Development of the human innate immune system throughout gestation

by

Ashish Arunkumar Sharma

B.Sc., The University of British Columbia, 2009

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

January 2016

© Ashish Arunkumar Sharma, 2016
Abstract

Almost four million neonates die of infectious and prematurity-related causes across the world annually. The innate immune system provides evolutionarily ancient first-line protection against most microbial pathogens. In contrast, the adaptive immune system is capable of developing an immunological memory that provides enhanced protection in vertebrates. A mechanistic understanding of the maturation of the human preterm neonatal immune system is lacking and this may limit our ability to develop more age-appropriate immunological therapies. In Chapter 1, I analyzed prototypic anti-microbial receptor responses in a clinically well-characterized cohort of premature infants to provide evidence that responses develop asynchronously and follow a developmental pattern that is independent of perinatal factors linked to the premature delivery. In Chapter 2, I dissected molecular rate-limiting steps along a major inflammatory pathway leading to the production of the interleukin-1β (IL-1β) cytokine, across development. The IL-1β cytokine is particularly important as its production serves to amplify innate immune responses. I show that premature neonates born early in the third trimester of gestation lack IL-1β responses due to a lack of activity in the caspase-1 enzyme. In the final chapter, I developed an efficient purification method to study the phenotype of neonatal invariant Natural Killer T (iNKT) cells. Using this method, I show that neonatal iNKT cells display heightened proliferative capacity compared to conventional T cells, consistent with an innate-like phenotype. Altogether, my work provides important insights into mechanisms and developmental characteristics of the fetus and early newborn immune system. This work forms the basis for future studies aimed at understanding how functional characteristics of the neonatal immune system can be therapeutically modulated to prevent neonatal infections.
Preface

All studies conducted in this thesis were approved by the University of British Columbia and Children’s and Women’s (C & W) Research Ethics Boards (UBC certificate #H05-70519 and #H07-02681).

The research questions proposed in this thesis were conceived by A.A. Sharma, with supervision and assistance from P.M. Lavoie. The research in chapter 3 was designed in collaboration with R. Jen.

All work in chapter 2 was conducted by A.A. Sharma, with the following exceptions. The collection of cord blood followed by the extraction and TLR-stimulation of mononuclear cells in was conducted by A.A. Sharma with help from M. Ladd and R. Jen. Similarly, RIG-I stimulation of these cells was conducted in collaboration with N. Marr, Y.S. Hu and S.E. Turvey. Placental histology for the classification of chorioamnionitis was conducted by A.A. Sharma and C. Senger. Whereas, the clinical data used to determine the exposure to antenatal corticosteroids was determined by A.A. Sharma and P.M. Lavoie. The statistical analysis conducted in chapter 2 was done with the help of R. Brant.

All work in chapter 3 was conducted by A.A. Sharma, with the following exceptions. Cord blood collection, mononuclear cell extraction and stimulation was conducted by A.A. Sharma, R. Jen and B. Kan. Routine flow cytometry and ELISA experiments throughout the chapter were conducted by A.A. Sharma and R. Jen. The assay used to detect active caspase-1 using the FLICA, on the Amnis Imagestream, was developed by R. Jen and A.A. Sharma in collaboration with A. Tang and S.E. Turvey. Western blotting experiments throughout the thesis were conducted by A.A. Sharma, R. Jen and B. Kan in collaboration with S.E. Turvey and L.M. Sly. Quantitative PCR experiments were done at the lab of H.Côté from the Department of
Pathology at UBC with help from Abhinav Sharma, R. Jen and I. Gadawski. As in chapter 2, histological analysis of placental slides for chorioamnionitis was conducted by C. Senger and A.A. Sharma. Post-natal collection and stimulation of blood was done by E. Marchant.

Flow cytometry based experiments in chapter 4 were mostly conducted by M. Ladd, with help from P.M. Lavoie. The sorting of iNKT cells in this chapter was conducted by L. Xu and A.A. Sharma. Whereas, the thymidine assay to study the proliferation of iNKT cells was conducted by A.Y. Wang in collaboration with M.K. Levings. Functional experiments looking at the effect of IL-2 blockade on proliferation of neonatal iNKT cells were conducted by A.A. Sharma with help from Q. Huang. Preliminary data looking at neonatal mouse iNKT cells was conducted by J. Ding with help from A.A. Sharma. The protocol for purification of iNKT cells (Section 4.2.3) was developed by A.A. Sharma, with help from L. Chew and R. Jen.

Most material in this thesis has been previously published, or has been adapted from published work. Section 1.3 has been adapted from a book chapter that has been recently published in eLS citable reviews. This chapter was written by A.A. Sharma and edited by P.M. Lavoie. Section 1.3.4 was adapted from a review published in Sharma et al (2012) Clin Immunol 145(1): 61-8. The manuscript of this review was written by A.A. Sharma with help from A. Butler, R. Jen and P.M. Lavoie. Chapter 2 has been adapted from Sharma et al (2014) Neonatology 106(1): 1-9. The manuscript for this chapter was written A.A. Sharma and was edited by P.M. Lavoie. RIG-I experiments performed in chapter 2 resulted from collaborating work published in Marr, Wang, Kam, Hu, Sharma et al (2014) J Immunol 192(3): 948-57. Chapter 3 was adapted from Sharma, Jen et al (2015) Eur J Immunol 45(1): 234-49. The manuscript for this publication was written by A.A. Sharma and R. Jen and was edited by P.M. Lavoie. The flow cytometry based detection of active capase-1 was developed as a result of
Table of Contents

Abstract.............................................................................................................................................. ii
Preface.................................................................................................................................................. iii
Table of Contents.............................................................................................................................. vi
List of Tables ........................................................................................................................................ x
List of Figures ....................................................................................................................................... xi
List of Abbreviations .......................................................................................................................... xiii
Acknowledgements ............................................................................................................................ xix

Chapter 1: Introduction ...................................................................................................................... 1

1.1 Neonatal mortality: where, why and how many? ................................................................. 1

1.1.1 Diseases of the premature neonates ................................................................. 2

1.2 The intertwining relationship between preterm births and infections ...................... 4

1.2.1 Intrauterine infections: chorioamnionitis ....................................................... 5

1.2.1.1 Classification of chorioamnionitis ............................................................. 6

1.2.2 Neonatal sepsis and impact of inflammation on immature organ development .... 7

1.2.2.1 Pathogens involved in early and late onset sepsis .................................... 9

1.3 The ontogeny of the immune system .................................................................................... 11

1.3.1 The embryonic immune system (0 to 8 weeks post conception) ......................... 13

1.3.2 The early fetal immune system (8 to 38 weeks post conception) ...................... 18

1.3.2.1 Innate immune cells of myeloid origin ................................................... 18

1.3.2.1.1 Fetal monocytes ................................................................................ 19

1.3.2.2 Cells of the adaptive immune system ..................................................... 21
3.2.5 Western blot experiments ........................................................................................................ 58
3.2.6 Statistical analyses ..................................................................................................................... 58
3.3 Results ........................................................................................................................................ 63
3.3.1 Immature monocytes in cord blood before the term of gestation .............................................. 63
3.3.2 Reduced TLR-induced IL-1β response in high CD14-expressing preterm cord blood monocytes .............................................................................................................................................. 68
3.3.3 Adult-like production of pro-IL-1β in high CD14-expressing preterm neonatal monocytes .............................................................................................................................................. 70
3.3.4 Lack of NLRP3 induction in preterm monocytes following TLR stimulation .................. 73
3.3.5 Developmental lack of caspase-1 activity early in the third trimester of gestation .. 76
3.3.6 Placental infection contributing to developmental lack of inflammasome activity during gestation .............................................................................................................................................. 79
3.3.7 Production of IL-1β rapidly matures after birth in preterm neonates ......................... 81
3.4 Discussion .................................................................................................................................... 81

Chapter 4: Natural Killer T cells constitutively expressing the IL-2 receptor α chain early in life are primed to respond to lower antigenic stimulation ........................................... 85
4.1 Background ................................................................................................................................ 85
4.2 Materials and methods ................................................................................................................. 87
4.2.1 Cells, reagents and antibodies .................................................................................................... 87
4.2.2 Mononuclear and T cell purification ............................................................................................ 88
4.2.3 Detailed iNKT cell purification .................................................................................................... 88
4.2.4 Activation and proliferation experiments .................................................................................... 91
4.2.5 PCR quantification of mRNA expression .................................................................................... 92
4.3 Results

4.3.1 Neonatal iNKT cells are highly abundant early in gestation and display a quiescent CD25$^{pos}$ memory T cell phenotype.

4.3.2 Expression of CD25 on neonatal iNKT cells is observed earlier in gestation and is not due to activation from labour.

4.3.3 CD25$^{+}$ neonatal iNKT cells are not functionally suppressive cells.

4.3.4 Enhanced proliferation threshold in neonatal iNKT cells.

4.3.5 Neonatal iNKT cells require de novo TCR/CD28 co-stimulation in order to proliferate.

4.3.6 “Priming” of neonatal iNKT cells due to de novo CD25-expression.

4.4 Discussion

Chapter 5: Conclusion

5.1 Interpretation

5.2 Significance

5.3 The contribution of the main findings to the literature

5.4 Limitations and future directions

Bibliography
List of Tables

Table 2.1 Clinical characteristics of preterm neonates included in the experiments conducted in this chapter............................................................................................................................................................................ 32
Table 3.1 Sequences of primers used for SYBR green based real-time PCR experiments........ 57
Table 3.2 Sequences of primers and probes used for Taqman based real-time PCR experiments57
Table 3.3 Clinical characteristics and caspase-1 activity in monocytes of preterm neonates with or without histological chorioamnionitis ............................................................................................................................................ 62
Table 4.1 Oligonucleotide sequences used for gene expression quantification by real-time PCR93
List of Figures

Figure 1.1 Embryo hematopoietic sites and the emergence of lymphopoiesis in mid-gestation embryos .......................................................................................................................................................................................... 16

Figure 1.2 Differentiation routes from the haemangioblast/haemogenic endothelium up to mature blood cells .................................................................................................................................................................................................................. 17

Figure 1.3 Developmental changes occurring in the human immune system early in life .......... 27

Figure 2.1 Low batch related variability in the cytokine production upon stimulation with TLR ligands .......................................................................................................................................................................................................................... 34

Figure 2.2 Lower cytokine production in preterms after stimulation with TLR ligands .............. 37

Figure 2.3 Developmental hierarchy in neonatal TLR response maturation ............................... 38

Figure 2.4 Influence of ANS exposure on TLR responses in preterm CBMCs ............................ 40

Figure 2.5 Influence of chorioamnionitis on TLR responses in preterm CBMCs ....................... 42

Figure 2.6 Contribution of prematurity, histological chorioamnionitis (maternal or fetal) and ANS to TLR responses ............................................................................................................................................................................. 44

Figure 2.7 Contribution of chorioamnionitis and prematurity to TLR-independent cytokine responses ........................................................................................................................................................................................................... 46

Figure 3.1 Kinetics and dose-dependent responses for IL-1β and IL-6 secretion in preterm, term neonatal and adult BMC ............................................................................................................................................................................................................ 53

Figure 3.2 Detection and kinetics of caspase-1 activation ............................................................ 55

Figure 3.3 Phenotype of human monocytes comparing cord blood to adults ......................... 65

Figure 3.4 Cell surface phenotype of cord blood and adult monocytes .................................... 66

Figure 3.5 TLR-induced IL-1β responses in high CD14-expressing monocytes ....................... 67
Figure 3.6 TLR-induced cytokine gene and protein expression are impaired in preterm neonates .......................................................... 69
Figure 3.7 Uncut Western blots for IL-1β, Caspase-1 and B-actin ........................................... 71
Figure 3.8 TLR-induced cytokine gene and protein expression are impaired in preterm neonates ................................................................................................................................. 72
Figure 3.9 Impaired NLRP3 induction in preterm neonatal monocytes ........................................ 75
Figure 3.10 Low levels of active caspase-1 in CD14-expressing preterm monocytes ................. 78
Figure 3.11 Intra-uterine and postnatal maturation in inflammasome activity ......................... 80
Figure 4.1 Multi-step protocol for purification of human iNKT cells ........................................ 90
Figure 4.2 Phenotype of iNKT cells in adults, neonates born at term and before the term of gestation .................................................................................................................................. 96
Figure 4.3 Representative staining of CD45RO and CD62L expression in adult iNKT, neonatal T or iNKT cells ........................................................................................................................................ 97
Figure 4.4 Phenotype of neonatal iNKT cells in absence or presence of labour ..................... 99
Figure 4.5 Neonatal and adult iNKT cell CD127 and FOXP3 expression, and suppressing effect of neonatal iNKT cells on polyclonal T cell responses ......................................................... 101
Figure 4.6 Activation and proliferation threshold in neonatal and adult iNKT cells ............... 103
Figure 4.7 Representative data set used to calculate thresholds for CD69 induction or proliferation (using CFSE dilution) in iNKT cells .................................................................................. 104
Figure 4.8 TCR/CD28 co-stimulation requirements and comparison of proliferation in neonatal iNKT, adult iNKT and T cells ...................................................................................... 107
Figure 4.9 Importance of CD25 expression and effect of early IL-2 blocking during the induction phase of iNKT cell proliferation ............................................................................. 110
List of Abbreviations

α  Alpha
α-GC  Alpha-galactosylceramide
β  Beta
γ  Gamma
δ  Delta
ACTB  Beta-actin
AGM  Aorta-gonad-mesonephros
AIRE  Autoimmune regulator
ANOVA  Analysis of variance
ANS  Antenatal steroids
APC  Allophycocyanin
ASC  Apoptosis-associated speck-like protein
ATP  Adenosine triphosphate
AUC  Area under the curve
BCR  B cell receptor
BMC  Blood mononuclear cells
BPD  Bronchopulmonary dysplasia
BW  Birth weight
C/EBP  CCAAT/enhancer binding protein
C57BL/6  Black 6 mouse strain
CA  Chorioamnionitis
CBMC  Cord blood mononuclear cells
CCL  Chemokine ligand
CCR  Chemokine receptor
CD25  Cluster of differentiation
cDC  Conventional dendritic cells
CDP  Common dendritic cell progenitors
CFSE  Carboxyfluorescein succinimidyl ester
CLP  Common lymphoid progenitor
cMoP  Common monocyte progenitor
CMP  Common myeloid progenitor
CoNS  Coagulase negative staphylococcus
C-section  Caesarean Section
CX3CL  Chemokine (C-X-C motif) ligand
CX3CR  Chemokine (C-X-C motif) receptor
DAMP  Danger associated molecular pattern
DNA  Deoxyribonucleic acid
ds  Double stranded
EBF  Early B-Cell factor
ELISA  Enzyme-linked immunosorbent assay
EOS  Early onset sepsis
FACS  Fluorescence-activated cell sorting
FBS  Fetal bovine serum
FIR  Fetal inflammatory response
FITC  Fluorescein isothiocyanate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL</td>
<td>Fetal liver</td>
</tr>
<tr>
<td>FLICA</td>
<td>Fluorescent labeled inhibitor of caspases</td>
</tr>
<tr>
<td>FMO</td>
<td>Fluorescent minus one</td>
</tr>
<tr>
<td>FOXP3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>GA</td>
<td>Gestational age</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GATA</td>
<td>Globin transcription factor</td>
</tr>
<tr>
<td>GBS</td>
<td>Group B streptococcus</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GMP</td>
<td>Granulocyte-monocyte progenitor</td>
</tr>
<tr>
<td>HC</td>
<td>Histological chorioamnionitis</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNKT</td>
<td>Invariant natural killer T</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinases</td>
</tr>
<tr>
<td>KLF</td>
<td>Krüppel-like factor 2</td>
</tr>
<tr>
<td>LOS</td>
<td>Late onset sepsis</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTR</td>
<td>Long-term reconstituting</td>
</tr>
<tr>
<td>Ly6C</td>
<td>Lymphocyte antigen 6C</td>
</tr>
<tr>
<td>MDP</td>
<td>Macrophage-dendritic cell progenitors</td>
</tr>
<tr>
<td>MEP</td>
<td>Megakaryocyte-erythocyte progenitors</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescent intensity</td>
</tr>
<tr>
<td>MIR</td>
<td>Maternal inflammatory response</td>
</tr>
<tr>
<td>MMP</td>
<td>Multipotential progenitors</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Matrix metallopeptidase 2</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix metallopeptidase 9</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>MZ</td>
<td>Marginal zone</td>
</tr>
<tr>
<td>NEC</td>
<td>Necrotizing enterocolitis</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NICU</td>
<td>Neonatal intensive-care unit</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T</td>
</tr>
<tr>
<td>NLR</td>
<td>Nod-like receptor</td>
</tr>
<tr>
<td>NLRP</td>
<td>NACHT, LRR and PYD domains-containing protein</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>NOMID</td>
<td>Neonatal onset multisystem inflammatory disease</td>
</tr>
<tr>
<td>P2X</td>
<td>Purinergic receptor</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>Pax-5</td>
<td>Paired box 5</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
</tr>
<tr>
<td>PROM</td>
<td>Premature rupture of membranes</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RDS</td>
<td>Respiratory distress syndrome</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid-inducible gene 1</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-I-like receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SD</td>
<td>Standard abbreviation</td>
</tr>
<tr>
<td>SEB</td>
<td>Staphylococcal enterotoxin B</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>STR</td>
<td>Short-term reconstituting</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline and tween 20</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>------</td>
<td>----------------------</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymicstromallymphopoietin</td>
</tr>
</tbody>
</table>
Acknowledgements

I would like to thank my graduate study supervisor, Dr. Lavoie for his ongoing support through my research endeavors in his laboratory. I would also like to thank my supervisory committee, composed of Drs. Peter van den Elzen, Laura M. Sly, Megan K. Levings and Jan A. Ehses for their input and guidance during my PhD. I would also like to thank all the other lab supervisors and trainees at CFRI for providing their expertise whenever help was needed. The community at CFRI and UBC has been helpful in several collaborations. I would like to thank Drs. H.C. Cote, S.E. Turvey, C. Senger and R. Brant for their essential collaboration, and I. Gadawski, A. Tang, N. Marr, A.Y. Wang and L. Xu for their technical help. The members of the Lavoie lab have been the most engaged in my research. I would like to thank the numerous students and staff I have had the pleasure of working at the lab. These people include M. Ladd, Q. Huang, R. Jen, B. Kan, E. Marchant, G. Boyce, J. Ding, L. Chew, A. Sharma, T. Khosravi, K. Chen, A. Butler, K. Lee and H. Razzaghian. I would also like to thank J. Priatel, B. Chung, L. Cooper, R. Delnavine, P. Cho and T. English for helping me edit grants and manuscripts.

This research was funded by the Canadian Institute of Health and Research (CIHR), the SickKids foundation and the Child and Family Research Institute (CFRI) granted to P.M. Lavoie. I would also like to acknowledge invaluable financial support from CFRI and the CIHR Transplantation Training programs during my graduate studies. Finally, I would like to thank the Experimental Medicine Program, Faculty of Graduate Studies and the University to British Columbia (UBC) for providing administrative support during the completion of this thesis.
Chapter 1: Introduction

1.1 Neonatal mortality: where, why and how many?

It can be estimated that at an annual death rate of 7.89/1000 people, 66 million human lives are lost each year\(^1\). These deaths are concentrated within high risk age groups, i.e. the elderly and the children. In 2012, 6.6 million deaths were reported below 5 years of age. These deaths can largely be attributed to 2 major causes: infectious diseases and prematurity. Neonatal deaths (under 28 days of age) account for 44% of all deaths under 5 years of age\(^2\). In addition to the 2.9 million neonatal deaths every year, 2.6 million stillbirths and 0.3 million maternal deaths also result from complications during pregnancy or delivery\(^3\). Despite being highlighted as a priority in the millennium development goals, the proportion of neonates and infants dying remains particularly high throughout the world\(^2\). These overwhelming numbers have led to an increasing amount of research into understanding the causes of neonatal mortality.

Preterm birth related complications account for a 1 million neonatal deaths and are the leading cause of neonatal deaths worldwide\(^4\). A premature delivery is defined as birth at less than 37 weeks of gestation\(^5\). Approximately 15 million preterm babies born each year, account for 11.1% of 135 million live births every year\(^4\). Neonatal complications resulting from prematurity cannot be explained by socioeconomic factors alone. In fact, preterm birth related issues continue to dominate neonatal care in developed countries, where 1.2 million preterm births occur each year\(^6\). In the USA, the preterm birth related complications account for 35% of neonatal deaths\(^5\). Further, estimates from 2005 show that medical, educational and productivity costs associated with preterm birth accounted for 26.2 billion U.S. dollars\(^7\). This reality has motivated me to help in the advancement of neonatal care by furthering the understanding of the neonatal immune system.
1.1.1 Diseases of the premature neonates

Diseases associated with prematurity have a profound effect on major organ systems. Of these, the study of neonatal brain remains a priority in the field of pediatric research. Nearly 50% of children with cerebral palsy are born preterm. Cerebral palsy is a disease that results from the improper neuronal development of the brain. Abnormal motor neuron development is associated with permanent movement disabilities. Further, communication ability and depth perception are also compromised. Traditionally, these ailments are relieved by occupational therapy. However, improvements in care for the newborns have decreased the rates of this disease. One of the primary preventative measures has been the antenatal use of tocolytic drugs (e.g. Magnesium Sulfate) that can prevent the adverse neurodevelopmental effects of a premature birth on the infant. These drugs delay preterm birth by reducing the frequency of contractions in the mother, thereby increasing the time available for the development of major organs like the brain and lungs.

