we cannot entirely exclude age-related effects on the ability of APCs to enhance production of IL-8 by naïve CD4 T cells, due to small sample size, these effects are likely to be marginal. A limitation of our approach is that we were only able to study circulating APCs and more detailed studies are required to determine the role of each type of APCs that may be present in tissues other than blood. Neutrophils are able to upregulate CD80 and CD86 receptors (Breedveld et al. 2017), which stimulate the naïve T cell if they encounter each other. The ability of neutrophils to act as APCs has been shown in memory T cells, but not in naïve T cells (Vono et al. 2017). Whether neutrophils can activate and induce IL-8 production in naïve CD4 T cells remains to be examined. Potentially, the T cell-produced

IL-8 could have a paracrine effect on these neutrophils by enhancing their cytokine production. Also, the co-stimulatory actions of the APCs led to the activation of the T cell and the downstream production of IL-8. Further investigation of the cytokine milieu produced by the APCs and their upregulation of the co-stimulatory receptors, such as CD80/86, could elucidate the physiological inducers of IL-8 in CD4 T cells.

Classically, IL-8 is regarded as a chemokine with potent neutrophil-recruiting capacity. Hence, we undertook studies to assess the potential of naïve CD4 T cell-secreted IL-8 to induce neutrophil migration. Concentration of naïve T cell supernatant and culturing of four million naïve CD4 T cells was insufficient to significantly increase the concentration of IL-8 in supernatants to be used for the migration assay, due to the relatively low amount of IL-8 produced by naïve CD4 T cells. The inability to detect neutrophil migration to IL-8 above background levels of migration underlines the conclusion that the amount of IL-8 produced by naïve CD4 T cells is much lower than the amount produced by monocytes (> 20,000 pg/ml for unstimulated 24hr monocyte supernatants) (data not shown). we hypothesize that the activity of naïve CD4+ T cell-derived IL-8 is largely localized *in vivo*, rather than the strong systemic recruitment of neutrophils out of circulation to a site of infection in the manner of tissue resident macrophages and endothelial cells. Naïve CD4 T cell-derived IL-8 may have other effects on neutrophils, such as inducing degranulation (Topham et al. 1998). Alternatively, the function of the naïve CD4 T cell-derived IL-8 may be confined to specific tissues. The amount secreted may be sufficient to recruit cells present in secondary lymphoid organs (e.g. a lymph node) where circulating neutrophils may travel. Perhaps in lymph nodes naïve T cells recruit neutrophils if there is a local infection or induce neutrophils to secrete cytokines to provide the third signal required for naïve CD4 T cells to successfully differentiate into a CD4 T helper cell.

Production of IL-8 by naïve CD4 T cells may serve to recruit other cells in addition to neutrophils. Data suggest that IL-8 can also be a chemoattractant for γδ T cells (Gibbons et al. 2009) and other cells, such as T cells and mast cells (Lippert et al. 2004), which express the receptors for IL-8 (CXCR1 and CXCR2). Of particular interest, CXCR1 is found on CD4 T cells (Lippert et al. 2004; Evgenievich et al. 2017) suggesting a potential autocrine effect of the secreted IL-8 from the naïve CD4 T cells.

In the future, to test the ability of the neutrophils to migrate towards IL-8 produced by other immune cells we could use supernatant from MNCs, but this will include a significant proportion produced by monocytes. To further elucidate the role of IL-8 on the neutrophils we could examine the activation of neutrophils and their other cellular functions when co-cultured with naïve CD4 T cell supernatant. The low concentration of IL-8 produced by the T cells is insufficient to induce migration in my assay, but may have other effects on neutrophils, such as inducing degranulation and ROS production.

It is important to consider the role of low-density neutrophils in the *in vivo* response to the IL-8 produced by the naïve T cells. Umbilical cord blood has higher levels of low-density neutrophils which are found in the MNC layer of a density separation (Rosales 2018). These low-density neutrophils are immature neutrophils and granulocytic myeloid-derived suppressor cells. To fully understand the innate-like production of IL-8 in T cells it will be important to further characterize the function of immature neutrophils, which are abundant in cord blood (personal communication, Amina Kariminia, Kirk Schultz's lab). In our examination of the function of IL-8 we only assessed the mature neutrophils of both neonates and adults. If the proportion of immature neutrophils in cord blood is significant and they are responsive to IL-8, then it would also be important to establish the migratory capacity of these cells.