Morbidities and their treatments in other major premature organ systems have been tightly linked to inflammation, raising concerns of a possible immune dysregulation in the fetus. However, relatively little research has been done in trying to understand the mechanisms regulating immune development in early humans. Let’s consider the primary disease pathology in an underdeveloped lung. The lack of surfactant production by preterm pneumocytes causes the collapse of the alveolar sacs in preterm lungs, resulting in diseases like respiratory distress syndrome (RDS). In order to induce lung maturation and increase oxygen supply to the body, clinicians and scientists have relied on two main strategies: the use of antenatal corticosteroids (ANS) and mechanical ventilation. Betamethasone and to a lesser extent dexamethasone are two glucocorticoids that are routinely administered antenatally to the mother if she is expected to
deliver before 34 weeks of gestations. The administration of these steroids has shown to better *in utero* lung maturation and surfactant production by the fetus\(^{10}\). Concurrently, proper ventilation has also been important for the survival of neonates with RDS\(^{5}\). Despite their benefits, these methods have been thought to be burdensome to the developing immune system. Aggressive mechanical ventilation of the babies is now known to be associated with bronchopulmonary dysplasia (BPD)\(^{11}\). The use of forced high oxygen ventilation during the neonatal period has been shown to result in inflammation in and around the alveolar sac. This in turn causes the scarring of the sac and reduction of oxygen absorption into the blood\(^{12}\). Where BPD is associated with increased pro-inflammatory responses\(^{13}\), the use of ANS in animal models has shown a reduction in innate immune cell function rendering them incapable of fighting off invading pathogens\(^{14}\).

Like the lung diseases, the improvement in oxygen supply has not eliminated prominent ailments of the gut. Necrotizing enterocolitis (NEC) remains the second most common cause of death within preterm infants in the USA\(^{15}\). NEC often takes place within the first few weeks of life and is characterized by the improper development of intestine. Clinically, a neonate with NEC experiences reduced tolerance to feeding, bloody stools and bloating. NEC pathogenesis is explained by the inability of the immune system to deal with the incoming microbial load. The resulting inflammation leads to the destruction of the epithelia lining the intestinal tract, further increasing the chance of acquiring a microbial infection\(^{16}\). Studies looking at the use of anti-inflammatory cytokines and growth factors have shown promise in the animal models for NEC\(^{17}\). However, these strategies are yet to be applied to human scenarios. In their absence, preventative measures such as less aggressive enteral feeding and the use of human milk have proven
beneficial\textsuperscript{16}. Given the role of inflammation in the above listed preterm disease pathologies, it is imperative that we comprehend the attributes of the preterm immune system.

1.2 The intertwining relationship between preterm births and infections

A substantial proportion of preterm births are associated with intra-uterine infections. However, other non-infectious causes can also be responsible. In the context of studying the immune system in preterm neonates, these causes will be briefly discussed here. The causes of preterm births can be categorized into 3 groups: births that happen prematurely yet spontaneously with intact membranes (or amniotic sac), births resulting from premature rupture of membranes (PROM), and births resulting from induced labour or Caesarean section (C-section) deliveries\textsuperscript{18}.

Spontaneous preterm births with labour and intact membranes account for 40-45% of all preterm deliveries\textsuperscript{18}. The reasons for activation of labour in these births remains poorly understood. It is speculated that a change in maternal hormonal patterns, compared to those seen at full gestational terms, might result in initiation of spontaneous labour\textsuperscript{19}. Further, studies in sheep have shown a role of fetal hormonal profile. The lack of fetal cortisol in these sheep due to ablation of the adrenal glands leads to reduced labour initiation\textsuperscript{20}. From a genetic point of view, it has been shown that white mothers having premature babies are more likely to undergo spontaneous preterm birth as opposed to their black counterparts. In spite of this, the number of preterm deliveries seems to be significantly higher in black mothers\textsuperscript{21}. Deliveries in the latter group make up most of the births attributed to preterm premature rupture of membranes (PROM)\textsuperscript{18}.
PROM can be defined as the rupture of the amniotic sac at least an hour before the initiation of labour. Unlike spontaneous births, PROM related births can be comprised of both vaginal and C-section deliveries and account for 25% of all preterm deliveries\textsuperscript{18}. PROM (especially when prolonged to a few days) has been associated with intrauterine infections\textsuperscript{18}. The presence of intrauterine infections is also a major reason for deliveries resulting from non-PROM based induced labour and C-section deliveries. These deliveries make up the last 30% of the causes of preterm birth\textsuperscript{18}.

1.2.1 Intrauterine infections: chorioamnionitis

The prevalence of intrauterine infections has been tightly linked with preterm births. It is estimated that 25-40% of premature births have an underlying presence of a microbial infection\textsuperscript{18}. Often times these infections are difficult to detect due to a lack of advancement in culture techniques and the asymptomatic nature of the pathogen itself. However, PCR based characterization of the microbiota in the amniotic fluid and the chorioamnionic membrane has led to the characterization of several pathogens\textsuperscript{22}. In addition to bacterial species like Mycoplasma and Ureaplasma, viral and fungal species (e.g. Candida) are also detectable here\textsuperscript{23}. Microbes can also enter the amniotic cavity from the maternal circulation directly through the placenta. A pathogen present either at the feto-maternal interface, in the amniotic sac or in the fetus is often faced with an immune response by the mother and/or the fetus. The inflammation resulting from this response is broadly called chorioamnionitis. Chorioamnionitis, the inflammation of the chorion and the amnion, is characterized clinically by monitoring symptoms like abdominal tenderness, increase in fetal/maternal heart rate and increased maternal leukocyte count\textsuperscript{23}. In the past, studies have attempted to improve their diagnosis of chorioamnionitis by
examining microbial load and inflammation at a histological scale. The results from these studies showed that the presence of microbial load does not always correlate well with clinical chorioamnionitis. However, the histological inflammation is a better predictor of the disease pathology\textsuperscript{24}. Furthermore, at least one study has shown that of a cohort of low-weight babies suffering from histological chorioamnionitis, only 30\% showed clinical symptoms\textsuperscript{25}. Therefore, well-defined histological criteria for chorioamnionitis are necessary. The current classification system is briefly explained below.

1.2.1.1 Classification of chorioamnionitis

Histological examination of the placenta remains the gold standard for characterizing chorioamnionitis. A closer examination of the placental tissues has led to the organization of the disease pathology into 2 major subdivisions: the maternal inflammatory response (MIR) and the fetal inflammatory response (FIR). Both these subdivisions can further be divided into 3 stages based on the level of neutrophil infiltration into the tissues. From the maternal immune systems point of view, there are two borders separating the mother from the fetus: the choriodecidual border and the membrane of the amniotic sac. The MIR is defined as stage 1 when there is a sparse accumulation of maternal neutrophils on the decidual side of the choriodecidual junction. As the response intensifies and acute chorioamnionitis begins, a much greater number of neutrophils accumulate and breach this barrier and find a niche in the chorioamniotic tissue. During the third and the most intense phase of MIR, quickly aging neutrophils fragment and apoptose during necrotizing chorioamnionitis\textsuperscript{26}. This stage is especially detrimental to the fetus, as the death of the neutrophils is accompanied by the shedding of the amniotic epithelium, further compromising the sterility of the environment around the fetus\textsuperscript{27, 28}. Apart from the
stages, the severity of the MIR can be broken down into 2 grades. The more severe of the two, grade 2, is characterized by formation of immune cell aggregates around the amniotic membrane. Although MIR is correlated with increased levels of preterm births, the incidence of FIR is far more detrimental for the fetus\textsuperscript{26}.

Like the MIR, the FIR can also be broken down into 3 stages based on the infiltration of neutrophils. The first stage of FIR is characterized by the presence of neutrophils in the fetal vessels, like the umbilical veins infiltrating the chorionic plate. The neutrophil infiltration intensifies in stage 2, where the neutrophils can be detected in the muscular wall of the umbilical artery\textsuperscript{26}. The movement of the neutrophils out of the artery is correlated with an increase in circulating interleukin-6 within the fetus, further indicative of fetal immune response\textsuperscript{29}. Stage 3 of the FIR is defined as the movement of neutrophils out of the fetal vessels. Histologically, this movement is easily visible in the gelatinous substance around the umbilical artery. It is known that the movement of neutrophils out of the fetal vessels is directed towards complexes made of maternal IgG and the microbial culprits. Like the MIR, the severity of the inflammatory response in FIR is quantified on a 2-point grading system, where the higher grade is indicative of an increased concentration of neutrophils around the fetal vessels\textsuperscript{26}. Given the proximity of the intrauterine inflammatory response to the fetus, it is not surprising that this response has detrimental impact on the development of multiple organs of the body\textsuperscript{28}.

### 1.2.2 Neonatal sepsis and impact of inflammation on immature organ development

With the decrease in asphyxia related deaths (e.g. RDS and Cerebral Palsy), infections have become the leading cause of mortalities in preterm. As described earlier, the acquisition of infection by the fetus can start \textit{in utero}. Once within the amniotic fluid, pathogens have easy
accessibility to fetal organs like skin, lungs and gut. In animal studies, one of the core centres of inflammation has been noted as the fetal lung. After stimulation with microbial products, fetal lungs have been shown to generate a rigorous pro-inflammatory response. The generation and amplification of an inflammatory response is associated with the maturation of alveolar macrophages and subsequent promotion of inflammation in other organ systems\textsuperscript{28}. Further, the production of pro-inflammatory proteins has been shown to promote lung maturation. The secretion of IL-1\(\alpha\), causes an increase in the generation of surfactant proteins A and B, which are essential for normal lung function by reducing surface tension in the alveoli\textsuperscript{30, 31, 32}. Additionally, IL-6 advances lung development by matrix metallopeptidase 9 (MMP-9) dependent bronchial remodeling\textsuperscript{33}. Like animal models, studies of bronchial aspirate and bronchoalveolar lavage have shown an increase in the levels of CD68 (a macrophage associated protein) and IL-6 respectively\textsuperscript{34}. Lower rates of RDS in neonates exposed to \textit{in utero} infections further confirm the improved maturation of lungs. However, animal studies have shown that lung maturation resulting from chorioamnionitis closely resembles the lung pathology during BPD\textsuperscript{28}. Neonates of lower gestational ages are also more likely to be exposed to intense ventilation and develop BPD\textsuperscript{11}. The complexity underlying the understanding of fetal lung maturation is further complicated by the use of antenatal steroids. These drugs act by improving surfactant production and lung remodeling in a manner similar to pro-inflammatory responses. However, interesting mechanistic differences have been observed. For instance, where IL-6 recruits MMP-9 for the bronchial remodeling, steroids rely on MMP-2\textsuperscript{33}. Consequently, a synergistic effect in the use of ANS and pro-inflammatory protein production has been shown in sheep models\textsuperscript{35}. Although ANS use has been heralded as a major breakthrough in preterm care, questions have been raised about the anti-inflammatory nature of steroids. Studies in animal models have demonstrated that
ANS use suppresses the pro-inflammatory capacity of neonatal immune cells for the first 5 days after administration. This suppression is short-lived and is superseded by a significant increase in pro-inflammatory protein production by day 14. This phenomenon remains poorly studied in humans, and will be discussed further in chapter 3.

Immune cell deposition in fetal skin begins early in gestation. Upon encountering an infection, the fetal skin upregulates pathogen recognition receptors (PRRs) like toll-like receptors 2 and 4 (TLR4). TLR2 and TLR4 are known to associate with pathogen associated molecular patterns (PAMPs) like Lipoteichoic acid (LPA) and lipopolysaccharide (LPS), respectively. It can be speculated that these molecules propagate the cutaneous and mucosal pro-inflammatory response observed in the skin. Unlike the skin, fetal gut immunology is more specialized. The presence of pathogens in the gut is followed by rapid downregulation of tight junction proteins, an important event in the pathogenesis of NEC. Indeed, clinical studies have confirmed that the presence of chorioamnionitis is a risk factor for the development of NEC.

1.2.2.1 Pathogens involved in early and late onset sepsis

The effect of in utero infections on solid organs is important in discerning the pathophysiology of BPD and NEC. The need to understand these diseases is currently trumped by the need to triumph over infections. Neonatal infections are the leading reason for death in preterm neonates. Neonatal infections can be divided in two major categories: early-onset neonatal sepsis and late-onset neonatal sepsis. Early onset neonatal sepsis (EOS) is usually acquired in utero and is most pathogenic in the first 72 hours after birth. Early onset sepsis is clinically characterized by the respiratory distress, abnormal heart rate, hypo/hyperthermia, seizures and poor feeding. Common pathogens associated with EOS include Group B
streptococci (GBS), *Escherichia coli* and *Listeria Monocytogenes*. Historically, the incidence of PROM and chorioamnionitis has been associated with increased GBS infections. In recent years, a dramatic reduction in GBS infections has been associated with use of maternal GBS prophylactic treatment. Additionally, neonates suffering from EOS are often treated with antibiotics like gentamicin and penicillin. These measures have helped in keeping the incidence of EOS below 1.5% in very low birthweight babies. In contrast, late onset sepsis (LOS) infects 20% of the same groups of babies. The mortality associated with LOS can be as high as 40-50% in preterm infants born below 25 weeks of gestation. LOS is characterized as an infection that occurs at least 72 hours post-birth resulting in symptoms like increased white blood cell count, hyperglycemia, apnoea and abdominal distension. Pathogens responsible for LOS include species of gram positive bacteria, gram negative bacteria and fungi. Infections due to gram negative bacteria (like E.coli) are rare compared to staphylococcal and fungal infections. Coagulase negative staphylococci (CoNS) represent 50% of all LOS infections. Like most pathological species, the presence of bacteria is determined by culturing neonatal blood. The small amount of blood available for culturing, compounded with the abundance of these bacteria in the environment greatly enhances the chance of a false positive culture, resulting in a high exposure of these infants to antibiotics.

LOS can also result from an infection acquired during invasive treatments of the neonates. Surgical apparatus, catheters and feeding tubes can be a site of biofilms that are resistant to common antimicrobials. These biofilms are composed of two common microbes: *Staphylococcus aureus* and *Candida albicans*. *S. aureus* infections are not as common as infections by CoNS and are relatively efficiently dealt with using antibiotics. On the other hand, fungal infections by *Candida spp* are a major source of mortality in the neonatal period. These
infections can be acquired either in utero or during the neonatal period. Early administration of antibacterial (and sometimes also antifungal) drugs to mothers at risk of having intrapartum infection is indicated when sepsis is clinically suspected\(^4\). In the neonatal period, treatment post-infection with antifungal imidazole compounds has proven effective. More recently there have been attempts at using prophylactic treatment to reduce the incidence of infection. Systemic use of medications like fluconazole has been shown to reduce the incidence of fungal infections\(^4\). Despite this, the exact impact of prophylactic treatment remains unclear.

The pathogens responsible for late onset sepsis are ubiquitously found in the natural and NICU environments. The adult immune system encounters and fights off these infections every day. However, risk of neonatal infection augments considerably with reducing gestational age indicating a potential role for incomplete immune maturation. In the following chapters, I’ll cover the known and the unknown of the neonatal immune system, followed by a description of studies that I have been involved in and their contribution to the understanding of the neonatal immune development.

### 1.3 The ontogeny of the immune system

A main purpose of the immune system is to discern and discard foreign threats and corrupted-self entities, while minimizing the damage to healthy self-structures. The immune response comprises an innate and an adaptive immune arm. The innate immune system is a first line of defence, able to respond to a broad range of molecular structures. These structures are called Pathogen-Associated Molecular Patterns (PAMPs), which are conserved within microbes. Furthermore, products of cellular damage called Danger-Associated Molecular Patterns (DAMPs) can also stimulate the innate immune system. The recognition of PAMPs and DAMPs
by specialized receptors leads to the production of chemokines/cytokines, and also to activation of complement proteins, which can enhance phagocytosis. Cytokines and chemokines result in increased trafficking of cells to the site of infection, including antigen-presenting cells, which can then carry antigens (e.g. microbial particles or corrupted cell components) to the lymph nodes and spleen, where a cognate interaction with T and B lymphocytes from the adaptive immune system occurs. Unlike the innate immune system, the adaptive immune system is able to develop an “immunological memory” to the antigens it recognizes. This immunological memory is characterized by an ability of cells to tailor the specificity of the receptors involved in pathogen recognition through the expression of specific recombinases and template-independent polymerase genes. The adaptive immune system is a unique feature of vertebrate organisms. It is notable for an extraordinary diversity of clonotypic receptors, including immunoglobulins (Igs) and T cell receptors (TCRs). These receptors are able to recognize specific antigenic determinants using receptors that have been selected for by a somatic mutation process. Within the adaptive immune system, discrete lymphoid cell subsets known as innate-like cells (B1 and marginal zone B cells, γδ and natural killer T (NKT) cells) display more limited antigenic receptor diversity and act as intermediates bridging the innate and conventional forms of adaptive immunity.

The human gestation and first few months of life correspond to the most rapidly changing period of immune development. During that period, primitive hematopoietic cells first originate in the yolk sac of the growing embryo. These cells are important for shaping the macroanatomy of the developing embryo. During fetal life, the diversity of immune cells increases considerably. Definitive hematopoietic cells develop in specific organs (e.g. liver and thymus). At this stage, fetal immune cells are also intimately involved with cells from the
maternal immune system to establish immunological tolerance necessary for preventing rejection of the fetal allograft\textsuperscript{47}. Additionally, animal studies have shown that the fetal immune response resulting from any major breach of sterility is significantly subdued\textsuperscript{48}. At the time of birth, the neonate becomes exposed to a horde of commonly available microbes requiring major transitional adaptation in the nature and specificity of the immune response. Yet, this period is also a critical one as the immune system needs to learn to rapidly respond to infectious threats. In the post-natal period, maternal antibodies transferred from the placenta \textit{in utero} and during post-natal life through breast-milk play an important role in protecting the immunologically developing organism until its full transition to an adult immune system. Despite being well adapted to its environment, the neonatal immune system remains predisposed to certain types of infections. Neonatal infections are responsible for the greatest burden of childhood mortality and morbidity across the world. This morbidity and mortality further increases with prematurity. The lack of a post-natal immune response in premature neonates can partly be attributed to a lack of fetal-immune development. To combat neonatal infections, therefore, it is necessary to understand immune immaturity associated with prematurity. In this section, we discuss the ontogeny of the immune response, emphasizing both major gaps in knowledge and how a more detailed understanding of the post-natal immune system may provide insights into improving health outcomes in human neonates.

1.3.1 The embryonic immune system (0 to 8 weeks post conception)

Hematopoietic cells originate from a group of stem cell progenitors called hematopoietic stem cells (HSCs). In adults, these cells reside in the bone marrow, where they undergo a long-term cycle of self-renewal and differentiation. Upon differentiation, these cells give rise to the
myeloid, lymphoid and erythroid lineages. The erythroid lineage gives rise to megakaryocytes and red blood cells, which play a major role in the circulation of oxygen and nutrients within the body. The myeloid and the lymphoid cells, on the other hand, together form the immune system.

In contrast to adults, the site of the embryonic and fetal hematopoiesis differs with gestational age (Figure 1.1). In the yolk sac of the mouse embryo, the earliest signs of the immune system can be seen at about 7 embryonic days (3-4 gestational weeks in humans). This period is characterized by the presence of red bodies in the yolk sac, known as blood islands. The blood islands are a site of limited hematopoiesis, giving rise to cells of erythroid lineage and embryonic macrophages. The development of this primitive erythroid compartment is essential for the circulatory system, which in turn is essential for seeding other sites of hematopoiesis in the embryo. Aside from the erythrocytes and megakaryocytes, the blood islands are also the site of myelopoiesis. In particular, the myeloid cells from this site differentiate into macrophages. Embryonic macrophages have been shown to have both immediate and long-term functional roles in ontogeny. Immediately after differentiation, these cells start to play an important role in the development of red blood cells by producing erythropoietin, a glycoprotein hormone that enhances erythropoiesis. In addition, they also help in the process of red blood cell differentiation by ingesting the nucleus expelled during the final stages of red blood cell maturation. Embryonic macrophages also play an important role in tissue remodeling, through phagocytosis of apoptotic cells. Upon the start of circulation, these cells travel from the blood islands to other organs including the liver, skin and brain. Here, they become self-sufficient long-term resident macrophages called Kupffer cells (in the liver), Microglia (brain) and Langerhans cells (skin). Unlike the skin and the liver macrophages
which are largely replaced by bone-marrow-derived macrophages from fetal liver, embryonic macrophages from the microglia reside in the brain throughout adulthood.

The limited embryonic hematopoiesis is replaced with the origin of true multipotent HSCs at 4-6 weeks of gestation, in a part of the splanchnopleura mesoderm tissue (which develops into the Aorta-Gonad-Mesonephros (AGM) region) \(^{60}\). The HSCs isolated from this region add to the immune cell repertoire by differentiating into lymphoid and myeloid progenitors (Figure 1.2). The vast majority of lymphoid progenitors go on to differentiate into T cells, B cells and Natural Killer (NK) cells. The commitment to the B-cell lineage strictly depends on the activity of EBF and Pax-5 transcription factors, whereas the establishment of T-cell lineages depends on Notch signalling after continual interaction with its Dll ligands. The emergence of NK lymphocytes is defined by the upregulation of the β chain of the IL-2R (CD122). These immune cells may derive from progenitors sharing either T- or B-cell potentials. In the absence of a thymus, lymphoid progenitors preferentially develop into B cell subsets. Like most early fetal lymphoid cells, CD19\(^{\text{pos}}\)CD45R\(^{\text{neg}}\) B cells have a limited antibody repertoire for antigen recognition\(^{61}\). At about 8 weeks of gestation, these cells and the HSCs both join the circulation. The HSCs start colonizing the fetal liver and thymus by the 10\(^{\text{th}}\) gestational week\(^{46,62}\). The fetal liver remains the primary site of hematopoiesis late into the second trimester of gestation, at which point production of hematopoietic cells occurs primarily in the bone marrow.
Figure 1.1 Embryo hematopoietic sites and the emergence of lymphopoiesis in mid-gestation embryos

The transversal view depicting AGM region includes subaortic and intraaortic hematopoietic cell clusters. FL is likely colonized by both YS and AGM-derived progenitors (1, 2). The embryo thymus is engrafted with AGM and FL-derived pre-committed precursors (3, 4). Whether hematopoietic progenitors migrate between both YS and AGM is unclear (5, 6). In red, embryonic hematopoietic sites. Neural tube, blue; dorsal aorta, green; mesonephros, brown; gonads, orange. This figure is an adaption of a previously published electronic book chapter in “eLS citable reviews in life sciences”.63
Figure 1.2 Differentiation routes from the haemangioblast/haemogenic endothelium up to mature blood cells

Full arrows indicate the scheme based on the existence of a primordial CLP/CMP branching. Dotted arrows show alternative roads of lymphoid differentiation. The cellular stages and pathways predominating in embryo are labelled in blue. ETP, early T-cell progenitors; GMP, granulocyte-macrophage progenitors; LTR, long-term reconstituting; MEP, megakaryocyte-erythrocyte progenitors; MMP, multipotential progenitors; MZ, marginal zone; STR, short-term reconstituting; other abbreviations, in the main text. This figure is an adaption of a previously published electronic book chapter in “eLS citable reviews in life sciences” 63.
1.3.2 The early fetal immune system (8 to 38 weeks post conception)

1.3.2.1 Innate immune cells of myeloid origin

The fetal liver is the main site of hematopoiesis until about 22 to 26 weeks of gestation. Fetal innate immune hematopoietic cells originate from either the common lymphoid or myeloid progenitors. The differentiation of the progenitors into myeloblasts is followed by further branching into specialized innate immune cell progenitors: granulocyte monocyte progenitors (GMPs) and the macrophage-dendritic cell progenitors (MDPs). As the name implies, the GMPs go on to differentiate into a group of cells called granulocytes. These cells are differentiated in the fetal liver at 5 weeks of gestation and include: neutrophils, eosinophils, basophils and mast cells. Neutrophil blood counts in the fetus are substantially lower than in adults, because of a lack of GM-CSF and G-CSF (growth factors for neutrophil expansion). On the other hand, the fetus has an abundance of eosinophils. As with adult eosinophils, the differentiation of fetal eosinophils is dependent on the cytokine IL-5. However, unlike adult cells, fetal eosinophils have a capacity to self-renew. Levels of eosinophils remain high during gestation and in the postnatal period, whereas levels of neutrophils increase to adult standards only by the time of delivery. The biological purpose of a high eosinophil count remains understudied. It can be speculated that the high eosinophil levels may contribute to a number of eosinophilia-related conditions observed in preterm neonates.

The MDPs further differentiate into common dendritic progenitors (CDPs) and common monocyte progenitors (cMoPs). The CDPs give rise to two subsets of dendritic cells: plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs). pDCs are long-lived cells that recognize viral patterns using TLR7 and TLR9, two members of the pathogen
recognition receptor (PRR) subfamily of Toll-Like Receptors (TLRs). Upon recognition of viral patterns, these cells produce high amounts of type I interferons (IFN-α and IFN-β). Dendritic cells can be further subdivided into subsets in humans according to expression of the surface molecules CD8α, CD103 and CD11b. CD8α and CD103-expressing cDCs orchestrate cell mediated immunity by presenting the antigens to CD8 T cells. The increased production of IL-12 upon the activation of these cells further activates type 1 helper T lymphocyte adaptive immune response. In contrast, CD11b positive DCs play a specific role in humoral immunity by activating CD4 T cells during a type 2 helper T lymphocyte immune response. Most DC subsets remain present in low numbers at birth and gradually mature thereafter.

1.3.2.1.1 Fetal monocytes

The cMoP subset of MDPs has a restricted differentiation capacity towards the monocyte lineage. Differentiation of monocytes is regulated by the master transcription factor PU.1. PU.1 activates interferon regulatory factor-8 (IRF8), kruppel-like factor 4 (KLF4) and Egr1. Furthermore, PU.1 enhances the growth capacity of the differentiating monocytes by increasing the expression of CD115 or macrophage colony-stimulating factor receptor (M-CSFR). Aside from the activation of these factors, PU.1 antagonizes the GATA-1, GATA-2 and C/EBPα, thereby restricting these cells from differentiating into erythroid and granulocyte lineages. In mice, two main subsets of monocytes, Ly6Chigh and Ly6Clow, have been described. Monocytes of the Ly6Chigh subset also express the C-C chemokine receptor 2 (CCR2) and low levels of CX3C chemokine receptor 1 (CX3CR1). The release of CCL2 results in the recruitment of this cell subset to the site of inflammation. Upon entering the site of inflammation, these cells display a robust pro-inflammatory phenotype characterized by the production of Tumor Necrosis Factor α.
(TNF-α), reactive oxygen species and nitric oxide. On the other hand, the Ly6C\textsuperscript{low} monocytes lack expression of CCR2, but express abundant levels of CX3CR1\textsuperscript{52}. Unlike CCL2, the CX3CL1 (fractalkine; the ligand for CX3CR1) is also expressed on endothelial cells. Consequently, Ly6\textsuperscript{low} monocytes play a role in patrolling the blood vessels and are considered first responders at the site of infection\textsuperscript{75}.

In humans, three subsets of monocytes have been identified. The CD14\textsuperscript{pos}/CD16\textsuperscript{neg} (also termed classical monocytes) and CD14\textsuperscript{pos}/CD16\textsuperscript{pos} (intermediate monocytes) subsets are the human equivalents of the mouse Ly6C\textsuperscript{high} subset. Of these, the CD14\textsuperscript{high}/CD16\textsuperscript{neg} monocytes make up >90\% of the monocytes in circulating blood. One of their many roles is to resolve inflammation by producing large amounts of IL-10\textsuperscript{76}. In comparison, the CD14\textsuperscript{high}/CD16\textsuperscript{pos} monocytes play a more intermediate role in inflammation\textsuperscript{77}. CD14\textsuperscript{neg}/CD16\textsuperscript{neg} precursors from the fetal liver can differentiate directly into CD14\textsuperscript{pos}/CD16\textsuperscript{pos} cells. These cells are thought to play an important role in processes such as angiogenesis and tissue remodeling during the development of the fetus\textsuperscript{44}.

In contrast, the smaller, non-classical CD14\textsuperscript{neg}/CD16\textsuperscript{pos} monocytes correspond to the Ly6C\textsuperscript{low} subset in mice. These cells have been characterized as particularly pro-inflammatory, with an ability to mount an immediate immune response during the development of sepsis\textsuperscript{78}. Furthermore, these cells show an enhanced expression of antigen presentation molecules (such as HLA-DR) on their surface\textsuperscript{79}, and the ability to produce large amounts of the inflammatory cytokine TNF-α\textsuperscript{80}. These properties make the CD14\textsuperscript{neg}/CD16\textsuperscript{pos} monocyte subset particularly pro-inflammatory. Major developmental and functional changes occur in the phenotype of monocytes during the fetal and perinatal period. However, how these changes contribute to developmental differences in immune responses in preterm neonates remains unclear.
1.3.2.2 Cells of the adaptive immune system

Unlike innate immune cells, adaptive immune cells harbor a capacity to develop tailored complementary responses towards specific antigens, a process called immunological memory. Two major cell types of the adaptive immune system are the T and the B lymphocytes. These cells are differentiated from the common lymphoid progenitors (CLP) in the presence of IL-7, in the adult bone marrow and the thymus. In contrast to adults, fetal lymphopoiesis is strictly dependent on the Ikaros transcription factor. The fetal equivalents of CLPs or other lymphoid precursors (present in the late fetal liver) are also capable of differentiating into myeloid cells. After differentiation, the mature B cells migrate to and reside in secondary lymphoid organs (e.g. spleen and lymph nodes) until they are recruited to the site of inflammation. During a humoral response against extracellular infections, B cells can function as antigen presenting cells by presenting peptide fragments to T cells. In addition, B cells can also adapt the structure of their antibody against pathogens during a process called somatic hypermutation. This process is aimed at increasing the specificity of the B cell repertoires (BCR) following rearrangements in the Variable(V), Joining(J) and Diversity(D) gene segments of the B cell receptor. The resulting antibodies, are capable of neutralizing an innumerable number of foreign particles.

B cell progenitors first appear in the fetus 3-6 days after the HSCs have colonized the fetal liver. In contrast with adult B cells, the differentiation of fetal B cells does not require the cytokine interleukin 7 (IL-7), since the latter can be differentiated using Thymicstromallymphopoietin (TSLP). Fetal B cell progenitors show transient expression of endothelial cell markers, and lack the expression of steroid hormone receptors; PLRLC (a lymphocyte-restricted myosin L chain); MHC class II; CD25 (IL-2Rα chain); and CD138 (Syndecan-1) surface receptors. Furthermore, the fetal B cell repertoire presents reduced...
antigenic specificity compared with adults. This is due to a lack of the enzyme Terminal deoxynucleotidylTransferase (TdT), which is responsible for the addition of nucleotides in the hypervariable region of the B cell receptor during VDJ rearrangement. The main role of TdT is to improve the antigenic diversity of the T/B cell receptor, through somatic hypermutation. During this process, TdT adds N-nucleotides to the V, D and J chains.

1.3.2.2.1 Fetal T cells

In adults, T cell precursors are generated in the bone marrow from common lymphoid progenitors. These cells then travel from the bone marrow to the thymus for the completion of their maturation. Thymic T cell precursors undergo somatic V(D)J recombination just like B cells. After somatic recombination, T cells undergo a sequence of positive and negative selection steps. Positive selection selects for T cells that show affinity to the self-antigens present on the thymocytes, while negative selection is aimed at eliminating the T cells that show too much affinity for the self-antigens presented by the epithelial cells in the medulla of the thymus. To prevent self-reactivity, the medullary thymic epithelial cells express proteins from various parts of the body under the control of the AIRE gene. The strong recognition of these peptides causes apoptosis in the developing T cells. However, a small proportion of self-reactive T cells do graduate from the thymus. T regulatory cells are released into the peripheral circulation to further prevent auto-reactivity by T cells that may have escaped the rigorous process of thymic selection.

As mentioned earlier, the thymus develops at about 10 weeks of gestation following its colonization by the progenitors from the AGM region. Upon further maturation, the thymus receives waves of lymphoid progenitors from the fetal liver and bone marrow respectively. Once
in the thymus, the progenitors become committed to the T cell lineage upon recognizing the Notch ligand\textsuperscript{85}. According to data in adults, about 3\% of progenitors are positively selected. After undergoing negative selection, these cells can be found in the peripheral lymphoid organs (and the blood) by 10-12 weeks of gestation. Unlike humans, the colonization of peripheral lymphoid organs by mouse T cells does not commence until very late in gestation\textsuperscript{86}.

1.3.3 **Innate-like T cell subsets**

Gamma-delta (γδ) T cells express a unique type of TCR composed of the γ and δ chains. They are amongst the first type of T cells to develop in both mice and humans. Unlike conventional alpha-beta (αβ) T cells, which recognize peptide antigens presented on major histocompatibility complex (MHC) molecules, γδ T cells recognize phospholipid antigens without the need for an MHC molecule (the use of CD1 class molecules has been shown in some cases)\textsuperscript{87, 88}. The restricted ability to recognize antigenic particles is further complemented by the lack of TdT in fetal cells, resulting in a repertoire of cells with limited antigen-specificity\textsuperscript{84}. In the adults, γδ T cells comprise two main subsets: Vδ2\textsuperscript{pos} and the Vδ2\textsuperscript{neg}\textsuperscript{89}. Vδ2\textsuperscript{pos} cells express a δ2 V chain and a γ9 J chain and are most abundant in the adult. These cells have an interesting dynamic during development. Studies of fetuses from the second trimester of gestation show that these cells make up the highest proportion of all γδ T cells. However, these cells are present in much lower proportions in cord blood\textsuperscript{90}. It is thought that during late fetal development, there is an increase in the maturation of the Vδ1\textsuperscript{pos} T cells\textsuperscript{91}. Vδ1\textsuperscript{pos}-cells have been shown to migrate to the gut and the skin and specialize in their defence. They are abundant at birth and produce high levels of IFN-γ, in contrast to most other conventional cord blood T cells\textsuperscript{92}. Within a year of
birth, however, a dominance of Vδ2pos cells can be seen in the γδ subset, stressing their consistent need in human life93.

Natural Killer T (NKT) cells are another subset of cells that display limited TCR diversity. NKT cells have distinct regulatory properties, and are innate-like lymphocytes that express the NK1.1 marker, commonly found on Natural Killer (NK) cells, and a T cell receptor. These cells are capable of quickly producing impressive amounts of IL-4 upon stimulation94,95. Studies into the phenotype of NKT cells have led to the characterization of 2 subsets. Type I NKT cells express an invariant TCR and have been the focus of the majority of NKT cell related research. In the mouse, the iNKT cell TCR is composed of a unique variable (V) α14 and junction (J) α18 chain and is paired with a β-chain of limited diversity96,97. In parallel, iNKT cells in humans express a structurally similar TCR α chain (Vα24/Jα18)98,99,100.

Human iNKT cell numbers peak in the thymus during the third trimester of gestation. Where cord blood iNKT cells make up about 0.1% of all T cells, the adult peripheral iNKT cells make up a much smaller and more variable compartment of CD3-positive cells (0.005 to 0.2%)101. The primary location of iNKT colonization in humans is the gut, where up to 5% of T cells can be iNKT cells102,103. In contrast, mouse iNKT cells first appear in the thymus about a week after birth. As the mouse ages, the iNKT cells largely localize in the liver where they make up about 20-40% of T cells. These cells can also be found in the spleen and peripheral blood, where they make up ~0.5-1% of all T cells104,105.

In humans, neonatal and adult iNKT cells display a memory phenotype, characterized by the expression of CD45RO on the surface of the cells (D’Andrea et al., 2000; Eger et al., 2006; van Der Vliet et al., 2000). Cord blood iNKT cells also constitutively express CD25, or the IL-2Rα chain. CD25 is the high affinity binding chain of the IL-2 receptor106,107. The binding of IL-
2 to the IL-2 receptor is critical for the proliferation of lymphocytes\textsuperscript{108, 109}. Consequently, CD25\textsuperscript{pos} iNKT cells show a substantially reduced threshold for proliferation, with an initial lack of requirement for IL-2 to drive them into cell cycle. The mechanisms of action of neonatal iNKT cells during infections are yet to be researched.

### 1.3.4 Post-natal immune response in preterm neonates

Neonatal adaptive immune compartment is considered naïve, as it has not been exposed to foreign pathogens\textsuperscript{110}. In addition to the protection provided by the maternal antibodies, a full term neonate is heavily dependent on its innate immune compartment\textsuperscript{111}. Furthermore, full-term neonates generally have a higher number of lymphoid and myeloid cells\textsuperscript{112, 113, 114, 115}. On the other hand, preterm neonates born before 30 weeks of gestation lack the protection provided by maternal antibodies\textsuperscript{116, 117}. The preterm immune system has reduced PRR function, reduced capacity for endothelial rolling/adhesion and lower ability to eliminate extracellular bacteria\textsuperscript{118, 119}.

PRRs such as Toll-like receptors (TLRs) and Nucleotide Oligomerization Domain (NOD)-like receptors (NLRs) are present on innate immune cells and are responsible for recognizing PAMPs. Various studies have now demonstrated that preterm innate immune cells show significantly lower production of pro-inflammatory cytokine after stimulation of the TLR pathways\textsuperscript{120}. Specifically, stimulation of preterm innate immune cells with LPS (lipopolysaccharide, a component of bacterial cell wall and stimulator of TLR4) led to a reduced pro-inflammatory cytokine production in preterm neonates\textsuperscript{121, 122, 123, 124, 125}. Previous studies from our lab have demonstrated that preterms secrete lower anti-viral interferon-\(\alpha\) despite having similar numbers of anti-viral pDCs\textsuperscript{126}. In contrast, preterm neonates produce high anti-
inflammatory responses\textsuperscript{124, 126, 127, 128, 129, 130} (Figure 1.3).

The fundamental mechanisms underlying the developmental attenuation of innate immune responses during gestation are not well understood. Part of the attenuation in pro-inflammatory cytokine production may be due to a reduction in surface expression of TLR4 and its co-receptor CD14,\textsuperscript{131, 132, 133} or proximal signalling molecules such as MyD88 and IRF5 (two key components of the TLR signalling cascade\textsuperscript{133, 134}). Monocytes also display a functional reduction in the activity and nuclear translocation of the NF-κB and p38/JNK transcription factors.\textsuperscript{133, 134} The attenuation of immune responses is not restricted to transcriptional and translational control. Newborns also secrete minimal amounts of IL-12 because of a lack of expression of the p35 subunit\textsuperscript{135}. This lack of IL-12 expression is compensated for by a high production of IL-23 (through pairing of p19 with the p40 molecular subunit) in term neonates\textsuperscript{136, 137, 138}. In contrast, preterm infants lack expression of p40 and therefore have a markedly reduced capacity to produce both IL-12 and IL-23, resulting in a high vulnerability to infection\textsuperscript{126}. Overall, the lack of fetal exposure to antigen and an immature innate immune response culminates in high rates of sepsis related mortalities in premature neonates. To fight infections in the neonates, we must understand the fetal and neonatal immune system.
Figure 1.3 Developmental changes occurring in the human immune system early in life

This figure illustrates maturational events occurring in major adaptive and innate immune functions as the human transitions from a fetal tolerance state and becomes exposed to microorganisms as well as other environmental antigens de novo after birth. **Adaptive immune functions [top panel]:** Maternal transplacental antibody transfer (IgG) mainly occurs during late gestation, followed by maternal antibody protection (IgA) acquired through breast-milk after birth. Infants’ own antibody response become fully mature later during early childhood. Neonatal T cells are largely biased towards helper type II responses and humans display high proportions of T regulatory and Natural Killer T cells at birth. **Innate immune functions [bottom panel]:** Pro-inflammatory (IL-1β, IL-6, TNF-α, IL-12, IL-23) and anti-viral (IFN-α) cytokine responses are largely attenuated in preterm infants, whereas production of the anti-inflammatory IL-10 cytokine is relatively high during late gestation and at birth.
1.4 Rationale and hypothesis

The high rate of infections in the preterm neonates has led to increased morbidity and mortality rates. The acquisition of these infectious pathogens can happen in utero, or in the hospital setting. A preterm neonate’s inability to mount a proper immune response against these fairly common pathogens, suggests that there is a lack of maturity in their immune system. Previous studies have proposed that extrinsic factors, like the presence of in utero infections or the use of antenatal corticosteroids, play a role in altering the immune response. On the other hand, an intrinsic immaturity in various branches of the immune system has also been characterized. Despite these efforts, the main culprit behind the immunological immaturity observed in a preterm neonate remains elusive. During my thesis, I identified some of the fundamental mechanisms underlying this immune immaturity.

The main hypothesis underlying my thesis work, is that the innate immunological hyporesponsiveness observed in infants born prematurely is due to developmental factors rather than extrinsic factors linked to the premature birth of these infants. An important corollary to this contention is that a better understanding of the molecular determinants of this immunological hyporesponsiveness might lead to new therapeutic targets amendable to interventions aimed at preventing the dreadful health consequence of infections in newborns. The overarching objective of this work is to determine at what levels the neonatal immune system functionally differs from adults. My specific objectives are:

1. **Objective 1 (Chapter 2).** To determine the relative contribution of development, versus extrinsic perinatal factors, to the attenuation of innate immune anti-microbial responses.
2. **Objective 2 (Chapter 3).** To localize the molecular defect resulting in the lack of interleukin-1β production in human neonatal monocytes with decreasing gestation.