While we have not proven that PKC is the only inducer of IL-8 in T cells, we have shown that the activation of PKC in conjunction with the calcium flux induced by an ionophore or TCR activation does lead to the differential production of IL-8 in neonates and adults. The next step would be to measure the PKC activity with inhibitors to determine which PKC isoform is involved in the signaling leading to IL-8 production. Also, a direct measurement of PKC within the naïve CD4 T cells may identify qualitative or quantitative differences in these T cells. This could provide information about the state of the naïve T cells. If there are different amounts of PKC present in the IL-8-producing cells this could explain why PMA stimulation acts to produce more IL-8 in neonates. If there are comparable amounts of PKC we should consider the activity state of the PKC in these cells. PKC is phosphorylated during TCR/CD28 signaling and the ability/speed at which this happens could explain the differences we see.

Two recent papers by different groups have investigated the role of these IL-8-producing cells. Adrian Hayday's group examined the ability of neonatal and adult IL-8+ naïve CD4 T cells to differentiate into Th1 cells and evaluated their ability to produce IL-8 and IFN-γ after 22-52 days in culture (Das et al. 2017). They found that upon re-stimulation with PMA/I most of these cells were able to produce IFN-γ (Das et al. 2017). Their conclusion was that IL-8-producing CD4 T cells represent precursors of Th1 cells and this is an advantage in neonates. However, they did not compare this capacity to naïve T cells from adults and neither did they show any data for the IL-8 negative cells to compare their ability to become IFN-γ-producing Th1-type cells, making it difficult to determine if this property represents a unique development stage.

In our attempt to understand the production of IL-8 and its function in the innate-like response in naïve CD4 T cells, we have confirmed the 'early' naïve phenotype of these CD4 T cells (van den Broek et al. 2016; Gibbons et al. 2014). CD31 and CD38 are present on a large

proportion of cells recently egressed from the thymus as well as IL-8 producing cells, but neither of these markers are unique to IL-8 producing cells. During our investigation others were also pursuing this novel innate-like function in naïve CD4 T cells. Van den Broek and colleagues investigated the function of naïve CD4 T cell compartment in thymectomized children (van den Broek et al. 2017). Those lacking part of their thymus had a reduced number of CD31+ naïve CD4 T cells (RTEs) and had fewer IL-8+ cells compared to age-matched healthy controls. They concluded that it is the CD31+ naïve T cells that have recently emigrated from the thymus which are producing IL-8 (van den Broek et al. 2016). This supports our findings that very early, naïve CD4 T cells produce IL-8. Crespo and colleagues' recent work supports our findings that these IL-8-producing cells are not Tregs as they do not express FoxP3 or CD25, and they also demonstrate a lack of activation receptors such as CD69, CD122, CD154 and CXCR3 (Crespo et al. 2018).

Our use of a single clone for our flow cytometry antibodies does not represent an extensive investigation of these markers on naïve CD4 T cells. For example, CD93 was found on monocytes (CD3- CD4- CD45RA-, monocyte position for FSC/SSC), but not our naïve CD4 T cells. However, this could indicate that the flow cytometry antibody does not adequately bind to the CD93 on T cells. Research performed by Hidetoshi Inoko's group to identify the presence of CD93 on immune cells in cord blood illustrates the limitations of using only commercially available antibodies (Ikewaki et al. 2010). They were able to identify CD93 on adult and cord blood monocytes and granulocytes using four antibodies (3 commercially available and 1 made in-house). The CD93 receptor was only detected on lymphocytes by their in-house designed antibody (clone: mNI-11) and was absent on adult lymphocytes for all antibodies (Ikewaki et al.

2010). Interestingly, their CD93 marker only worked for cord blood lymphocytes further emphasizing the limitations of antibody science.

The discovery of the production of IL-8 by this subset of T cells in an ‘effector-like’ manner raises the question as to why these cells might expend energy to do this. These IL-8 producing naïve T cells may have a role in immune responses early in the life of a neonate that requires further investigation. The neonatal immune system relies heavily on the innate immune cells and their PRR responses prior to the development of immunological memory provided by the adaptive immune response. T cell research has focused on the number and type of T cells and their ability to perform traditional T helper functions and our knowledge about the function of neonatal T cells has increased in the last few years. As our understanding of the human immune system has shown that our restrictive definition and categorization of T cells limits our understanding of the synergistic properties of the immune system and this will change as more functional human studies are performed.

Chapter 5: Discussion

When I first started this thesis, the premature neonate's immune system was described in the literature as diminished and impaired compared to the term baby. Since then I and others have shown that the immune system of premature neonates is not deficient but represents a developmentally-regulated stage that that is unprepared for life outside the uterus. At birth, new requirements are imposed on the immune system as the preterm encounters colonizing microbes and pathogens. This thesis has examined the ability of premature infants to respond to infections at a cellular level. The immune response of neonates has evolved to enhance survival. The early entry into the world of preterm babies interrupts the timing of important developmental events. This thesis contributes to our understanding of the immune system of premature neonates during the postnatal period examining innate immune cell responses (chapter 2), whole blood transcriptomic profiles (chapter 3) and innate-like production of IL-8 by CD4 T cells in neonates (chapter 4). The poor response to PRR stimulation in the preterm should not be seen as merely a failure to respond, but as an attenuation of the immune response. A reduction of inflammatory responses is helpful in the developing fetus in the comparatively sterile *in utero* environment, to prevent rejection of maternal cells and damage to developing organs and tissues. However, when the fetus exits this protected environment, as a premature neonate, it must combat commensal and pathogenic microbes. In its attenuated immunological state the premature neonate is unable to mount an adequate immune response to infections. The work in this thesis has investigated the immune development of the premature neonate to describe and understand the changes of the immune response after birth.

5.1 Interpretation

5.1.1 Chapter 2

Research conducted prior to this work investigated the expression of TLR receptor expression (TLR2 and TLR4) in preterms during the first few months after birth and observed an increase in receptor expression within the first couple of weeks (Shen et al. 2013). The increased receptor expression did not correlate with increased pro-inflammatory cytokine production upon TLR4 stimulation compared to term newborns during the first 5 days after birth (Shen et al. 2013). Strunk et al (2012) examined the immune responses to *Staphylococcus epidermidis*, a leading cause of late-onset sepsis in preterm babies, in mononuclear cells extracted from umbilical cord blood. They observed that the TLR expression, phagocytosis and intracellular killing capacity of neonatal monocytes (<30 weeks to term) were comparable, but there was gestational age-dependent maturation of intracellular cytokines (IL-1β, IL-6, IL-8, and TNF-α) (Strunk et al. 2012). Other work from this group showed that preterm neutrophils and monocytes were capable of phagocytosis, but there were fewer of these cells in preterms than terms (Prosser et al. 2013). Azizia et al. also utilized cord blood to examine TLR4 responses, using whole blood cytokine response as a surrogate for cytokine production in monocytes. They observed that preterms secreted less TNF-α than term newborns (Azizia et al. 2012). All of these previous reports indicated that our study on the postnatal blood of premature neonates adds a significant dimension to the understanding of the preterm immune system during the vulnerable postnatal period. Our examination of babies on the far ends of gestational age (<30 and >37 weeks GA) using cord blood as well as peripheral blood collected up to 42 days postnatally, covers a wider range of postnatal development. With this cohort we have been able to confirm

the reduced production of pro-inflammatory cytokines in response to TLR4 stimulation at birth, but have also demonstrated an increase in cytokine production in the postnatal period.

In chapter 2 of my thesis, I have examined how PRR responses mature using TLR4 and TLR 7/8 stimulation to examine extracellular bacterial and intracellular viral responses, respectively. To explore immune maturation, we measured the ability of preterm neonates to produce pro-inflammatory and anti-inflammatory cytokines, which are produced by innate immune cells, in a normal response to infection. The innate immune response is the primary mechanism of protection at this age and this was the reason we examined the immune response to TLR-stimulation. I have shown that there is some increase in immune responses, as denoted by the increased production of pro-inflammatory cytokines. My work also examined the single-cell cytokine production showing that innate immune cells were present in preterms at comparable numbers to terms and adults, but fewer monocytes were producing IL-6 and TNF-α. These data suggest that the immune cells are present, but they may not be fully developed or functional in the preterm during the postnatal period. This was an important study characterizing the postnatal development of the preterm immune responses.