3. **Objective 3 (Chapter 4).** To characterize the functional impact of the constitutive interleukin-2 alpha (CD25) receptor expression on the activation of neonatal invariant Natural Killer T cells (iNKT cells).
Chapter 2: Hierarchical maturation of innate immune defences in very preterm neonates

2.1 Background

The innate immune system provides the primary barrier in the defence against foreign particles. The detection of these pathogens by PRRs initiates a pro-inflammatory immune response. Of the many different type of PRR responses, TLR responses have been best studied and are markedly attenuated in preterm neonates$^{139,140}$. Prematurity has been broadly implicated in the attenuation of these immune responses. However, this attenuation could also result from a number of factors in utero, which may also broadly suppress innate immune responses. Specifically, chorioamnionitis, commonly associated with prematurity, can induce a state of immune tolerance$^{26}$. The use of antenatal corticosteroids (ANS) in mothers of preterm infants delivered before 34 weeks of gestation is standard practice in perinatal medicine$^{141}$. Significant immune attenuation can also be observed in mononuclear cells exposed in vitro to corticosteroid equivalents comparable to levels measured in serum of pregnant women after a standard ANS treatment$^{123,142,143,144}$. When examining infants exposed to ANS, however, no effects were detected on cord blood IL-6 responses$^{144}$. One major limitation of previous studies is the lack of rigorous definitions of chorioamnionitis and of the timing of ANS exposure, which can vary substantially among subjects. Moreover, we have recently shown that RIG-I responses are also attenuated in human preterm neonates, indicating a more global immaturity$^{145}$. However, it is unclear to what extent exogenous perinatal factors may contribute to such global attenuation of innate immune responses in neonates born early in gestation.

Better understanding of the development of innate immune defences at this age requires a more rigorous examination of perinatal exposures to common factors such as ANS or
chorioamnionitis. Here, we applied robust experimental procedures to determine the contribution of prematurity, ANS and chorioamnionitis to the extent of innate immune attenuation observed in preterm neonates born early in gestation.

2.2 Materials and methods

2.2.1 Study population

After written consent, cord blood samples were collected in sodium heparin-anticoagulated Vacutainer tubes (BD Bioscience) from 43 preterm neonates born before 33 weeks of gestation, and from 20 healthy neonates born at term by Caesarean section delivery at the Children’s & Women’s (C&W) Health Centre of British Columbia between July 2009 and July 2012. Clinical characteristics of preterm neonates are shown in table 2.1. Labour was generally defined by vaginal cervical changes in presence of regular uterine contractions preceding delivery. Exposure to ANS was defined according to the timing of the last maternal dose received. Chorioamnionitis was determined by a blind histological examination of at least 5 micro-dissection slides by a medical pathologist, and defined as maternal stage 1 or greater according to validated criteria\textsuperscript{26} described in section 1.2.1.1. Based on i) the half-lives for serum levels and receptor occupancy of acetate betamethasone of up to 9.0+/-2.7 and 14 hours, respectively, in pregnant women \textsuperscript{146, 147, 148}, and on ii) an estimated fetal-to-maternal serum drug ratio at delivery between 0.3 and 0.5 \textsuperscript{146, 147, 148}, and on data in preterm neonates confirming virtual plasmatic drug clearance beyond 48 hours \textsuperscript{149}, the direct effect of a short-term exposure to ANS was assessed by comparing four subgroups: exposed to ANS for less than 12 hours, 12 to 72 hours or more than 72 hours.
Clinical characteristics

<table>
<thead>
<tr>
<th>Clinical characteristic</th>
<th>Preterm neonates (n = 43)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age, average ± SD (weeks)</td>
<td>29 ± 3.0</td>
</tr>
<tr>
<td>Birth weight, average ± SD (g)</td>
<td>1182 ± 480</td>
</tr>
<tr>
<td>Male gender (%)</td>
<td>63</td>
</tr>
<tr>
<td>Histological chorioamnionitis† (%)</td>
<td>28</td>
</tr>
<tr>
<td>Antenatal corticosteroid exposure (n)</td>
<td></td>
</tr>
<tr>
<td>&lt;12 hours</td>
<td>8</td>
</tr>
<tr>
<td>12-72 hours</td>
<td>8</td>
</tr>
<tr>
<td>&gt;72 hours</td>
<td>25</td>
</tr>
<tr>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>Caesarean deliveries (%)</td>
<td>70</td>
</tr>
<tr>
<td>Labour at delivery (%)</td>
<td>58</td>
</tr>
<tr>
<td>Post-natal infection (%)*</td>
<td>35</td>
</tr>
</tbody>
</table>

Table 2.1 Clinical characteristics of preterm neonates included in the experiments conducted in this chapter

2.2.2 Cord blood processing, stimulation and cytokine measures

We used strict procedures for processing of blood samples, TLR stimulation and cytokine measures. To minimize experimental variability, blood was processed within 2 hours of collection. Cord blood mononuclear cells (CMBC) were isolated from whole blood using Ficoll-based density centrifugation. 500,000 CMBCs/well were then stimulated in 96-well, round-bottom plates containing pre-mixed concentrations of TLR4 (LPS, Invivogen #tlrl-3pelps), TLR5 (flagellin, Invivogen #tlrl-pstfla) and TLR7/8 (R848, Invivogen #tlrl-r848) agonists, for 24 hours in a 37°C incubator, at 5% CO₂. Pre-made plates containing batched dilutions of TLR agonists were stored at -80°C for a maximum of 6 months. For standardization, significant batch-to-batch variability over a relatively long study period was excluded by ensuring IL-6 and IL-1β...
responses in term neonates remained within one standard deviation of each other (Figure 2.1). In addition, an equivalent proportion of preterm versus term samples were tested within each of the batches. Finally, responses from each batch of plates were benchmarked on a reference group of healthy adult subjects (data not shown).

RIG-I-like receptor (RLR) dependent responses were determined following stimulation of CBMC from a subgroup of neonates born between 28 and 33 weeks of gestation (n = 13-20 per group) in RPMI-1640 medium supplemented with 10% FBS using 1 μg/mL of the synthetic RIG-I agonist 5’ppp-dsRNA delivered into the cytosol by transfection (LyoVec). To control for RIG-I independent responses, we transfected cells with a similar dsRNA-19mer, which lacks the 5’tri-phosphate group (ctl-dsRNA) required for RIG-I activation. After stimulation, cell culture supernatants were stored at -80°C and cytokines (IL-1β, IL-6, TNF-α, IL-12/23p40) were measured using enzyme-linked immunosorbent assays (ELISA).
Figure 2.1 Low batch related variability in the cytokine production upon stimulation with TLR ligands

IL-1β and IL-6 secretion between 3 batches of pre-made TLR plates in term neonates, after 24 hour stimulation with LPS (TLR4), R848 (TLR7/8) and flagellin (TLR5). The X-axis shows ligand concentrations (ng/mL), whereas the Y-axis shows the concentration of cytokines (ng/mL). Bars represent one standard deviation around the mean.
2.2.3 Statistical analyses

Based on our previous studies, we estimated that a minimum of 6 subjects per chorioamnionitis or ANS exposure subgroup would provide 80% power to detect differences in either IL-1β, IL-6 or IL-12/23p40 LPS responses between preterm and term neonates (p<0.01). In order to obtain this number of subjects in each subgroup, we projected to recruit 42 preterm neonates. Relationships between non-transformed cytokine responses were first examined graphically. In subsequent analyses, peak responses and area under the dose-response curve (AUC) yielded similar results. Therefore, only the AUC-based data are presented.

Differences in TLR responses between term neonates and preterm neonates according to subgroups of ANS and chorioamnionitis exposure were assessed using a Mann-Whitney U test. Differences between TLR/cytokine dose-responses were determined using mixed-model ANOVA. Sex-related differences in responses were determined using an ANOVA. Comparisons between ligand- and cytokine- specific maturation effects were adjusted for multiple comparisons. The effect of gestational age, birth weight and mode of delivery on TLR responses within the preterm group was assessed using correlations. Contributions of prematurity, ANS and maternal/fetal chorioamnionitis were determined separately, using adjusted correlations with responses in a linear regression model for each TLR/cytokine response (using AUC) as predictors, and 95% confidence intervals calculated using the bootstrap. ANS exposure was analysed as a continuous variable (hours) after transformation to log(1/ANC hours) to reduce skewness. For neonates with no ANS exposure ANS, hours was set to the maximum of observed values + K, with K chosen to normalize the resultant distribution on the log scale.
2.3 Results

2.3.1 Hierarchical, gestational age-dependent maturation in TLR responses

To assess the maturation of TLR responses at low gestation, we first compared dose-responses between preterm and term neonates. Most TLR responses were diminished in preterm neonates compared with term neonates (Figure 2.2). Generally, responses to the TLR7/8 agonist R848 were also stronger, except for IL-6, followed by responses to the TLR4 agonist LPS; responses to the TLR5 agonist flagellin were weakest. In all neonates, sex did not significantly affect responses using an analysis of variance and in group comparisons (p>0.1; data not shown). Moreover, within the group of preterm neonates, no significant effect of gestational age, birth weight and mode of delivery was detected on TLR responses (data not shown).

When the contribution of prematurity was considered, we observed a distinct developmental hierarchy (Figure 2.3). When comparing individual responses according to each TLR ligand, R848 cytokine responses were more mature or “term-like”, followed by LPS responses and by flagellin responses. Likewise, when comparing cytokine responses, TNF-α responses were significantly more mature compared with IL-1β and IL-6 responses. Together, these data indicate that neonatal TLR responses follow a distinctive, asynchronous development before 33 weeks of gestation.
Figure 2.2 Lower cytokine production in preterms after stimulation with TLR ligands

Secretion of IL-1β, IL-6, TNFα and IL12p40 was measured after a 24h stimulation of preterm CBMCs with LPS (TLR4), R848 (TLR7/8) and flagellin (TLR5). Cytokine responses were compared between preterm (white symbols) and term neonates (grey symbols). The X-axis
shows ligand concentrations (ng/mL), whereas the Y-axis shows the concentration of the cytokines. Bars represent interquartile range around the median; *p<0.05; **p<0.01; ***p<0.001.

Figure 2.3 Developmental hierarchy in neonatal TLR response maturation

*Immaturity* coefficients (y-axis) are expressed for each ligand-cytokine combination as the difference in the mean area under the curve of dose-response curves in Figure 2.2 between term and preterm neonates, transformed on the log(x + 1) scale. The data represent averaged *Immaturity* coefficients for (A) TLR ligand-specific or (B) cytokine-specific responses between term and preterm neonates with 95% confidence intervals (error bars) calculated using the bootstrap; *p<0.05 after adjusting for multiple comparisons using methods described in ref#151.
2.3.2 Contribution of ANS and chorioamnionitis

To determine the contribution of exogenous perinatal factors to the developmental attenuation in TLR responses, we stratified subgroups of preterm neonates according to rigorous exposure definitions for chorioamnionitis and ANS. Significant differences were observed in TLR responses between term neonates and each of the preterm ANS (Figure 2.4) and chorioamnionitis (Figure 2.5) exposure subgroups, whereas responses were comparable among preterm neonates. We used adjusted correlations to quantify the contribution of exposure to ANS, and maternal or fetal chorioamnionitis to each TLR/cytokine response within 95% confidence intervals. For IL-1β and IL-6, prematurity contributed significantly to all individual TLR responses, whereas, again, the contributions of ANS and either fetal or maternal histological chorioamnionitis were negligible (Figure 2.6). For TNF-α responses as well as for the IL-12/23p40 response to R848, neither prematurity, ANS, nor maternal or fetal chorioamnionitis significantly contributed to the attenuation in preterm neonates. This finding is expected, considering that both TNF-α and R848-induced IL-12/23p40 responses are mature in preterm infants, resulting in very small variability. Overall, we detected a substantial contribution of prematurity whereas no separate contributions of ANS and chorioamnionitis were detected.
Figure 2.4 Influence of ANS exposure on TLR responses in preterm CBMCs

Secretion of IL-1β, IL-6, TNFα and IL12p40 was measured after a 24h stimulation of preterm CBMCs with Median LPS (TLR4), R848 (TLR7/8) and flagellin (TLR5). Data are presented as
box-and-whisker plots (bars represent range) of the area under the curve (AUC; Y-axis) for each of the TLR/cytokine dose-responses from figure 2.2. The groups on the X-axis represent the amount of ANS exposure received by preterms. The number of samples per group are detailed in table 2.1. *p<0.05; **p<0.01; ***p<0.001.
Figure 2.5 Influence of chorioamnionitis on TLR responses in preterm CBMCs

Secretion of IL-1β, IL-6, TNFα and IL12p40 was measured after a 24h stimulation of preterm CBMCs with Median LPS (TLR4), R848 (TLR7/8) and flagellin (TLR5). Data are presented as
box-and-whisker plots (bars represent range) of the area under the curve (AUC; Y-axis) for each of the TLR/cytokine dose-responses from figure 2.2. X-axis is comprised of preterm samples grouped based on the presence or absence of chorioamnionitis. The number of samples per group are detailed in table 2.1. *p<0.05; **p<0.01; ***p<0.001.
Figure 2.6 Contribution of prematurity, histological chorioamnionitis (maternal or fetal) and ANS to TLR responses

Partial correlations adjusted for other factors (i.e.: prematurity, ANS exposure, maternal and fetal chorioamnionitis) (y-axis) with 95% confidence intervals (bars). Calculations for this figure are based on the data from figures 2.2, 2.4 and 2.5.
2.3.3 Attenuation in RIG-I-like receptor responses

Next, we asked whether non-TLR responses were also affected by an exposure to ANS or chorioamnionitis. To this end, we compared cytokine responses to cytosolic delivery of a synthetic ligand of RIG-I (5’ppp-dsRNA), and of a RIG-I-independent control ligand (ctl-dsRNA). We chose these ligands because their signalling pathways are TLR-independent\(^\text{153}\). Responses to these ligands were also profoundly attenuated in preterm neonates. Moreover, this attenuation in RIG-I responses was also largely unaffected by exposure to chorioamnionitis (Figure 2.7A), or to ANS (Figure 2.7B). Interestingly, IL-1β, IL-6 and TNF-α (Figure 2.7) and IFN-α (data not shown) cytokine responses were even more attenuated than TLR responses despite preterm neonates’ being more mature in this analysis (mean GA+/−SD = 30+/−1.6 weeks).
**Figure 2.7 Contribution of chorioamnionitis and prematurity to TLR-independent cytokine responses**

RIG-I-dependent (5’ppp-dsRNA) and control RIG-I-independent (ctl-dsRNA) responses (boxes and whisker graphs) in term (white bars) and preterm neonates (grey bars) (A) with or without histological chorioamnionitis (HC) and (B) exposed to different levels of antenatal corticosteroids (ANS). CBMCs were exposed to 24 hours of stimulation in conditions described in section 2.2. *p<0.05; **p<0.01; ***p<0.001.

### 2.4 Discussion

In order to assess the developmental impact of chorioamnionitis and ANS on innate immune responses, we quantified multiple TLR responses in a sizeable cohort of clinically well-characterized preterm neonates born early in gestation, using highly-standardized experimental procedures and rigorous exposure definitions. Overall, we demonstrate that the maturation of TLR responses before 33 weeks of gestation does not follow a linear continuum. Rather, these
responses mature asynchronously, according to a distinct developmental hierarchy. Moreover, we detect a predominant contribution of prematurity to the extent of developmental attenuation observed in TLR response, whereas the influence of chorioamnionitis and of a short-term exposure to ANS appears negligible. To our knowledge this is the largest dataset reporting on multiple TLR function in very preterm neonates. Our data have important implications for understanding of the specific vulnerabilities to infection observed in preterm neonates.

The hierarchy observed in the maturation of preterm TLR responses is reminiscent of the maturation of other immune functions during development. For example, the development of TLR functions also progresses asynchronously in early childhood\textsuperscript{137}. Likewise, placental transfer of maternal antibodies occurs selectively during pregnancy\textsuperscript{116}. This conserved pattern may reflect a high degree of functional specialization required in humans exposed to an evolving microorganism environment throughout development. The recognition of pathogen using PRRs is a key event in the defence against infections. Consistent with our findings, we observed even greater attenuation in response to LPS in neonates born before 29 weeks of gestation, which may explain the high occurrence of gram-negative bacterial infections in this age group\textsuperscript{139}. In contrast, the more mature R848 responses may indicate potential avenues to enhance immune responses at this early age\textsuperscript{154}.

To our knowledge, our study is the first examining TLR5 (flagellin) responses in preterm neonates. We recently also reported diminished RIG-I responses in preterm neonates born at 28 to 32 weeks of gestation\textsuperscript{145}. Engagement of RIG-I triggers activation of transcription factor NF-κB, and of interferon regulatory factor 3 (IRF3) and IRF7 through a distinct, dsRNA-dependent protein kinase R pathway, independent of TLRs\textsuperscript{155}. The more global attenuation in both TLR and
TLR-independent PRR responses indicate a central regulation either at the level or more downstream of NF-κB.

The marginal influence of chorioamnionitis is consistent with other studies in humans showing that exposure to prolonged rupture of membranes did not affect LPS-induced TNF-α and IL-6 production\(^{156}\). In contrast, exposure to intra-amniotic infection in preterm sheep increases inflammatory responses upon \textit{ex vivo} re-stimulation of cord blood cells\(^{157}\). Likewise, a short-term exposure to ANS in this model profoundly reduced endotoxin-stimulated IL-6 responses\(^{158}\). Several reasons might explain the discrepancies between human studies and animal models, including differences in the type and chronicity of infection. Nonetheless, data pragmatically obtained in human populations are important for interpretation of neonatal immunology studies that mainly use cord blood, as a less invasive source of biological sample from small preterm neonates. \textit{In vitro}, corticosteroids directly affect the function of immune cells as a dexamethasone drug equivalent to the concentration of corticosteroids found in cord blood during maternal treatment with ANS can suppress TLR-induced cytokine responses\(^{143}\). In human preterm neonates, ANS have been associated with lower HLA-DR expression on monocytes\(^{149}\).

However, according to our study such transient decrease in antigen presentation function in cord blood due to ANS exposure is unlikely to be accompanied by significant TLR hypo-responsiveness. The negligible effect of chorioamnionitis and ANS on cord blood responses also implies that exposure to these factors should not be a systematic exclusion in studies, since neonates represent a considerable proportion of the “normal” population.

Our study has limitations. Despite its being the largest study of its kind in very preterm neonates, it was not designed to detect subtle effects of ANS or chorioamnionitis (or a major effect on a minority of preterm neonates) on innate immune responses. This undoubtedly
requires considerably larger sample size, as our present dataset informs us. Furthermore, because only two neonates were not exposed to ANS as a comparison group, we were unable to assess the possibility of sustained effects of ANS exposure; for example, on the differentiation of immune cells, beyond the predicted plasmatic clearance of the drug\textsuperscript{149, 159}. However, the lack of major sustained effect of ANS on preterm immune functions is less likely, considering the results of clinical trials, which demonstrated a reduction (rather than a worsening) of the incidence of neonatal sepsis in neonates born from mothers who received ANS\textsuperscript{141}. Finally in the clinical setting, a number of variables not captured in our analysis may also have affected the maturation of innate immune responses. Specifically, our preterm and term neonates differ substantially in the presence of maternal labour. Previous studies have reported differences in TLR responses with labour in term neonates\textsuperscript{160, 161}. However, no substantial effect was detectable in preterm neonates in our study (data not shown). Severe growth restrictions may also affect innate immune responsiveness in a subset of preterm neonates over the attenuation already resulting from prematurity\textsuperscript{162}.

In conclusion, our study reveals an important developmental characteristic of the neonatal innate immune system. In preterm neonates, PRR responses mature asynchronously, according to a developmental hierarchy that is largely independent of perinatal exposures such as chorioamnionitis and ANS. This study represents a more explicitly defined step forward in our understanding of why human preterm neonates are so vulnerable to infection. Future studies are required to identify potential ways to promote the maturation of neonatal innate immune defences to prevent infections.
Chapter 3: Impaired NLRP3 inflammasome activity during fetal development regulates IL-1β production in human monocytes

3.1 Background

Newborns are at high risk of infections, in part due to attenuated innate immune defences\(^{163}\). The cytokine interleukin-1β (IL-1β) is an important inflammatory mediator in response to infections\(^{164}\). Mice lacking IL-1β display impaired acute phase and pyrogenic responses\(^{165}\), and increased susceptibility to pathogens commonly encountered in the neonatal period\(^{166,167}\). In contrast, high levels of IL-1β in a fetus can result in autoimmune organ damage\(^{164}\), as well as lethal metabolic disturbances including severe weight loss and hypoglycemia\(^{168}\). Together, these data illustrate the evolutionary importance of a tight regulation of IL-1β in order to avoid inflammation-mediated organ damage, neurological injury\(^{169}\) or even premature birth in a developing human\(^{164,170}\).

Monocytes are primarily responsible for the production of IL-1β in circulating blood\(^ {171,172}\). Three subsets predominate in humans: ‘Classical’ monocytes express CD14, but lack expression of the immunoglobulin receptor CD16. These CD14\(^{\text{high}}\)/CD16\(^{\text{neg}}\) monocytes make up the majority of monocytes in peripheral adult blood, and have been proposed to be capable of producing high amounts of IL-10\(^ {76}\). In contrast, the smaller, non-classical CD14\(^{\text{low}}\)/CD16\(^{\text{pos}}\) monocytes express high levels of the antigen-presenting molecule HLA-DR\(^ {79}\), as well as cytokine TNF-α\(^ {80}\). This latter subset has been proposed to play an important immediate responder ‘proinflammatory’ role in sepsis\(^ {76,78,173}\). Finally, a third subset of monocytes expressing high levels of CD14, as well as CD16 (CD14\(^{\text{high}}\)/CD16\(^{\text{pos}}\)) may play a more intermediate role in inflammation\(^ {77}\).
Production of IL-1β differs from that of most other inflammatory cytokines as it requires a dual signal for its activation \(^{164}\). First, intracellular pro-IL-1β is produced following stimulation of pattern-recognition receptors (PRRs) such as the Toll-like receptors (TLRs). Second, in order to be functional pro-IL-1β requires proteolytic cleavage, predominantly by caspase-1, a component of the NLRP3 inflammasome multi-protein complex, resulting in secretion of mature, biologically active IL-1β \(^{174}\). Activation of the NLRP3 inflammasome can be triggered by local mediators of host cell damage in vivo (e.g. free radicals, DNA or adenosine triphosphate: ATP, through the purinoceptor P2X7)\(^{175}\). Earlier studies showed that IL-1β production is markedly reduced in human cord blood monocytes in response to endotoxin (LPS)\(^\text{140,176}\). Caspase-1 processing was also reduced in term neonates\(^{177}\). However, the functional consequence of this reduced caspase-1 processing on neonatal IL-1β responses has never been functionally characterized.