5.1.2 Chapter 3

Our preterm postnatal samples are a snapshot into the function of these immune cells at the time of sampling. The readout of cytokine production is only one measure of a cell's reaction to stimulation. Before proteins are made the cell must transcribe its DNA into mRNA, which is then translated into the requested protein. Measuring the presence of mRNA transcripts at any given time can indicate the cells' current state and future actions. To further characterize the immune responses of our postnatal preterm cohort, I examined the baseline transcriptome of cells

in whole blood for a subset of preterms (chapter 3). Qualitative and quantitative changes in preterm and term newborns' cell composition have been shown to follow a similar pattern during the neonatal period (Xanthou 1970) and white blood cell counts are regularly measured today in the NICU. While this can provide information about the absolute numbers of known immune cells types, it does not detect progenitor and hematopoietic cells. Early studies on fetal blood cell composition (< 22 weeks) identified a high level of circulating hematopoietic progenitor cells (Linch et al. 1982). The enrichment in HSC and erythroid progenitor cells in premature neonates at birth and subsequent decreases during the postnatal period follows the development of immune cells. This study characterizes premature neonates as being in an immunologically immature state due to an enrichment of progenitor cell-types in addition to their attenuated immune function described in chapter 2. This work illustrates the need for research into the role of immature, progenitor cell-types in the function of the preterm immune response and their association with a risk of sepsis.

5.1.3 Chapter 4

Chapter 4 was an investigation into the phenotype and TCR-induced production of the chemokine IL-8 by neonatal naïve CD4 T cells. During these preliminary studies other groups also identified these cells as being predominantly CD31+ RTE cells (van den Broek et al. 2016; Das et al. 2017). The purpose of the production of IL-8 is hypothesized by Das and colleagues to be related to their ability to more rapidly become Th1 cells, evidenced by their production of IFN-γ after differentiation (Das et al. 2017). This is demonstrated in both adults and cord blood, but they only sorted CD4 T cells, and therefore could have captured non-naïve CD4 T cells in the adults or memory T cells that have reverted to expressing CD45RA (terminally differentiated T

cells). Their examination of extensively cloned and expanded cells is less relevant to the immediate production of IL-8 by naïve CD4 T cells. It may be that these cells have other capabilities upon differentiation, but the production and secretion of IL-8 is metabolically expensive for the cell and I believe it should have a more immediate purpose in the immune response. In studying this subset of T cells, I have determined that they are RTE cells that are more abundant in neonates due to their age and thymic output. Future research on these cells should focus on physiologically relevant induction of IL-8 and its potential role in early immune responses in neonates.

5.2 Significance of this work

The study of the immune response of preterm babies was initially to better understand the fetal development of the immune system (Gasparoni et al. 2003), but as a disruption of *in utero* development we must understand the unique immunological state they exist in and the effect of this interruption. The immune system of the fetus must prevent rejection by the mother and limit excessive inflammation in developing organs and tissues of the fetus. At birth a newborn encounters new sources of infection and inflammation and a failure of the immune system to respond becomes a disadvantage when it prevents an adequate response to an infection after birth. Premature infants may share many immunological characteristics with the fetus that are beneficial *in utero*, while at the same time the premature newborn is faced with the same environmental cues experienced by the term neonate. The preterm immune system's attempt to reconcile these two paradigms is the junction where my research has the greatest contribution. The major objective for the entire work within this thesis was to characterize the immune system of preterm neonates during the postnatal period and understand the functional capacities of their

immune cells. There are two areas where this information will add to the knowledge of the field; immunological reasons for the susceptibility to infections in the NICU population and how naïve CD4 T cells (RTEs) may have a role in the early immune response.

5.2.1 Chapter 2

Chapter 2 of this thesis examined the immune response of uninfected preterms in the postnatal period. We have shown that innate responses of the premature infant remain attenuated during the postnatal period and this might account for their continued susceptibility to infections. This concept had never been demonstrated on postnatal blood and is crucial to support the premise that immaturity of the preterm immune system plays an important role in their susceptibility to infections. The ability to secrete the appropriate cytokines to direct immune cells is important in the early immune response and is the basis for the differentiation of CD4 T cells and the development of effector and memory cells characteristic of efficient responses in adulthood. The developmental regulation of innate immune responses in premature neonates raises more questions about the controls and regulatory mechanisms for the observed attenuated immune responses.