In order to understand the molecular basis for the regulation of IL-1β secretion in monocytes in humans at an early stage in life, we analyzed the developmental variability in the TLR and NLRP3 inflammasome pathways in neonates born late in gestation. Given the need for a tight regulation of IL-1β responses in order to avoid major metabolic disturbances in the fetus, we hypothesized that the NLRP3 inflammasome is regulated in a discrete manner before the term of gestation.

### 3.2 Materials and methods

#### 3.2.1 Recruitment of human subjects and blood sample collection

Cord blood was collected from either healthy neonates born at term or from preterm neonates born between 24 and 32 weeks of gestation at the Children’s & Women’s Health Centre
of British Columbia (C&W). Peripheral blood was collected from healthy adult volunteers and preterm neonates 1 to 28 days of age. Due to ethical limitations, peripheral blood was not obtained from term neonates for comparison of postnatal responses. All blood samples were collected in sodium heparin-anticoagulated Vacutainers (BD Biosciences) and processed within 2 hours of collection. Chorioamnionitis was determined by a blind examination of microdissection slides by a medical pathologist, and was defined as maternal stage 1 or greater. Informed consent was obtained for all subjects. Our study was approved by the C&W Research Ethics Board.

3.2.2 Cell purification, stimulation and cytokine detection

Cord blood and peripheral blood was collected in sodium heparin tubes and processed within 2 hours of collection. CBMC and PBMC were isolated from whole blood using Ficoll-based density centrifugation. High CD14-expressing monocytes were purified from fresh BMCs by positive selection using EasySep™ for Human Monocyte (StemCell, Canada). Each aliquot of purified monocytes was analyzed by flow cytometry using a CD14-PeCy7-conjugated antibody to ensure >95% enrichment. Purified BMC (5.0 x 10⁵ cells/well) or monocytes (5.0 x 10⁵ cells/well) were stimulated for 5 hours with or without lipopolysaccharides (LPS, 10 ng/mL), with or without ATP (5 mM, MP Biomedical), or nigericin (30 μM, Invivogen) added during the last hour of culture, as indicated. These conditions were chosen based on pilot experiments where we determined that pro-IL-1β cytokine expression started as early as 1 hour and peaked ~5 hours after stimulation in all three age groups (data not shown). Secreted IL-1β experiments comparing age groups were also carried out at 24 hours in order to exclude kinetic effects (Figure 3.1A and B). PAM-CSK (Pam3Cys-SKKKK, TLR1/2 agonist) and R-FSL-1
(TLR2/6 agonist) were obtained from EMC microcollection (catalog#L2000 and L7022, respectively). LPS (TLR4 agonist) and R848 (TLR7/8 agonist) were obtained from InvivoGen. Following stimulation, cells were stained for flow cytometry analyses. Cultured cells were washed and stained, in PBS, for 30 minutes with the fluorescent-conjugated antibodies listed in section 3.2.3. This step was carried out in the dark\textsuperscript{139}. IL-1β and IL-6 in culture supernatants were quantified in batches by Enzyme-Linked Immunosorbent Assay (ELISA, eBioscience), in duplicate with coefficients of variability \(<20\% \) (not shown).

![Graphs showing kinetics and dose-dependent responses for IL-1β and IL-6 secretion in preterm, term neonatal and adult BMC](image)

Figure 3.1 Kinetics and dose-dependent responses for IL-1β and IL-6 secretion in preterm, term neonatal and adult BMC

To establish the optimal stimulation time and dose of LPS for this study, BMC were stimulated with LPS (A) for varying times and (B) with varying concentration of LPS, followed by ATP. IL-1β secretion was detected using ELISA. Peak response was obtained at 5 hours of stimulation using 10 ng/mL LPS. Preterm subjects in (A) and (B) were 26 to 30 weeks of gestation. (C) IL-6 protein secretion (ELISA) in BMC culture supernatants after LPS stimulation. Preterm subjects for this figure were $28.7 \pm 1.8$ weeks of gestation (mean $\pm$ SD). Data represent mean $\pm$ SEM ($n = 6$ subjects per group); \textbf{**p}<0.01.
3.2.3 Detection of intracellular IL-1β production and caspase-1 activation

Subsets of monocytes were characterized by staining fresh BMCs (stimulated or controls) with fluorescent-conjugated monoclonal antibodies against the cell surface markers CD14 (PE-Cy7), CD16 (eFluor 450), CD33 (PE; all from eBioscience), HLA-DR (PerCp-Cy5.5, BD Biosciences), CD33 (PE, eBioscience), CD11c (AF 700, BD Pharmingen), a rabbit polyclonal antibody against the P2X7-receptor (FITC; Alomone Labs, Israel), or against intracellular IL-1β (FITC- or Alexa fluor-647-conjugated, eBioscience). In preliminary experiments, caspase-1 activation peaked within 60 min of ATP stimulation in preterm, term and adult monocytes; therefore, all caspase-1 activation was assayed after no more than one hour in order to maximize response but minimize apoptosis (not shown). For detection of intracellular IL-1β, cells were stained using staining buffer (eBioscience). For caspase-1 activation (expressed as percentage activated cells), cells were stained using the FITC-conjugated Fluorescent-Labeled Inhibitor of Caspase-1 Activity (FLICA) Z-YVAD (ABD Serotec) (Figure 3.2). FLICA was added to the cell culture 60 min before the end of stimulatory period. After post-stimulation washing, FLICA stained cells were surface stained. Data were acquired immediately after staining of cells on an LSR-II™ flow cytometer (Becton Dickenson) or on an ImageStreamX imaging instrument (Amnis Corporation). For data acquisition, standard voltage settings were set for each experiment using cells, including fluorescence-minus-one (FMO) controls. Signals were compensated using single color positive and negative control CompBeads (BD Biosciences). Flow cytometry data were analyzed with FlowJo vX.0.7 (TreeStar Inc., OR) for Windows (Microsoft, WA).
Figure 3.2 Detection and kinetics of caspase-1 activation

(A) Representative flow microscopy visualization (40X magnification) of intracellular IL-1β (red) and caspase-1 activity (green) in high CD14-expressing adult monocytes (BMC) after stimulation with 10ng/mL of LPS (5mM of ATP added in the last hour); (B) Representative flow
cytometry histograms of caspase-1 activity after LPS stimulation (5 hours) with ATP added for
the last hour of culture.

3.2.4 Real-time PCR experiments

Primer sequences used for PCR amplification are provided in Table 3.1 and 3.2. For
*NLRP3* gene expression, experiments were carried out in CD14-magnetic column purified
monocytes. For *IL1B* gene expression, because expression of the IL-1β protein was only
significantly detected within high CD14-expressing monocytes and due to often limited
obtainable blood volumes, expression experiments were carried out in whole blood. In this case
whole blood was mixed 1:1 with RPMI medium. Following stimulation, samples (cell pellets)
were immediately frozen (dry ice) and stored at -80°C for batch analyses. Cycloheximide (10
μg/mL, Sigma Aldrich) was added at the beginning of stimulation as indicated. Upon analysis,
mRNA was extracted using the TRIzol LS method (Invitrogen), followed by an RNeasy column
cleanup step (Qiagen). DNase (Qiagen) I-treated RNA was reverse transcribed into cDNA using
the High Capacity cDNA RT kit (Applied Biosystems). Real-time PCR was performed on a
LightCycler® 480 System (Roche), using the LightCycler® SYBR Green I Master (Roche). All
gene amplifications were performed in triplicates. Sample cDNA copy numbers were calculated
based on the standard curve generated by serial dilutions of DNA plasmids containing each gene
studied, using the second derivative amplification threshold values (Ct). For *NLRP3*, real-time
PCR was performed on a ViiA 7 System (Applied Biosystems). PCR efficiencies were similar
(>1.95) for all genes. Inter-replicate coefficients of variability were <10%. Gene expression
levels were normalized to that of β-actin (*IL1B: ACTB*). Similar results were obtained whether
normalizing gene expression to *ACTB* or *GAPDH* (not shown).
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1B Forward</td>
<td>5’- GGC CCT AAA CAG ATG AAG TGC TCC TCC-3’</td>
</tr>
<tr>
<td>IL1B Reverse</td>
<td>5’-GGT GCT CAG GTC ATT CTC CTG G-3’</td>
</tr>
<tr>
<td>BACT Forward</td>
<td>5’-TCC TAT GTG GGC GAC GAG G-3’</td>
</tr>
<tr>
<td>BACT Reverse</td>
<td>5’-GGT GTT GAA GGT CTC AAA CAT G-3’</td>
</tr>
<tr>
<td>GAPDH Forward</td>
<td>5’-CGT CAA GGC TGA GAA CGG GA-3’</td>
</tr>
<tr>
<td>GAPDH Reverse</td>
<td>5’-ATC AGC AGA GGG GGC AGA GA-3’</td>
</tr>
</tbody>
</table>

Table 3.1 Sequences of primers used for SYBR green based real-time PCR experiments

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer and Probe sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLRP3 Forward</td>
<td>5’- GTG TTT CGA ATC CCA CTG TG-3’</td>
</tr>
<tr>
<td>NLRP3 Reverse</td>
<td>5’- TCT GCT TCT CAC GTA CTT TCT G-3’</td>
</tr>
<tr>
<td>NLRP3 Probe</td>
<td>56-FAM-TCCTCTTCA/ZEN/ATGCTGTCTTCCCTGGC-3IBkFQ</td>
</tr>
<tr>
<td>BACT Forward</td>
<td>5’- ACC TTC TAC AAT GAG CTG CG-3’</td>
</tr>
<tr>
<td>BACT Reverse</td>
<td>5’- CCT GGA TAG CAA CGT ACA TGG-3’</td>
</tr>
<tr>
<td>BACT Probe</td>
<td>56-FAM-ATCTGGGTC/ZEN/ATCTTCTCGCGTGTG-3IBkFQ</td>
</tr>
</tbody>
</table>

Table 3.2 Sequences of primers and probes used for Taqman based real-time PCR experiments
3.2.5 Western blot experiments

Following incubation, cells (5.0 x 10^5 cells) were washed once with PBS and lysed in 10% SDS with β-mercaptoethanol (Sigma Aldrich) followed by immediate boiling at 95°C for 2 min before storage at -80°C. Samples were run on SDS-PAGE electrophoresis, wet-transferred onto polyvinylidene fluoride (PVDF) membranes (BioRad) that were then blocked in skim milk for 1h\textsuperscript{178}. After blocking, membranes were incubated with 1:1000 dilution (5% bovine serum albumin in TBS-T) of primary antibodies against cleaved (Cell Signalling), whole IL-1β (Santa Cruz), caspase-1 (Cell Signalling), NLRP3 (Adipogen), ASC (Adipogen) or β-actin (Cell Signalling; used at 1:2 000 dilution) overnight at 4°C. After 3 washes, the blots were probed with anti-mouse goat IgG (LI-COR) or anti-rabbit donkey IgG (LI-COR) secondary antibodies (1:10 000 in TBS-T), as indicated, conjugated with fluorescent dyes (IRDye 680 or IRDye 800 CW; LI-COR Biosciences), for 1 hour at room temperature. Blots were imaged using the LI-COR Odyssey infrared imaging system\textsuperscript{178}.

3.2.6 Statistical analyses

A two-sided Mann-Whitney U Test was used for all group comparisons, assuming a non-parametric distribution. In univariate analyses of clinical factors associated with caspase-1 activity, we included 21 subjects with a minimum of 6 subjects per group (clinical characteristics of subjects have been provided in table 3.3) for binary variables (i.e. exposure to antenatal corticosteroids, multiple gestation, histological chorioamnionitis) to provide 80% power (p<0.05). For multivariate analyses of factors influencing caspase-1 activity, adjustment for the following co-variables: presence of maternal or fetal chorioamnionitis stage one or greater,
gestational age was performed using SPSS Statistics 20 (IBM, CA), with ‘caspase-1 activity’ (%-positive cells) as the dependent variable.
<table>
<thead>
<tr>
<th>ID</th>
<th>GA (weeks)</th>
<th>BW (g)</th>
<th>Gender</th>
<th>Mode of delivery</th>
<th>Twin?</th>
<th>ANS</th>
<th>CA</th>
<th>Caspase -1 (%)</th>
<th>Reason for delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28</td>
<td>128</td>
<td>F</td>
<td>C/S</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>34</td>
<td>Preterm labour due to cervical incompetence</td>
</tr>
<tr>
<td>2</td>
<td>28.7</td>
<td>114</td>
<td>F</td>
<td>Vaginal</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>6</td>
<td>Preterm labour with prolonged rupture of membranes</td>
</tr>
<tr>
<td>3</td>
<td>29.4</td>
<td>985</td>
<td>M</td>
<td>C/S</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>39</td>
<td>Pregnancy-induced hypertension</td>
</tr>
<tr>
<td>4</td>
<td>29.3</td>
<td>143</td>
<td>F</td>
<td>C/S</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>71</td>
<td>Prolonged rupture of membranes</td>
</tr>
<tr>
<td>5</td>
<td>27.9</td>
<td>123</td>
<td>M</td>
<td>C/S</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>47</td>
<td>Undetermined fetal distress</td>
</tr>
<tr>
<td>6</td>
<td>27</td>
<td>103</td>
<td>F</td>
<td>C/S</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>21</td>
<td>Preterm labour with prolonged rupture of membranes</td>
</tr>
<tr>
<td>7</td>
<td>31.4</td>
<td>191</td>
<td>F</td>
<td>C/S</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>74</td>
<td>Idiopathic preterm labour</td>
</tr>
<tr>
<td>ID</td>
<td>GA (weeks)</td>
<td>BW (g)</td>
<td>Gender</td>
<td>Mode of delivery</td>
<td>Twin?</td>
<td>ANS</td>
<td>CA</td>
<td>Caspase -1 (%)</td>
<td>Reason for delivery</td>
</tr>
<tr>
<td>----</td>
<td>------------</td>
<td>--------</td>
<td>--------</td>
<td>------------------</td>
<td>-------</td>
<td>-----</td>
<td>----</td>
<td>----------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>8</td>
<td>31.4</td>
<td>1825</td>
<td>M</td>
<td>C/S</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>75</td>
<td>Idiopathic preterm labour</td>
</tr>
<tr>
<td>9</td>
<td>30.7</td>
<td>1460</td>
<td>F</td>
<td>Vaginal</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>69</td>
<td>Idiopathic preterm labour</td>
</tr>
<tr>
<td>10</td>
<td>31</td>
<td>1090</td>
<td>M</td>
<td>C/S</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>60</td>
<td>Pregnancy-induced hypertension</td>
</tr>
<tr>
<td>11</td>
<td>29</td>
<td>1482</td>
<td>F</td>
<td>Vaginal</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>16</td>
<td>Preterm labor with clinical chorioamnionitis</td>
</tr>
<tr>
<td>12</td>
<td>28.3</td>
<td>1100</td>
<td>F</td>
<td>Vaginal</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>55</td>
<td>Preterm labour post-twin reduction</td>
</tr>
<tr>
<td>13</td>
<td>26.6</td>
<td>860</td>
<td>M</td>
<td>C/S</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>35</td>
<td>Preterm labour with prolonged rupture of membranes</td>
</tr>
<tr>
<td>14</td>
<td>32.7</td>
<td>1150</td>
<td>F</td>
<td>Vaginal</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>7</td>
<td>Placental abruption</td>
</tr>
<tr>
<td>ID</td>
<td>GA (weeks)</td>
<td>BW (g)</td>
<td>Gender</td>
<td>Mode of delivery</td>
<td>Twin?</td>
<td>ANS</td>
<td>CA</td>
<td>Caspase -1 (%)</td>
<td>Reason for delivery</td>
</tr>
<tr>
<td>----</td>
<td>------------</td>
<td>--------</td>
<td>--------</td>
<td>-----------------</td>
<td>-------</td>
<td>-----</td>
<td>----</td>
<td>----------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>15</td>
<td>30.7</td>
<td>143</td>
<td>M</td>
<td>Vaginal</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>57</td>
<td>Idiopathic preterm labour</td>
</tr>
<tr>
<td>16</td>
<td>30.7</td>
<td>120</td>
<td>F</td>
<td>Vaginal</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>72</td>
<td>Idiopathic preterm labour</td>
</tr>
<tr>
<td>17</td>
<td>26</td>
<td>919</td>
<td>F</td>
<td>C/S</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>65</td>
<td>Placental abruption</td>
</tr>
<tr>
<td>18</td>
<td>25.4</td>
<td>650</td>
<td>M</td>
<td>C/S</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>40</td>
<td>Fetal compromise/IUGR due to placental insufficiency</td>
</tr>
<tr>
<td>19</td>
<td>31.9</td>
<td>179</td>
<td>F</td>
<td>C/S</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>50</td>
<td>Idiopathic preterm labour</td>
</tr>
<tr>
<td>20</td>
<td>32.1</td>
<td>193</td>
<td>M</td>
<td>C/S</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>54</td>
<td>Idiopathic preterm labour</td>
</tr>
<tr>
<td>21</td>
<td>32.1</td>
<td>166</td>
<td>M</td>
<td>C/S</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>53</td>
<td>Idiopathic preterm labour</td>
</tr>
</tbody>
</table>

Table 3.3 Clinical characteristics and caspase-1 activity in monocytes of preterm neonates with or without histological chorioamnionitis

GA: Gestational age; BW: Birth Weight; CA: Chorioamnionitis; ANC: Antenatal corticosteroids received <24 hours prior to delivery, defined from the timing of last dose; Caspase-1 activity was measured using flow cytometry, as percentage of high CD14-expressing monocytes.
3.3 Results

3.3.1 Immature monocytes in cord blood before the term of gestation

Data are greatly lacking regarding the ontogeny of monocyte subsets in humans. To address this, we first analyzed subsets of monocytes in preterm, term and adult subjects, using standard flow cytometry gating strategy, as defined previously (Figure 3.3A). Expectedly, cumulative proportions of monocytes were generally preserved in cord blood relative to blood mononuclear cells (BMC) (Figure 3.3B). Also, monocytes from all three age groups displayed comparable expression of the myeloid markers CD33 and CD11c, whereas expression of HLA-DR was reduced in preterm cord blood (Figure 3.4). Notably, we observed a predominance of CD14<sub>low</sub>/CD16<sub>pos</sub> monocytes in preterm CBMC (Figure 3.3C), and this subset of monocytes did not express any detectable intracellular pro-IL-1β upon LPS stimulation, suggesting that they represent an immature form (Figure 3.3D).