5.2.2 Chapter 3

The data in Chapter 3 have shown that there is more to the susceptibility of preterm neonates to infections than just a failure to produce cytokines. Previous transcriptomic studies on preterm babies have examined sick vs. healthy babies of various gestational ages (Wynn et al. 2015; Cernada et al. 2014; Smith et al. 2014). Each preterm population is heterogeneous and there are multiple factors that could be confounding the sepsis profile identified in these studies.

In Chapter 3, our nested, case-control study matched preterm babies who had a subsequent sepsis event with those who did not. By sequencing whole blood from neonates across gestational and postnatal ages we have identified an enrichment of progenitor cell types in preterm cord blood and during the early postnatal period in preterms. This work has unveiled developmental changes in cell proportions in the baseline transcriptome of whole blood that occur during the postnatal period. Interestingly, the immature, progenitor profile in cord blood was shown in this work to be associated with a risk of sepsis if present during the postnatal period.

The data generated from our entire cohort will help to clarify this immature phenotype and assess more precisely how it changes over the postnatal period. The presence of immature, HSC and progenitor erythroid cell types, based on identified surface marker combinations (Novershtern et al. 2011), could be a means of identifying preterms at greater risk of developing sepsis. Identifying preterm babies with an increased risk of infection could encourage extra monitoring in the NICU for signs of infection. Currently regular white blood cell counts performed in hospitals do not identify progenitor cells, but future development of a PCR assay for a pre-determined set of genes or cell-types could use a small sample of whole blood to identify at risk preterms. The use of whole blood to examine both the immune responses and transcriptome illustrates that future studies on preterm cohorts can be done with a minimum volume of blood, while providing extensive information about the immune system.

5.2.3 Chapter 4

My investigation into the production of IL-8 by naïve CD4 T cells clarifies and corroborates emerging information that the population of naïve CD4 T cells producing IL-8 is not unique to neonates, but is instead a very early, mature CD4 T cell recently exited from the thymus. The frequency of these cells is greater in neonates as fewer of their naïve CD4 T cells have undergone homeostatic proliferation in the periphery, continuing to possess CD31 and CD38, which are the most useful receptors for the identification of the majority of the IL-8 producing cells. However, I have not yet determined the physiological reason for the production of this IL-8 or its purpose during early immune responses. A recent review remarks that these RTEs possess a unique role in the T cell family and are not flawed or immature conventional T cells (Cunningham et al. 2018) supporting the need for further research into these IL-8-producing cells.

5.3 Limitations

For ethical reasons, I was restricted in the volume of blood available from the postnatal preterms. Therefore, I examined a whole blood model instead of purified immune cell subsets in preterm neonates. The use of this model provides a broad understanding of what will happen if an infection enters the bloodstream. However, we fail to capture the antigen presentation to lymphoid cells in the lymph nodes and the subsequent differentiation and development of effector and memory T cell responses. We examined pathogen recognition of the innate immune cells in this whole blood model. The use of *ex vivo* stimulation of whole blood is a relatively physiological method to examine the immune response in the bloodstream; however, pathogenic ligands are utilized at supra-physiological levels of purified proteins expressed by pathogens. It

would be more physiological to use whole pathogens, as more than one PRR is likely to be stimulated. It is an imperfect system due to the absence of cell migration out of circulation to the site of infection, the migration of APCs to the secondary lymphoid tissues and their interaction with T cells. These are important events that could enhance our understanding of the preterm immune responses.

One of the great limitations in all measurements of cytokine production is that one relies on *a priori* knowledge of what to examine. Though we can perform a cytokine detection assay we could not investigate the presence of uncommon or unknown proteins. Therefore, other methods, such as RNA sequencing, could indicate if a cytokine's mRNA is present or increases in abundance upon certain stimulations. However, caution must be taken to avoid assuming that the presence of transcript is correlated with the production and secretion of a protein.