In contrast, when testing the functional capacity of the cord blood high CD14-expressing subset of monocytes, both CD16<sub>pos</sub> and CD16<sub>neg</sub> subsets expressed levels of intracellular pro-IL-1β comparable (if not higher than) to that of adult peripheral blood (Figure 3.3D). In these cells, IL-1β secretion was also profoundly reduced at decreasing gestation and was not restored following co-activation of the inflammasome (Figure 3.5A). To exclude a contribution of low CD14-expressing monocytes to IL-1β secretion, negatively purified monocytes were similarly assayed, and yielded identical results (not shown). This lack of mature IL-1β secretion at decreasing gestation was also observed regardless of the TLR engaged (Figure 3.5B). Altogether, these data demonstrate a profound impairment of IL-1β production in high-CD14-expressing preterm monocytes despite intracellular detection of pro-IL-1β.
Figure 3.3 Phenotype of human monocytes comparing cord blood to adults

(A) Representative flow cytometry gating strategy used to identify monocytes subsets. (B) Cumulative percentage of monocytes relative to BMCs in preterm, term and adult subjects. (C) Proportion of monocytes subsets according to CD14 and CD16 expression in BMCs. (D) Intracellular pro-IL-1β production following LPS induction, in each monocyte subset; top panels display representative pro-IL-1β flow cytometry histograms for each subset. (B-D) Preterm subjects were 28.6±2.6 weeks of gestation (mean±SD). (B-D) Data are representative of at least 2 independent experiments performed using batch reagents and standardized flow cytometry voltage settings, where n = 6-12 subjects were included per age group. Bars represent medians. MFI: Mean Fluorescence Intensity; *p<0.05; **p<0.01; ***p<0.001, Mann-Whitney U test.
Figure 3.4 Cell surface phenotype of cord blood and adult monocytes

(A) Representative flow cytometry histogram of CD11c, HLA-DR and CD33 expression (all monocyte gate) ex vivo in preterm, term and adult subjects. (B) CD11c and HLA-DR expression in high CD14- expressing monocytes. Preterm subjects tested for (B) were 28.6 ± 2.6 weeks of gestation (mean ± SD). Bars represent median. MFI: Mean Fluorescence Intensity.
Figure 3.5 TLR-induced IL-1β responses in high CD14-expressing monocytes

(A) Secreted IL-1β in culture supernatants of positively selected CD14-expressing monocytes after stimulation with LPS ± ATP was measured by ELISA. Box and whiskers graph showing median + interquartile range. (B) Secreted IL-1β in culture supernatants of BMCs after stimulations with other TLR agonists [in brackets]. Data are shown as mean±SEM and are representative of one experiment performed, which included 4-6 subjects per group. (A and B) Preterm subjects were 28.8±1.8 weeks of gestation (mean±SD) and between 26 and 30 weeks, respectively. *p<0.05; **p<0.01, Mann-Whitney U test.
3.3.2 Reduced TLR-induced IL-1β response in high CD14-expressing preterm cord blood monocytes

As shown above, pro-IL-1β was detectable in high CD14-expressing preterm monocytes. To better understand the molecular basis for the developmental inability of monocytes to secrete IL-1β, we examined whether expression of the *IL1B* transcript was induced following LPS stimulation. Indeed, expression of the *IL1B* gene was significantly reduced at lower gestation (Figure 3.6A). In parallel experiments, secretion of IL-1β in whole blood was also markedly impaired indicating that the gestational age-dependent attenuation in IL-1β secretion is not due to a lack of serum factor(s) excluded during the BMC purification (Figure 3.6B). Treatment of cells with cycloheximide prior to LPS stimulation also completely abrogated secretion of IL-1β, consistent with a *de novo* requirement for protein expression following LPS challenge, in order to produce mature IL-1β in all three age groups (Figure 3.6C). Moreover, production of the inflammasome-independent cytokine IL-6 was similarly impaired, consistent with a hyporesponsive response intrinsic to the TLR component of the TLR/inflammasome pathway in cord blood monocytes (Figure 3.1C).
Figure 3.6 TLR-induced cytokine gene and protein expression are impaired in preterm neonates

(A) Kinetics of *IL1B* mRNA expression following LPS stimulation was measured by real-time PCR. Data represent the ratio of *IL1B* to *ACTB* (β-actin) transcript copy numbers of experiments performed on whole blood samples in duplicates (solid lines = LPS stimulation; dashed lines = unstimulated cells). (B) Kinetics of IL-1β protein secretion in culture supernatants from subjects simultaneously tested in (A) was measured by ELISA. Levels of statistical significance were only indicated for comparisons with preterm subjects. (A and B) Data are shown as mean ± SEM (n=6 subjects per group) and are representative of 2 independent experiments performed. Preterm subjects for this figure were 28.7±1.8 weeks of gestation (mean±SD). (C) Box and whiskers graph showing median + interquartile range of LPS-induced IL-1β secretion with or without cycloheximide (CH) added at the beginning of stimulation. IL-1β concentration was measured by ELISA. Preterm subjects were 28.8±1.8 weeks of gestation. Data are shown as mean ± SD (n=4-6 subjects per group) and are representative of one experiment performed. *p<0.05; **p<0.01, Mann-Whitney U test.
3.3.3 Adult-like production of pro-IL-1β in high CD14-expressing preterm neonatal monocytes

To further validate the production of pro-IL-1β in high CD14-expressing cells, despite their lack of IL-1β secretion, we used a combination of western blot (full uncut western blots are provide in Figure 3.7), flow cytometry and ELISA experiments. Western blot experiments confirmed a reduction in secreted IL-1β in culture supernatants after LPS/ATP stimulation (Figure 3.8A). Western blot experiments also confirmed the marginal reduction in pro-IL-1β in LPS-stimulated preterm monocytes, compared to term and adult monocytes (Figure 3.8A), after normalization to β-actin (Figure 3.8B). However, because western blots are not ideal for quantifying proteins, we combined this method with the more quantitative flow cytometry detection. Importantly, when combining age groups and stimulation conditions in parallel experiments, we confirmed a high correlation between the amount of precursor pro-IL-1β protein detected in purified monocytes by western blot, and the pro-IL-1β protein detected by flow cytometry (Spearman’s coefficient $r = 0.88; p<0.0001; $ Figure 3.8C). With this result, we also demonstrate that our flow cytometry experiment is indeed detecting mainly pro-IL-1β in its precursor form. Then, using flow cytometry, we confirmed that LPS-induced preterm monocytes can produce levels of pro-IL-1β comparable to adults (Figure 3.8D, E), despite a complete lack of secreted IL-1β at the lower gestational ages tested (Figure 3.8F). These results confirm that pro-IL-1β accumulates intracellularly and is unable to be secreted in preterm cord blood monocytes.
Experiments were done in parallel and represent the blot presented in (A) figure 3.8A (pro-IL-1β/IL-1β and β-actin) and (B) figure 3.9A (proCaspase-1/Caspase-1). (A) First (top) panel shows pro-IL-1β and cleaved IL-1β in cell lysates from positive depleted CD14-monocytes. Second (middle) panel shows the levels of pro-IL-1β and cleaved IL-1β within supernatants from the same monocytes. Third (bottom) panel represents re-hybridization of the blot used in the first (top) panel with β-actin. (B) First (top) panel shows the levels of procaspase-1 and cleaved caspase-1 in cell lysates from monocytes. Second (middle) panel shows the levels of cleaved caspase-1 within supernatants from the same monocytes. Third (bottom) panel represents re-hybridization of the blot used in the first (top) panel with β-actin. U: Unstimulated, L: LPS, LA: LPS and ATP. Preterm subject in this figure was 30 weeks of gestation.
Figure 3.8 TLR-induced cytokine gene and protein expression are impaired in preterm neonates

(A) Representative western blot showing pro-IL-1β/IL-1β production in cell lysates of positively selected monocytes (top panel) or in culture supernatants (bottom panel) after stimulation with LPS±ATP, or in unstimulated. Results are from one experiment representative of three independent experiments performed. β-actin was used as a loading control. (B) Densitometry of pro-IL-1β normalized on β-actin from all three western blots including 3 preterm subjects born at 24, 26 and 30 weeks of gestation. Data represent 3 independent experiments. (C) Correlation between pro-IL-1β detected by western blot versus flow cytometry in positively selected monocytes based on parallel experiments combining preterm, term and adults (Spearman’s coefficient r=0.88; p<0.0001). (D) Detection of intracellular pro-IL-1β (FITC) and (E) detection
of intracellular pro-IL-1β (Alex Fluor-647; dashed line = detection in fluorescence minus-one staining controls) after LPS stimulation, in high CD14-expressing monocytes, by flow cytometry. (F) Secreted levels of IL-1β (ELISA) after LPS/ATP stimulation in preterm subjects (BMCs) categorized by gestational age. (D-F) Data are representative of at least 3 independent experiments performed, where up to 12-25 subjects per group were included. Bars represent medians. MFI: Mean Fluorescence Intensity; *p<0.05; **p<0.01; ***p<0.001, Mann-Whitney U test.

3.3.4 Lack of NLRP3 induction in preterm monocytes following TLR stimulation

We next asked whether the activity of the NLRP3 inflammasome was impaired in preterm cord blood monocytes. First, we determined whether both pro-caspase-1 (Figure 3.9A, B) and the upstream P2X7 receptor, whose activation is required for assembly of the NLRP3 inflammasome, were expressed in comparable levels in high CD14-expressing preterm, term and adult monocytes; and this was the case (Figure 3.9C). In addition, secretion of IL-1β by preterm monocytes was not restored using nigericin, which bypasses the need for activation of the P2X7 receptor for IL-1β production\textsuperscript{180}, suggesting that the developmental limitation in production of this cytokine lies downstream of the purinoceptor (Figure 3.9D). Others have shown that NLRP3 expression is inducible following TLR stimulation and its upregulation is necessary in order to activate caspase-1 and to produce mature IL-1β\textsuperscript{181}. When analyzing preterm cord blood monocytes, TLR-induced NLRP3 upregulation was substantially impaired upon activation of cells with LPS at lower gestation; this finding was evidenced using real-time PCR, where induction of NLRP3 after LPS versus unstimulated was significantly greater in adults compared to preterm subjects (Figure 3.9E). Results were also validated at the protein level in western blot
(Figure 3.9F). In comparison, expression of ASC was maintained (Figure 3.9F). These results suggested that the TLR-mediated induction of NLRP3 in high CD14-expressing monocytes is limiting activation of the caspase-1 NLRP3 inflammasome during gestation. Furthermore, our data provide a novel regulatory mechanism potentially limiting IL-1β responses downstream of TLR/NF-κB activation and pro-IL-1β protein expression, during gestation.
Figure 3.9 Impaired NLRP3 induction in preterm neonatal monocytes

(A) Representative western blot of pro-caspase-1 (Pro-Casp-1) processing into active caspase-1 (Casp-1) after LPS±ATP in preterm, term and adult positively selected CD14-expressing monocytes. Results are from one experiment representative of three independent experiments performed. β-actin was used as a loading control. (B) Ratio of pro-caspase-1 over β-actin band intensity as detected by western blot in three independent experiments, including preterm
subjects born at 24, 26 and 30 weeks of gestation. (C) Expression of P2X7R on high CD14-expressing monocytes after LPS induction; Representative flow cytometry histogram (lower panel in C); MFI: Mean Fluorescence Intensity. (D) IL-1β secretion (ELISA) after LPS±ATP or nigericin. (E) Real time PCR analysis of NLRP3 mRNA expression (fold-change compared to unstimulated) after stimulation of positively selected CD14-expressing monocytes with or without LPS; Data is normalized against ACTB. Data is shown as mean±SEM and is representative of one real-time PCR experiment performed in triplicates, including 8 adults, 8 term and 6 preterm subjects born between 24 to <28 weeks of gestation. (F) Representative western blot showing protein validation of NLRP3 and ASC expression following LPS stimulation in positively selected CD14-expressing monocytes (from a preterm neonate 27 weeks of gestation). Results are from one experiment representative of three independent experiments performed (left panel). β-actin was used as a loading control. Densitometry of NLRP3 (middle panel) and ASC (right panel) quantification by western blots (normalized on β-actin) in positively selected CD14-expressing monocytes with or without LPS. Data represent 3 independent experiments performed. Bars represent medians, n = 3 different subjects per group. *p<0.05; **p<0.01; ***p<0.001, Mann-Whitney U test, except for (E) where an ANOVA was used with a posttest Bonferroni correction.

3.3.5 Developmental lack of caspase-1 activity early in the third trimester of gestation

We use flow microscopy to determine the impact of the developmental lack of TLR-mediated NLRP3 induction on the levels of active caspase-1 (Figure 3.2). In parallel experiments, LPS/ATP-mediated activation of caspase-1 was well correlated with IL-1β secretion in BMC culture supernatants in all three age groups (Spearman’s coefficient r = 0.69;
p<0.0001; Figure 3.9A). On the other hand, in preterm neonates there was no correlation between LPS-mediated production of pro-IL-1β and LPS/ATP-mediated IL-1β secretion. IL-1β secretion remained low even at high levels of intracellular pro-IL-1β, suggesting a lack of active caspase-1 (Figure 3.9B). To confirm this hypothesis, we quantified both production of intracellular pro-IL-1β and levels of active caspase-1 on a per-cell basis, gating on high CD14-expressing monocytes. In term neonates, peak active caspase-1 levels were comparable to adult levels. However, these levels became significantly impaired below 29 weeks of gestation, reaching undetectable levels at 24 to <27 weeks of gestation (Figure 3.10C, D). Overall, our results show that activation of caspase-1 is profoundly limited in high CD14-expressing monocytes from preterm cord blood, leading to accumulation of pro-IL-1β at lower gestation.
Figure 3.10 Low levels of active caspase-1 in CD14-expressing preterm monocytes

(A) Linear regression (solid line; dashed lines = 95%CI) between caspase-1 activity (flow cytometry, gated on high CD14-expressing cells) and secreted IL-1β (ELISA), after stimulation with LPS+ATP. (B) Linear regressions (solid lines; dashed lines with shaded areas = 95%CI) between LPS-stimulated pro-IL-1β in high CD14-expressing monocytes and LPS+ATP-stimulated IL-1β production in culture supernatants (ELISA). (A and B) Symbols represent individual subjects. (C) Flow microscopy detection (40X magnification) of active caspase-1 (green) following LPS+ATP (representative neonatal and adult subjects) and unstimulated control cells (gated on high CD14-expressing monocytes). Third column shows caspase-1 activity superimposed on bright field cell image. (D) Caspase-1 activity after LPS+ATP stimulation. Bar represents median. Box and whisker graph shows median and interquartile range, n = 6-13 subjects per group. Data are pooled from independently performed experiments.
*p<0.05, Mann-Whitney U test. (A, B) Data are pooled from 7 independent experiments where pro-IL-1β (flow cytometry), IL-1β (ELISA) and caspase-1 activity (flow cytometry) were measured in parallel. MFI: Mean Fluorescence Intensity.

3.3.6 **Placental infection contributing to developmental lack of inflammasome activity during gestation**

In the next series of experiments, we sought to identify clinical factors that may impact the levels of active caspase-1 in human cord blood monocytes. To do so, we measured caspase-1 activation in a larger group of preterm neonates (n = 21). Upon examination of factors commonly associated with prematurity (i.e. chorioamnionitis, twin delivery, use of antenatal corticosteroids), we found significantly lower active caspase-1 levels in monocytes from preterm subjects with histological chorioamnionitis (Figure 3.11A), whereas the former was not affected by twin delivery or use of antenatal corticosteroids (p>0.2; details of cases provided in Table 3.3). In regression analyses, the association between levels of active caspase-1 and chorioamnionitis in preterm subjects remained statistically significant after adjusting for gestational age, indicating an independent effect (r² = 0.69; p<0.0001). Our results implicate a role for fetal infection regulating activity of the NLRP3 inflammasome in utero.
Figure 3.11 Intra-uterine and postnatal maturation in inflammasome activity

(A) Levels of active caspase-1, in monocytes (gated on high CD14-expressing cells; using FLICA), of preterm subjects with or without histological chorioamnionitis. Caspase-1 activity was measured after stimulation of BMCs with LPS+ATP. For this panel, preterm subjects were 28.2±1.0 and 29.5±2.1 weeks of gestational age (mean±SD) with and without chorioamnionitis, respectively (flow cytometry). (B) IL-1β secretion in whole peripheral blood stimulated with a supra-saturating dose of LPS (100 ng/mL) (ELISA). The blood for these studies was obtained from preterm subjects born 24 to <29 weeks of gestation (white squares), sampled at 7 to 28 days of age, compared to healthy adults. Data are pooled from 5 independent experiments performed. Symbols represent individual subjects, bar represents median, n = 5-10 subjects per group

***p<0.001, Mann-Whitney U test.
3.3.7 Production of IL-1β rapidly matures after birth in preterm neonates

Finally, we asked how long does the developmental attenuation in IL-1β secretion persists after birth? To this end, we directly examined the most downstream functional outcome of IL-1β secretion as the main outcome, on peripheral blood of preterm neonates (mean±SD: 15±11 [range 7-35] days of postnatal age). Responses obtained similarly in adults were used as a reference. In neonates born between 24 and 29 weeks of gestation, secretion of IL-1β, upon LPS/ATP stimulation, was heterogeneous but similar to adults during the neonatal period (Figure 3.11B). These data suggest that the developmental impairment in IL-1β secretion is restored after birth.

3.4 Discussion

Earlier studies have established that cord blood monocytes are impaired in their ability to produce IL-1β upon endotoxin stimulation\textsuperscript{176}. However, due to significant ethical challenges in studying neonatal subjects, the developmental aspects, as well as the mechanism(s) regulating IL-1β responses in humans remained unexplored at this early stage of life. In this study, we systematically investigated main rate-limiting components along the TLR and inflammasome pathways leading to the processing and secretion of IL-1β. The reduction in IL-6, IL1B gene and NLRP3 gene expression suggest a general decrease in TLR signalling (signal 1) due to a gestational age-dependent immaturity of transduction pathway(s) or deficient TLR expression, as reported\textsuperscript{133}. However, our results implicate an additional level of regulation in the inflammasome due to a lack of NLRP3/caspase-1 activation (signal 2). Overall, our data provide novel insights
into key mechanisms regulating IL-1β responses during fetal life. To our knowledge, our study is the first to functionally assess the inflammasome developmentally in human neonates.

During development, monocytes appear in circulating blood as early as the first trimester of gestation. Our findings that cord blood CD16\textsuperscript{pos} monocytes are abundant in late (third trimester) gestation are consistent with data from others\textsuperscript{182, 183}. Yet, we provide new evidence for a functional immaturity of monocytes at this stage. High expression of CD16 on monocytes has been shown to correlate with increased clearance of pathogens at the site of infection\textsuperscript{76, 78, 79, 80}, which may help compensate for the lack of IL-1β in the early neonatal period. On the other hand, the lack of LPS-mediated pro-IL-1β response in CD14\textsuperscript{low} monocytes potentially represents an early developmental stage at which monocyte IL-1β responses are dispensable. Of note, CD14 is required for TLR4 signalling, which may, in part, explain the lack of response of CD14\textsuperscript{low} monocytes to LPS\textsuperscript{184, 185}. Our data may indicate potential developmental preferences for certain responses at the expense of others, in order to maximize survival of the fetus.

Recently, processing of the caspase-1 enzyme was shown to be reduced in term neonates, although without a functional characterization of the inflammasome activity it was impossible to determine its impact on the production of IL-1β\textsuperscript{177}. In fact, adult-like levels of caspase-1 activity were detected in term neonates in our study, suggesting that the reduction in caspase-1 cleavage previously reported in term neonates is functionally negligible\textsuperscript{177}. A key finding of our research is that preterm monocytes express levels of the pro-IL-1β precursor protein similar to adult monocytes upon TLR stimulation, despite a marked reduction in IL1B gene expression. Previous studies have shown dissociation between pro-IL-1β protein expression such that protein levels can be maintained in human monocytes over important variations in IL1B transcript levels due to
posttranscriptional regulatory events\textsuperscript{186, 187}. This characteristic likely warrants an additional level of suppression of IL-1\(\beta\) responses through a regulation of caspase-1, as shown in our study.

Indeed, excessive inflammation can have major adverse health consequences in developing fetuses\textsuperscript{163}. In sheep, intra-amniotic endotoxin causes an increase in proinflammatory gene expression in the lungs\textsuperscript{188}. This strong inflammatory response parallels an increase in local inflammatory cell infiltration, resulting in IL-1-mediated organ injury\textsuperscript{188}. Hence, responses triggered by microbial pathogens during fetal life, may cause tissue damage and oxidative stress which can be equally damaging during critical phases of organ development\textsuperscript{164}. The harmful effects of an excessive production of IL-1\(\beta\) at this age is also evidenced from humans with rare mutations causing over-activation of the NLRP3 inflammasome as the Neonatal-onset multisystem inflammatory disease (NOMID). Subjects with this condition often develop severe arthritis, chronic meningitis leading to neurologic damage\textsuperscript{189}. Because NLRP3 is a key player in the regulation of caspase-1-mediated IL-1\(\beta\) secretion\textsuperscript{190}, centrally limiting induction of this protein provides a unifying mechanism through which fetuses can potentially limit cellular IL-1\(\beta\) responses to a variety of stimuli. Indeed, caspase-1 is not solely activated through the NLRP3 inflammasome. However, its induction is critical for maximal IL-1\(\beta\) secretion early on (<12 hours)\textsuperscript{191, 192}. Caspase-8 is also known to be involved in the late, non-canonical processing of pro-IL-1\(\beta\), although its contribution to TLR-mediated responses is more marginal\textsuperscript{193, 194}. More recently, cathepsin C has also been implicated in processing of pro-IL-1\(\beta\) in mice \textit{in vivo}, but whether this is also true of humans’ remains is unclear\textsuperscript{195}.

Another key result of our study is the demonstration that preterm neonates’ ability to produce IL-1\(\beta\) was restored to adult levels quickly after birth, within 2 weeks of age. This indicates a rapid maturation of this pathway during the neonatal period. Based on previous
observations, we speculate that the high production of placental-derived prostaglandins play an important role in suppressing IL-1β responses before birth. Our finding that chorioamnionitis negatively regulates levels of caspase-1 is also of clinical relevance, as delivery in conditions of high prematurity is frequently associated with infections. In mice, chronic inflammation triggers the ubiquitin-mediated targeted degradation of the NLRP3 complex through autophagy. Fetal control over the inflammasome activity in presence of infection has, to the best of our knowledge, not been previously reported in humans. Again, given the potentially harmful effect of IL-1β in a fetus, inhibition of this cytokine in the context of an infection in utero may represent an evolutionary advantage.

In conclusion, we identify a central developmental role for NLRP3 in regulating IL-1β during gestation, and potentially also after birth. A better understanding of the mechanisms regulating inflammatory responses in early life may provide critical insights into our understanding of the dysregulation of IL-1β pathways in human diseases as well as shed light into therapeutic exploitation of these mechanisms in prevention of neonatal sepsis.
Chapter 4: Natural Killer T cells constitutively expressing the IL-2 receptor α chain early in life are primed to respond to lower antigenic stimulation

4.1 Background

Much remains to be known about the development of human iNKT cells early in life. In mice, iNKT cells develop after birth whereas in humans, iNKT cells are detectable earlier during gestation and already comprise about 0.1% of blood CD3-positive (abbreviated CD3pos) lymphocytes at birth. Human peripheral iNKT cells display two main CD4pos and CD4neg subsets, functionally differing in their helper cytokine expression profiles and homeostatic requirements. CD4pos iNKT cells dominate in fetal and neonatal blood (>90% iNKT population) and their proportion decreases with age. Evidence also suggests that CD4pos iNKT cells directly expand from the thymus, whereas peripheral expansion of CD4neg iNKT cell may be mainly driven through homeostatic proliferation. The relatively stringent antigenic receptor combinatorial rearrangement, resulting in a bottleneck effect on thymic output, implies the potential existence of mechanisms to maintain iNKT cell repertoire diversity upon repeated antigen-driven cell proliferation.

Unlike the vast majority of fetal or neonatal T cells, both neonatal and adult iNKT cells predominantly express the CD45RO isoform memory T cell marker. However, neonatal iNKT cells display a specific and unique phenotype compared to their adult counterpart. Indeed, freshly isolated ex vivo human neonatal iNKT cells constitutively express CD25, the high affinity IL-2 receptor α chain. This characteristic was initially interpreted to be the result of antenatal recognition of a yet unidentified endogenous CD1d-ligand. However, constitutive CD25 expression may also reflect a discrete developmental stage that plays an important role in immunity in early life. The notion that neonatal CD25-expressing iNKT cells are not simply
activated cells, but rather represent a developmentally distinct subset is supported by the absence of other markers of recent T cell activation including CD69 and HLA-DR\textsuperscript{98,106}. Furthermore, even though it has long been recognized that neonatal iNKT cells express CD25, the functional consequence of this constitutive CD25 expression and its precise significance in neonatal NKT cell physiology remain unclear. In normal circumstances, CD25 is only expressed by resting or activated Tregs, or transiently following activation of conventional T cells\textsuperscript{106,107}. CD25 is necessary for signalling through the IL-2 receptor complex, which also includes the $\beta$ and common $\gamma$ chains\textsuperscript{210}. In activated T cells, TCR and CD28 co-stimulation triggers the expression of both IL-2 and CD25\textsuperscript{211,212}, CD25 expression is further driven by an IL-2-mediated positive-regulatory loop. Therefore a sustained interaction between IL-2 and CD25 is important to drive T cells through cell cycle progression following activation\textsuperscript{213}. Whether the constitutive expression of CD25 confers any proliferative advantage to neonatal iNKT cells is unknown.

In this chapter, we investigated the functional consequences of constitutive expression of CD25 on neonatal iNKT cell responses. We demonstrate that neonatal iNKT cells have a substantially reduced proliferation threshold compared to adult iNKT, conventional neonatal T or adult T cells. This lower proliferation threshold is independent of CD4 expression, intrinsic to neonatal iNKT cells and is most dramatic upon stimulation with low potency CD1d ligation, implying that this heightened proliferative capacity may critically help maintain TCR diversity in the iNKT cell compartment in limiting antigenic conditions. Finally, IL-2 blocking experiments suggest an important role for de novo IL-2 receptor $\alpha$ chain expression in bypassing the initial requirement for IL-2 in driving CD25 expression and lowering the proliferation threshold following activation. In light of this data, we propose a potential role for the constitutive
expression of the high affinity IL-2 receptor α chain in ensuring survival, stability and expansion of a structurally diverse antigenic receptor iNKT repertoire early in life.

4.2 Materials and methods

4.2.1 Cells, reagents and antibodies.

Blood samples were obtained following written informed consent from cord blood of neonates delivered at Children’s & Women’s Health Centre of BC (Vancouver, Canada) or from healthy adult peripheral control subjects. All cord or peripheral adult blood samples were collected in sodium heparin anti-coagulated Vacutainers™ (Becton Dickenson) and processed within one hour of collection. Placental histology was reviewed for clinical signs of chorioamnionitis by our institutional clinical pathologist. This study protocol was approved by the University of British Columbia Clinical Research Ethic Board.

Flow cytometry staining antibodies against human CD3, CD19, CD45RO, CD62L, HLA-DR, CD25, CD69, CD122, CD127, CD4 and CD161 were purchased from BD Biosciences. Flow cytometry antibodies against human FOXP3 and CD132 were purchased from eBioscience. OCH, and APC- or PE-conjugated, PBS57-loaded or unloaded CD1d MHC tetramers were supplied through a non-commercial contractual agreement with the National Institute of Health Tetramer Facility. α-galactosylceramide (α-GC) was provided by Dr. Peter van den Elzen (Child & Family Research Institute, Canada). OKT3 (stimulating anti-CD3 antibody) was produced from hybridoma cells by the University of British Columbia antibody core facility. The concentration of PBS57-loaded CD1d-tetramers used for staining of iNKT cells was regularly determined to obtain maximal specific mean fluorescence intensity signal over background when comparing with an unloaded CD1d-tetramer molecule and was stable throughout the study.
The CD1d-transfected K562 myelogenous leukemia cell line was generously obtained from Dr. D. Branch Moody (Brigham and Women's Hospital, Boston USA, unpublished data).

4.2.2 Mononuclear and T cell purification.

Cord blood or peripheral blood mononuclear cells were extracted from whole blood by Ficoll-Paque (Amersham) gradient centrifugation. When excessive, the higher density cord blood reticulocytes remaining following the Ficoll-Paque gradient centrifugation were negatively depleted using CD235a (glycophorin A) microbeads (Myltenyi Biotech). Purified T cells were obtained by cell sorting on CD3^pos^ cells from adult peripheral or cord blood mononuclear cells, using flow cytometry. iNKT cells were obtained by magnetic bead separation (positive extraction on MACS columns; Myltenyi Biotech) of APC-CD1d-PBS57-loaded tetramer-labeled mononuclear cells using anti-APC microbeads and subsequent cell sorting according to a CD3^pos^PBS57-loaded CD1d-tetramer^pos^ surface expression using flow cytometry. All cultures were carried out in RPMI 1640 medium (Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS; Fisher Scientific), 100 units/ml penicillin and 100 mg/ml streptomycin (Invitrogen).

4.2.3 Detailed iNKT cell purification

To circumvent the low occurrence of iNKT cells in human blood, we used a multi-step procedure for efficient purification of a relatively large amount of these cells ex vivo. Mononuclear cells were initially extracted using Ficoll-hypaque gradient centrifugation, followed by two positive selection steps using first, magnetic beads and second, fluorescent-
activated cell sorting (FACS; Figure 4.1). The initial magnetic bead enrichment of iNKT cells considerably reduced the time required for high purity FACS sorting of these cells (≫ 98%).

Ficoll-hypaque purified mononuclear cells were resuspended (20–50 × 10^6 cells/mL) in Dulbecco’s Phosphate Buffered Saline (dPBS) containing 2% Fetal Calf Serum (FCS) and labeled with a fluorescent-conjugated antigen-loaded human CD1d-tetramer or a biotinylated 6B11 antibody (Miltenyi Biotech), for 30 min at 4 °C. Stained cells were then washed twice in dPBS/2%FCS, followed by resuspension in cold (4 °C) MACS buffer (Miltenyi Biotech) and incubation for 15 min at 4 °C with MACS magnetic beads (4 μL of beads/10^7 cells, Miltenyi Biotech) conjugated to an antibody directed against the same fluorescent marker used for labelling of cells (when using CD1d-tetramers) or streptavidin (when using the biotin-6B11 antibody). Labeled iNKT cells were then passed onto a positive selection MACS MS column (Miltenyi Biotech) and the column was washed again twice with 500 μL of cold MACS buffer. After washing, cells were eluted from the column using 1 mL of cold MACS buffer. Following magnetic bead purification, cells were re-suspended in dPBS/2%FCS at a concentration of 10–20 × 10^6 cells/mL and stained with a conjugated anti-CD3 antibody at 4 °C for 30 min. Cells were then washed twice, resuspended in dPBS/2%FCS and sorted at 4°C on double CD3^{pos}/CD1d-tetramer^{pos} cells by fluorescence-activated cell sorting (FACS). This second positive selection step yielded > 98% iNKT cell purity.
Figure 4.1 Multi-step protocol for purification of human iNKT cells

The low proportion of iNKTs present in human mononuclear cells (A) can be enriched using an initial magnetic bead column purification step followed by (B) Fluorescent Activated Cell Sorting (FACS).
4.2.4 Activation and proliferation experiments.

For CFSE proliferation experiments, mononuclear cells were stained with 2 to 10 μM carboxyfluoresceindiacetesuccinimidyl ester (CFSE; Sigma-Aldrich) for 5 minutes in PBS/5%FBS at 37°C. After staining, the cells were washed once in PBS/5%FBS and once in PBS/2%FBS. For antigen presenting cell-free stimulation experiments, 1x10^5 T cells were cultured with (plate-bound) stimulating anti-CD3 (OKT3) ± anti-CD28 (BD Biosciences) ± recombinant IL-2 (eBioscience) ± anti-human IL-2 (BD Biosciences; clone MQ1-17H12) ± anti-CD25 (BD Biosciences; clone M-A251) as specified. Unless otherwise mentioned, the concentration of OKT3 used for these experiments was sub-saturating, as determined by proliferation among several neonatal and human donors, and in order to avoid excessive TCR down-regulation interfering with detection of cells (not shown). For T cell suppression experiments, 5x10^4 T cells were stimulated with anti-CD3 (1 μg/ml) in presence of 5x10^4 irradiated allogeneic feeder cells ± purified iNKT cells ± specified ratios of sorted CD19^{neg}CD25^{high}CD4^{pos}CD3^{pos}Tregs as specified. Stimulation with CD1d-transfected K562 cells was carried out using 2.5x10^4 K562 cells cultured with 1x10^5 purified T cells in the presence of anti-human CD28 (2 μg/ml). For IL-2 inhibition experiments, 1x10^5 T cells were cultured with an excess of anti-human IL-2 antibody was used (corresponding to a 5-fold greater concentration than required to completely inhibit detectable T and neonatal iNKT cell proliferation using CFSE; data not shown) in presence of anti-CD3, anti-CD28 ± recombinant IL-2 or anti-human IL-2 for the specified period of time. Following stimulation, cells were washed three times in dPBS/2%FBS to remove excess antibody and re-cultured in presence of anti-CD3, anti-CD28 and recombinant IL-2 at the same concentrations. After stimulation, cell viability was assessed by propidium iodide (PI) uptake in parallel experiments. Flow cytometry data acquisition was
carried out on LSRII and FACSCalibur. Cell sorting was carried out on a FACS-Aria instruments (Becton Dickenson). Data was analyzed using FlowJo (Tree Star Inc., Oregon). CFSE-time courses were analyzed using a method detailed in a previous study\textsuperscript{215}. \textsuperscript{3}H-Thymidine incorporation was measured in similar proliferation conditions and was added in the final 12-18 hours of a 72 hour culture.

4.2.5 \textbf{PCR quantification of mRNA expression.} 

For mRNA expression procedures were carried out at 4°C and cells were collected in RNAProtect (Qiagen) to minimize RNA degradation. Total mRNA was extracted using TRIzol (Invitrogen), from adult (resting) CD19\textsuperscript{neg}CD14\textsuperscript{neg}CD3\textsuperscript{pos}CD25\textsuperscript{neg} T cells, neonatal or adult (CD19\textsuperscript{neg}CD14\textsuperscript{neg}CD3\textsuperscript{pos}CD1d-PBS57-tetramer\textsuperscript{pos}) iNKT cells purified by cell sorting (>98\% purity), adult CD45RO\textsuperscript{pos}CD25\textsuperscript{neg} memory T cells or sorted adult T cells activated using phytohemagglutinin (PHA) for 72 hours or using staphylococcal enterotoxin B (SEB) for 5 days. cDNA reverse transcription was carried out using the Superscript VILO cDNA synthesis kit (Invitrogen). Quantitative PCR analyses were carried in triplicates using Express SYBR Green qPCR supermix (Invitrogen) according to manufacturer’s protocol on a 7300 real-time PCR instrument (Applied Biosystems Inc., CA). Gene expression was normalized over expression of the housekeeping \(\beta\)-actin gene using the Livak method\textsuperscript{216}. The oligonucleotide sequences used for PCR amplification are provided in Table 4.1.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25 Forward</td>
<td>5’-GATGGATTACATACCTGCTGATG-3’</td>
</tr>
<tr>
<td>CD25 Reverse</td>
<td>5’-TTGGTTTCGTTGTGTTCCGAGTGGC-3’</td>
</tr>
<tr>
<td>KLF2 Forward</td>
<td>5’-AAAGACCACGATCCTCCTTGACGA-3’</td>
</tr>
<tr>
<td>KLF2 Reverse</td>
<td>5’-TTCTCACAAGGCATCACAAGCCTC-3’</td>
</tr>
<tr>
<td>BACT Forward</td>
<td>5’-GTTGCGTTACACCCTTTCTT-3’</td>
</tr>
<tr>
<td>BACT Reverse</td>
<td>5’-ACCTTCACCGTTCCAGTTT-3’</td>
</tr>
</tbody>
</table>

Table 4.1 Oligonucleotide sequences used for gene expression quantification by real-time PCR
4.3 Results

4.3.1 Neonatal iNKT cells are highly abundant early in gestation and display a quiescent CD25\textsuperscript{pos} memory T cell phenotype.

As previous studies addressing the phenotype and abundance of iNKT cells in cord blood were based on a limited number of human subjects\textsuperscript{106,107}, we first confirmed the frequency of iNKT cells at term, as well as earlier in gestation in mononuclear cells \textit{ex vivo} (Figure 4.2A). Invariant NKT cells were identified by flow cytometry using fluorescent antigen-loaded PBS57-CD1d-tetramers to detect cells within CD19\textsuperscript{neg}CD3\textsuperscript{pos} cells. The proportion of neonatal iNKT cells in human neonatal (cord blood) mononuclear cells was 0.12% [95%CI 0.10% to 0.15%] (geometric mean; n=53). Furthermore, by comparing a large number of donors in each age group, we found that this proportion was significantly higher in cord blood obtained from infants born <28 weeks of gestation and decreased towards the term of gestation and in adulthood, even more so in male compared to female subjects (p<0.0004 by Kruskal-Wallis test). CD25 expression was also detectable on iNKT cells much earlier in gestation, as indicated by staining cord blood mononuclear cells from preterm infants born early in the third trimester of pregnancy (<28 weeks of gestation; Figure 4.2B).

Previous investigators showed that both neonatal and adult iNKT cells express the CD45RO isoform, indicating a memory T cell phenotype\textsuperscript{106,107}. Data with regard to the expression of CD62L, which is also generally expressed on naïve T cells, however, were conflicting\textsuperscript{106,107,209,217}. As the identification of iNKT cells requires at least three phenotypic surface markers in flow cytometry, potential for spectral cross-interference between fluorescent detectors may confound the determination of a positive surface expression. In order to address the question of CD62L expression on neonatal iNKT cells, cord blood cells were stained fresh \textit{ex
in vivo using antibodies against CD62L and CD45RO labeled with fluorescent markers having minimal emission overlap with one another (i.e. FITC and PECy7, respectively) and no emission overlap with other iNKT cells identification markers (i.e. APC-labeled CD1d-tetramer and Pacific Blue-labeled staining anti-CD3 antibody). Our results clearly demonstrate that neonatal iNKT expressed variable, but generally higher levels of CD62L compared to adult iNKT cells (Figure 4.3 and see Figure 4.4 for more representative datasets out of 10 subjects tested). We also confirmed that neonatal iNKT cells express the IL-2 receptor common γ chain (CD132) (Figure 4.2C), normally expressed on resting T cells and necessary for IL-2 receptor signalling. On the other hand expression of the IL-2 receptor β chain (CD122) was undetectable in flow cytometry (Figure 4.2C), even though the CD122 mRNA was detected by real-time PCR (not shown).

As the pattern of expression of surface markers, including CD69 and HLA-DR, on neonatal iNKT cells is indicative of a resting rather than activated T cell memory phenotype, we tested whether they expressed KLF-2, a transcription factor highly expressed in quiescent, non-proliferating T cells and down-regulated following activation. Pure (>98%) populations of T or iNKT cells were obtained by flow cytometry cell sorting and mRNA expression was measured by real-time PCR. Neonatal iNKT cells clearly expressed ex vivo KLF-2 mRNA levels comparable to resting T (CD3<sup>pos</sup>CD25<sup>neg</sup>) cells or resting memory (CD45<sup>pos</sup>CD25<sup>neg</sup>) T cells. In contrast, T cells activated following exposure to PHA or staphylococcal enterotoxin B and sorted by positive expression of CD25 expressed substantially less KLF-2 mRNA (Figure 4.2D), consistent with previous reports. Altogether, our results demonstrate that iNKT cells are abundant earlier in gestation and display a quiescent, rather than activated memory T cell phenotype.
Figure 4.2 Phenotype of iNKT cells in adults, neonates born at term and before the term of gestation

(A) Proportion of CD1d-restricted iNKT cells in preterm (born<28 weeks of gestation), term cord blood or adult (F: female; M: male) peripheral mononuclear cells, based on the number of CD19negCD1d-tetramerpos cells among CD3pos cells. Decrease with aging was statistically significant (P<0.001 by Kruskal-Wallis). (B) Expression of CD25 in neonatal iNKT cells from preterm neonates (representative from 3 donors tested). (C) Expression of IL-2 receptor components (CD122: β chain and CD132: common γ chain) (clear area) on neonatal iNKT cells from infants born at term. Fluorescence-minus one staining controls (gray area) yielded results similar to isotype controls (not shown). (D) Krüppel-Like Factor 2 (KLF-2) and CD25 mRNA expression (real-time PCR) in T cells activated either with PHA or staphylococcal enterotoxin B (SEB) and sorted by CD25 positive expression, neonatal iNKT, adult resting memory (CD45posCD25neg)T cells and adult iNKT cells (error bars = SEM among up to three different donors per condition). mRNA expression was normalized on levels measured in adult naïve (CD25neg) T cells.
Figure 4.3 Representative staining of CD45RO and CD62L expression in adult iNKT, neonatal T or iNKT cells

*Ex vivo* mononuclear cells were stained with fluorescent antibodies against CD3, Cd1d-tet, CD45RO and CD62L. Using flow cytometry, the expression of CD45RO and CD62L markers were analyzed and an increased expression of CD62L was observed on the surface of neonatal iNKT cells. All stained samples were compared to staining controls using the same combination of fluorescent-labeled antibodies except for inclusion of isotype controls (fluorescent-minus one plus isotype control).
4.3.2 Expression of CD25 on neonatal iNKT cells is observed earlier in gestation and is not due to activation from labour.

Invariant NKT cells are highly abundant at the materno-fetal placental interface and potentially involved in the induction of labour\cite{220,221}. To exclude the possibility that expression of a memory T cell phenotype could be consequence of recent activation from labour, we analyzed cord blood mononuclear cells collected from women undergoing elective “cold” cesarean section deliveries in the absence of clinically detectable labour. As shown in Figure 4.4, iNKT cells from three representative donors in each group reproducibly expressed CD45RO, CD62L and CD25. Importantly, expression of CD45RO and CD25 on neonatal iNKT was comparable between neonates born by vaginal delivery following normal labour and neonates who were delivered by cesarean section without labour and in absence of documented placental infection (i.e. chorioamnionitis; Figure 4.4). These results confirm that expression of CD25 is not due to a recent activation from labour.
4.3.3 **CD25+ neonatal iNKT cells are not functionally suppressive cells.**

Constitutive expression of CD25 on CD4\(^{\text{pos}}\) T cells is correlated with immunoregulatory functions and expression of the transcriptional regulator FOXP3\(^{222}\). We therefore investigated whether neonatal iNKT cells may share other phenotypic markers in common with Tregs and suppress immune responses. CD25-expressing neonatal iNKT cells did not express FOXP3, normally highly expressed in Tregs (Figure 4.5A), but did express high levels of CD127 (the IL-7 receptor α chain), which is absent on Tregs and also characteristic of naïve T cells\(^{223,224}\). To formally exclude the possibility that CD25-expressing neonatal iNKT cells are suppressive, CD3\(^{\text{pos}}\)CD1d-tetramer\(^{\text{pos}}\) neonatal iNKT cells were sorted and compared with different
proportions of CD4\textsuperscript{pos}CD25\textsuperscript{high} Tregs for their ability to suppress polyclonal T cell responses. As shown in Figure 4.5B and C, even at a 1:1 ratio, neonatal iNKT cells did not suppress T cell proliferation, but rather resulted in a 15-fold increase in proliferation. As expected, addition of Tregs suppressed T cell proliferation (Figure 4.5B).
Figure 4.5 Neonatal and adult iNKT cell CD127 and FOXP3 expression, and suppressing effect of neonatal iNKT cells on polyclonal T cell responses

(A) Expression of Foxp3, CD127 (IL-7 receptor α chain) in CD1d- tetramerposCD3pos iNKT or T cells (middle four panels are gated according to upper left panel). Expression of CD25 in FOXP3/CD127-gated cell populations (right histograms). (B) Suppression of proliferation (measured by ³H-thymidine incorporation) in T cells stimulated by anti-CD3 in presence of irradiated allogeneic antigen presenting cells plus either a 1:1 ratio of neonatal iNKT cells or different ratios of Treg:T cells. (C) Proliferation (CFSE dilution measured at 120 hours) in unstimulated T cells (shaded area; results were identical to T cells stimulated in presence of a 1:1 ratio of Tregs) or in T cells stimulated by anti-CD3 in presence of irradiated allogeneic antigen presenting cells plus an equal ratio of neonatal iNKT cells (clear area).
4.3.4 Enhanced proliferation threshold in neonatal iNKT cells.