There are limitations to studying the immune system of premature infants. The first is the many causes of preterm birth include maternal and perinatal infections, which could be modeled in an animal model, but would be representative of only a subset of all human preterm births as most premature births are idiopathic. Secondly, there can be significant human variability in immune cell proportions and immune responses even in healthy adult populations (Brodin & Davis 2017), and premature infants have confounding factors that can influence their response to our *in vitro* experiments. There are perinatal drugs, such as dexamethasone and corticosteroids, administered to improve the survival of the premature infants, which are known to suppress immune responses. Morphine sulfate is given to neonates to reduce pain in neonates placed on mechanical ventilation, and has been shown to impair cord blood neutrophil chemotaxis (Yossuck et al. 2008). Another confounder is a previous infection or sepsis episode, including intra-uterine infections, a cause of preterm birth (Azizia et al. 2012) and sub-clinical infections,

which could alter the ability of their immune system to respond to subsequent infections causing immune paralysis, the inability to mount an immune response to a secondary stimulus. However, it has also been shown that EOS does not increase the risk of developing sepsis later (Wynn et al. 2013). All these factors can influence the immune function of the premature neonate at any given sampling point and influencing the *ex vivo* results. Without fully understanding the etiology of premature birth it is difficult to understand its effect on immune system development in humans.

5.4 Future directions

The ability to study the immune response of preterm infants in whole blood using minimal blood volumes is very important for the field. I have shown that even with small blood volumes it is possible to investigate postnatal changes of the immune system. Our postnatal study was a snapshot of the immune response of preterms at a single point in time and future studies should develop large, longitudinal cohorts, with multiple sampling, using "omics" to study the preterm population. Using whole blood transcriptomics, I identified an overrepresentation of progenitor cells in preterm cord blood. Ideally, we would examine the phenotype and function of these immune cells, and with the development of technologies such as CyToF and high-parameter flow cytometry systems, more variables can be examined on whole blood to determine the function of distinct subsets of immune cells with limited sample material. CyToF and flow cytometry can only be used to assess *a priori* defined proteins of interest and may therefore be unsuitable for identifying novel, immature fetal-specific immune cell subsets. Single cell RNA-sequencing can overcome part of this limitation and would be an ideal method to corroborate our findings in a future preterm cohort.

A more immediate task is completing the RNA-sequencing of the entire postnatal preterm cohort from chapter 2. This would be an invaluable data set of the changes in the transcriptomic profile along a spectrum of gestation and postnatal ages in uninfected preterm neonates and with *ex vivo* bacterial and viral stimulations (LPS and R848, respectively). By characterizing both the baseline transcriptome of a subject and the changes seen during TLR stimulation this could identify transcripts that are upregulated during an infection regardless of gestational or postnatal age. This information could be used to provide a better and faster diagnostic tool to confirm sepsis in neonates. By removing the influence of the profile that is representative of the baby's age and using their stimulated profile it might be possible to detect the changes occurring during the first 24h after infection, a window where clinical signs may be unclear. In the NICU clinicians are weary of missing a bloodstream infection and prophylactically prescribe antibiotics to babies at risk, premature birth itself being a major risk. The speed at which an infant can succumb to sepsis means that waiting for culture results prior to administering antibiotics could be fatal. The presentation of sepsis in preterm babies is often through clinical signs of deterioration and there is currently no consensus definition of preterm sepsis (Wynn 2016).

5.5 Conclusion

The major goal of researching the immune function of neonates is to understand how their immune system works and how we can manipulate or enhance the immune response. By understanding how it works there is hope that one day we will develop vaccines that activate immune cells in the premature neonate to produce long lasting memory T and B cells early after birth. The work in this thesis enhances our understanding of age-specific characteristics of the immune system of neonates, especially those born prematurely. Since some vaccinations are less effective at generating long term immunity in this group, a better understanding of the 'immune state' of premature neonates will help us determine what adjuvants could direct the immune system to produce the best immunological response and memory cells to protect neonates in the postnatal period. Identifying the complex features and functions of the immune system on encountering a pathogen is not the sole objective of immunology research. Each and every researcher hopes that their contribution to the field will enhance the lives of patients. Ideally, this work will contribute to the improved identification of infections in preterm neonates.

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