Immunological activation can be defined as a state of heightened capacity to proliferate and mediate effector functions. We tested whether CD25-expressing neonatal iNKT cells display a heightened proliferative capacity. To this end, we activated mononuclear cells with two prototypic CD1d glycolipid antigens: α-GC and OCH, which is a less potent structural derivative of α-GC\textsuperscript{225}, and measured expression of CD69 used here as an early indicator of activation (Figure 4.6 and Figure 4.7A for a representative dataset), as well as late proliferation events detected using CFSE dilution (Figure 4.6B and Figure 4.7B for a representative dataset). When compared among donors, activation thresholds were similar between neonatal and adult iNKT cells (Figure 4.6C). However, substantially lower amounts of agonists were required to trigger proliferation in neonatal iNKT cells compared to adult iNKT cells (Figure 4.6D). As shown in Figure 4.6D, this difference was even more remarkable with OCH. Importantly, proliferation thresholds were comparable in both CD4\textsuperscript{pos} and CD4\textsuperscript{neg} adult iNKT cells (Figure 4.6E).

Differences in iNKT cell proliferation threshold were not due to enhanced CD1d-restricted presentation by neonatal antigen presenting cells or due to a differential requirement for CD28 co-stimulation, as demonstrated using purified T cells from either neonatal or adult donors exposed to α-GC presented by a common MHC\textsuperscript{neg}, CD1d-transfected K562 myelogenous leukaemia cell line. Indeed, a substantially lower proliferation threshold was again seen in neonatal iNKT cells stimulated by α-GC in presence of a soluble anti-CD28 antibody, when compared to their adult counterparts (Figure 4.6F and 4.6G). A similar difference in proliferation threshold was also obtained in absence of exogenous anti-CD28 co-stimulation, although proliferation was generally lower in both age groups (not shown).
Figure 4.6 Activation and proliferation threshold in neonatal and adult iNKT cells

(A) Activation (measured by CD69 expression at 48 hours and presented as percentage CD69-expressing cells) and (B) proliferation (measured by CFSE dilution at 72 hours and presented as an average number of cell divisions) compared between neonatal and adult iNKT cells stimulated with increasing concentrations of α-galactosylceramide (α-GC) or OCH (each line represents a different donor). Concentration of ligand required to achieve half-maximal CD69 expression (C) or proliferation (D) in neonatal (dark circles) or adult (shaded circles) iNKT cells (bar represent median). (E) Proliferation threshold to α-GC or OCH in adult CD4^{pos} and CD4^{neg} iNKT cells. (F) Representative CFSE-dilution profiles in CD3^{pos}CD1d-tetramer^{pos} iNKT cells stimulated with α-GC presented by the CD1d-expressing myeloid leukemia cell line K562, in presence of anti-CD28. Background stimulation by the equivalent non-CD1d-transfected cell line was <10% (not shown). NS=unstimulated. (G) Concentration of α-GC required to achieve half-
maximal proliferation in iNKT cells from neonatal (dark circles) or adult (shaded circles) donors.

*P<0.05 and **p<0.01 by Mann-Whitney U.

Figure 4.7 Representative data set used to calculate thresholds for CD69 induction or proliferation (using CFSE dilution) in iNKT cells

Peripheral or cord blood mononuclear cells were stimulated with graded concentration of α-galactosylceramide or OCH. The proportion of (A) CD69-expressing or (B) proliferating cell in each division from a normal fit of data were determined using FlowJo (Tree Star, Inc.; Oregon).

The data presented in this figure are representative of data in figures 4.6A-D.
4.3.5 Neonatal iNKT cells require de novo TCR/CD28 co-stimulation in order to proliferate.

Substantial inter-donor variability was observed in iNKT cell activation and proliferation thresholds (Figure 4.6A-D), presumably due to intrinsic differences in avidity for CD1d-restricted antigens. Therefore in order to obviate some of this potential variability, we used an “antigen-presenting cell-independent” assay. We reasoned that this system would allow to better evaluate whether this heightened proliferation threshold is “intrinsic” to neonatal iNKT cells. Sorted T cells (including iNKT cells) were stimulated with plate-bound stimulating anti-CD3 (i.e. OKT3) and soluble anti-CD28, in presence of an excess of recombinant IL-2 (50 U/mL) to avoid effects due to limiting IL-2 concentrations in the cell culture (Figure 4.8A, B, C, D). In this system, we confirmed the strict requirement for TCR and CD28 co-stimulation in both neonatal and adult iNKT cells (Figure 4.8A). Activated T cells express CD25 and readily proliferate in presence of IL-2. We therefore investigated whether this may also hold true for neonatal iNKT cells. Addition of IL-2, however, did not stimulate neonatal iNKT cell proliferation (Figure 4.8A). Notably, iNKT cell proliferation was strictly dependent on CD25 as shown by a complete inhibition of proliferation in cells stimulated in presence of a blocking anti-human CD25 antibody (Figure 4.8A). Using CFSE-time series models, we detected no statistically significant difference in the time of initiation of the first cell division or rate of subsequent divisions (p>0.05 between trend lines, by linear regression) between neonatal iNKT, adult iNKT and T cells (Figure 4.8B). However, a significantly greater proportion of neonatal iNKT cells reproducibly progressed into cell cycle in comparison to adult T, iNKT or neonatal T cells (Figure 4.8C; results are representative of 5 donors tested in each age group). Notably, we also confirmed the substantially greater proliferation in neonatal iNKT compared to adult iNKT,
neonatal T or adult T cells by measuring thymidine incorporation in purified cell sub-populations (Figure 4.8D).
Figure 4.8 TCR/CD28 co-stimulation requirements and comparison of proliferation in neonatal iNKT, adult iNKT and T cells

(A) T cells obtained from either a neonate or an adult were cultured (120 hours) in absence of stimulation (unstim) or exposed to anti-CD3, IL-2 only, anti-CD3 and anti-CD28 stimulation ± an excess recombinant IL-2 (50 U/mL) or anti-CD3 and anti-CD28 stimulation in presence of a blocking anti-CD25 antibody (gated on iNKT cells). (B) Time of initiation (intercept of line of best fit at mean division one) and rate of cell division (slope of line of best fit) in neonatal iNKT (dark grey circles), adult iNKT (light grey circles), neonatal T (dark grey squares) or adult T (light gray squares) cells. (C) Proportion of proliferating (CFSE<sup>low</sup>) iNKT cells or T cells (at 72 hours) following stimulation with anti-CD3 and anti-CD28. Results of panel A, B and C are
representative of 5 donors in each age group. (D) Thymidine incorporation (72 hours) in sorted neonatal (dark bars) and adult (grey bars) iNKT or T cells stimulated by anti-CD3 and anti-CD28 in presence or absence of IL-2. Cells (5,000(condition)) were tested in duplicates (bars represent SEM). Results of panel D are representative of two independent experiments.

4.3.6 “Priming” of neonatal iNKT cells due to de novo CD25-expression.

Antigen-driven activation and proliferation of T cells generally occurs in two IL-2-dependent steps. First, continuous engagement of the TCR and CD28 molecules is required to drive the production of both IL-2 and CD25 expression (referred to here as the early induction phase of proliferation). Second, continuous exposure to IL-2 interacting with the IL-2 receptor complex (inclusive of CD25 molecule), drives T cells through the cell cycle - referred to herein as the maintenance phase of proliferation; see reference\(^{210}\). In the early induction phase of proliferation, IL-2 is also critical in driving CD25 expression, therefore acting as a positive-regulator of proliferation\(^{228}\). To test whether de novo CD25-expression on neonatal iNKT cells could obviate the early requirement for IL-2 in driving cells into cell cycle, we stimulated T cells with plate-bound anti-CD3 and soluble anti-CD28 and measured the effect of early versus late IL-2 inhibition on the induction phase of proliferation. Expression of CD25 increased in both neonatal and adult iNKT in response to CD3/CD28 co-stimulation and was partially blocked by an anti-IL-2 blocking antibody added at the time of stimulation (Figure 4.9A). Expectedly, we only detected proliferation (i.e. CFSE dilution) in CD25-expressing cells (not shown).

More importantly, proliferation could be completely inhibited by IL-2 blockade in both neonatal iNKT cells as well as in T and adult iNKT cell cultures (Figure 4.9B, bottom panels), confirming the importance of IL-2 in driving T cells into cell cycle during the induction phase of
proliferation. Remarkably, blocking of IL-2 only during the induction phase of proliferation (i.e. by washing out the IL-2 blocking antibody after a specific time period, followed by exposure to recombinant IL-2) completely inhibited proliferation in T and adult iNKT cells, but not in neonatal iNKT cells (Figure 4.9B). These data, which are representative of 4 donors tested in each age group, confirmed that IL-2 is required early in the induction phase of proliferation in both T and adult iNKT cells, but not in neonatal iNKT cells.
Figure 4.9 Importance of CD25 expression and effect of early IL-2 blocking during the induction phase of iNKT cell proliferation

(A) CD25 expression (at 48 hours) in unstimulated (dark grey area) or stimulated (light grey area) neonatal and adult iNKT cells is partially blocked by an anti-IL-2 antibody (clear area). (B) Stimulation in presence of anti-IL-2 washed out after the specified number of hours (∞ = control condition where the antibody was not washed out; bottom four panels) abrogated proliferation in neonatal or adult T cells, but not in neonatal iNKT cells. CFSE histograms are gated on live CD3posCD1dneg or CD3posCD1dpos cells at 120 hours of stimulation. Bar graphs represent overall percentage of viable cells in each condition. Proliferation was identical in cells stimulated in presence of recombinant IL-2, or in presence of anti-IL-2 washed immediately after its addition (not shown). Results are representative of 4 independent experiments in each age group.
4.4 Discussion

Natural killer T cells play an important immune regulatory role in autoimmune diseases and malignancies, although their ontogeny in humans is insufficiently understood. In this study, we expand on previous findings that neonatal natural killer T cells constitutively express the high affinity IL-2 receptor α chain (CD25). However, in these earlier studies authors have not elucidated the functional impact of this CD25-expressing phenotype and in the absence of this important piece of data, may have precipitously concluded that this phenotype simply reflected activation from a previous encounter with an undefined endogenous ligand in utero\textsuperscript{106, 107}. CD1d is expressed on human fetal trophoblastic placental cells and iNKT cells are abundant at the decidual materno-fetal interface, comprising about 0.5% of T cells\textsuperscript{220}. Stimulation of iNKT cells by α-GC during pregnancy triggers cytokine-mediated CD1d-dependent fetal abortion in C57BL/6J mice\textsuperscript{221}. Although the role of iNKT cells in the physiology of normal labour is not entirely elucidated, the possibility that CD25 expression on neonatal iNKT could be related to activation in this context needed to be addressed. Alternatively, iNKT cell activation might have also been due to activation by ascending intra-uterine micro-organisms which are well-reported to be associated with labour\textsuperscript{221, 229}. CD25 expression in neonatal iNKT cells, however, is clearly not due to labour or placental infection as evidenced by a consistent expression in cord blood samples obtained from either preterm or full-term placenta without evidence of labour or detectable inflammation in the mother.

We demonstrate that neonatal iNKT cells, in fact, are not activated in spite of their constitutive CD25 expression. It is not clear what drives the constitutive expression of CD25 on neonatal iNKT cells. Nonetheless, neonatal iNKT cells present several features that make their phenotype clearly distinct from other CD25-expressing activated T cells and Tregs. These
features include the absence of detectable proliferation upon exposure to IL-2 alone, a strict requirement for co-stimulation of the TCR and CD28 for activation and proliferation de novo, the expression of other quiescent T cell markers CD127, CD62L and the transcription factor KLF-2, the absence of other markers of recent activation, such as CD69 and HLA-DR\textsuperscript{98, 106} and finally, the lack of suppression of polyclonal T cell responses. Altogether, these findings are more consistent with the theory that expression of CD25 by iNKT cells reflects a distinct early life developmental stage.

Despite this non-activated state, CD25-expressing neonatal iNKT cells proliferate with substantially lower antigenic stimulation following activation, thereby attenuating avidity differences observed among adult iNKT cells. Cord blood T cells are largely naïve and generally require greater antigenic stimulation to proliferate compared to adult peripheral blood T cells\textsuperscript{230, 231}. Therefore, the heightened proliferation threshold in neonatal iNKT cells clearly distinguishes them from other cord blood T cells. Both antigenic receptor affinity maturation and structural reorganization of the antigenic receptor mechanism contribute to lowering the activation threshold in (secondary) memory T cell responses\textsuperscript{232}. However, the heightened proliferation threshold in neonatal iNKT is also clearly independent of their CD45RO-expressing memory T cell phenotype.

The biological impact of the heightened proliferation threshold in neonatal iNKT cells is not clear. Neonatal iNKT cells display a more plastic cytokine program at birth and generally show a diverse antigenic receptor repertoire\textsuperscript{106, 209, 233}. In presence of limiting antigen stimulation, this mechanism may be particularly important in a rapidly growing fetus or neonate in humans in order to facilitate expansion of lower affinity iNKT cell clones and ensure stability in the iNKT antigenic repertoire upon repeated antigenic challenge. Also, it is not known whether a certain
degree of diversity in the iNKT repertoire is required for maintaining immunological function, although there is evidence for increased relapses of multiple sclerosis in subjects with a more limited iNKT cell repertoire\textsuperscript{234}.

Our findings that both neonatal and adult iNKT cells display similar activation thresholds, but that a greater proportion of neonatal iNKT cells proliferate following activation is in keeping with the existing role for early, sustained IL-2/CD25 interactions in the induction of T cell proliferation. Although we cannot completely exclude that other unidentified factors might contribute to lowering the proliferation threshold in neonatal iNKT cells, our findings are highly indicative that their increased antigenic sensitivity is primarily due to a constitutive CD25 expression. The importance of sustained exposure to IL-2 in the early phase of proliferation is further demonstrated \textit{in vitro} in models showing a lower proportion of precursor cells undergoing cell cycle and a slower rate of subsequent divisions in the presence of limiting IL-2 concentrations\textsuperscript{213}. In humans, CD25 expression is also critical to T cell proliferation \textit{in vivo}, as evidenced by markedly reduced polyclonal T cell responses in CD25-deficient patients\textsuperscript{108, 109}. Following induction of proliferation, high levels of CD25 are detectable in both neonatal and adult iNKT cells, likely explaining why we did not detect significant difference in division rates after initiation of cell cycle.

High levels of CD25 expression were comparably detectable in both CD4\textsuperscript{pos} and CD4\textsuperscript{neg} neonatal iNKT cells (data not shown), implying a similarly low threshold for proliferation between the two iNKT subsets. Because of the very low abundance of CD4\textsuperscript{neg} iNKT cells in cord blood, we were not able to accurately determine proliferation thresholds in neonatal CD4\textsuperscript{neg}iNKT cells, although we clearly demonstrate that both adult CD4\textsuperscript{pos} and CD4\textsuperscript{neg}iNKT subsets displayed comparable proliferation thresholds, as also reported by others\textsuperscript{206}. Therefore, the heightened
proliferation threshold we report is independent of a CD4-expressing phenotype and is not due to an age-related decline in CD4\textsuperscript{pos}:CD4\textsuperscript{neg}iNKT cell ratios. Given a structurally restricted combinatorial iNKT cell receptor rearrangement, CD25 expression may be important for repertoire stability particularly in the CD4\textsuperscript{neg} iNKT subset which appears to primarily expand through peripheral homeostatic proliferation\textsuperscript{206}.

Remarkably, CD25 expression has also been detected in a high proportion of circulating fetal T cells, suggesting that this phenotype may predominate in other early life T cell subsets\textsuperscript{235, 236}. Parallels can be made with a recently identified subset of polyclonal CD25\textsuperscript{pos}CD45RO\textsuperscript{pos}CD8\textsuperscript{pos} memory T cells presumably constituting a peripheral reservoir of antigenic receptor diversity in aging individuals with reduced thymic output\textsuperscript{237, 238}. However, a major phenotypic difference with neonatal iNKT cells is the fact that the latter do not spontaneously divide in the presence of IL-2 alone\textsuperscript{238}. This lack of spontaneous response to IL-2 in the absence of TCR stimulation may reflect a low IL-2R\beta chain expression which is essential for IL-2 receptor signalling\textsuperscript{210}. Alternatively, IL-2 receptor signalling may be functionally silenced in resting neonatal iNKT cells.

In conclusion, we demonstrate that CD25-expressing neonatal iNKT cells are able to proliferate with remarkably reduced antigenic threshold following activation. Our experiments further indicate a role for the constitutive CD25 expression in priming neonatal iNKT cells to sidestep the initial IL-2 requirement and proliferate with remarkably greater sensitivity following TCR activation. Further studies are required to clarify the role of this unique phenotype in iNKT cell ontogeny and its significance in human health and diseases.
Chapter 5: Conclusion

5.1 Interpretation

The main hypothesis of this thesis is that the functional immunological characteristics observed in infants born prematurely are due to developmental factors rather than to the premature birth of these infants. In order to address this hypothesis, I provided data linking prematurity, relative to the contribution of exogenous perinatal factors to functional responses observed in two main classes of anti-microbial receptor pathways: the TLR and RIG-I-stimulated cytokine responses (Chapter 2). Using statistical modeling, I detected no significant contribution of either histological chorioamnionitis or antenatal corticosteroids on the attenuation in cytokine response (when compared to full-term neonates). Moreover, I provided data supporting the novel concept that the developmental attenuation across distinct receptor-cytokine responses varied across gestation indicative of a hierarchal maturation in anti-microbial recognition pathways.

In chapter 3, I characterized the molecular mechanisms underlying the lack of IL-1β responses in neonatal monocytes obtained from neonates born early in the early third trimester of gestation. Interestingly, I found remarkable heterogeneity in surface receptor expression within the preterm monocyte population. Unlike adults, the neonates had an increased proportion of monocytes that expressed lower CD14 and higher CD16 molecules. Studies comparing gene expression signatures between human and mouse monocyte subsets, have shown that mouse Ly6hi and Ly6low subsets are equivalent to human CD14pos and CD14lowCD16pos subsets respectively. Both subsets of mouse monocytes can be differentiated from either MDPs or cMoPs. Furthermore, it has been shown that the Ly6low monocytes makeup the terminally differentiated blood monocyte subset, and are preceded by Ly6hi monocytes. In humans, the CD16pos monocytes are known to have higher phagocytic activity239. Although never tested, it
can be speculated that the high level of CD16\textsuperscript{pos} monocytes in neonates may be responsible for normal phagocytotic responses seen early in life. It is interesting to note that their decline with gestation, correlates with the shift in the site of monocyte development from the fetal liver to the bone marrow. The reason behind the abundance of CD16\textsuperscript{pos} cells in preterm cord blood and their biological role during ontogeny remains a subject of investigation. When investing pro-inflammatory cytokine production by preterm monocytes, I found that both neonatal CD14\textsuperscript{pos} and CD14\textsuperscript{low} monocytes secreted significantly lower IL-1\textbeta upon stimulation with LPS. The lack of expression in the latter subset can be attributed the fact that these cells would not be able to respond to LPS in the absence of CD14-\textsuperscript{TLR4} interaction. Investigation of the former subset, helped in the identification of NLRP3 as a main regulator of IL-1\textbeta secretion in preterm infants. Physiologically, it can be speculated that low response to LPS may assist in creating a more tolerant environment when the newborn is colonized by the environmental microbiota. However, more investigation into the exact reason behind this low response is necessary.

In Chapter 4, I provided evidence of a developmentally unique functional phenotype for neonatal iNKT cells, highlighting the broad nature of developmental differences in the immune system during ontogeny. Like the neonatal monocytes, neonatal iNKT cells display unique functional heterogeneity when compared to adults. Unlike adults, these cells are vastly CD4\textsuperscript{pos} and constitutively express CD25. Further studies need to be done to discover the purpose of CD25 expression on these cells. However, I was able to clearly demonstrate that unlike Tregs, neonatal iNKT cells do not suppress T cell proliferation. In fact, the constitutive expression of CD25 on these cells increases their ability to proliferate with low antigenic stimulation.

Overall, the data presented in my thesis provides unique functional characteristics of the neonatal immune system that could be exploited to enhance the immunogenicity of current
vaccines, which are largely based on our knowledge of the adult immune system, or to enhance the response of the vulnerable neonate to infections.

5.2 Significance

Based on my data, one may ask the question: Why is the neonatal immune system different from its adult counterpart? According to my results, the immune system of the preterm infants is not just “weak” compared to term infants or adults, as it may be stated in some literature. Rather, I identified specific characteristics that are independent from the perinatal conditions in which the premature infant is born, and which makes the preterm or neonatal immune system “different” and age-adapted instead of “weak”.

In light of my findings, it is legitimate to ask why functional differences exist during fetal life. I speculate that at least some of these developmental mechanisms are critical to protect the developing fetus against untoward immune activation, especially during a period in life where self/non-self antigenic discrimination from the adaptive immune system is incompletely established. Studies have shown that excessive production of IL-1β can have serious detrimental metabolic consequences, resulting in life-threatening damage to tissues that characterize some of the clinical complications observed in preterm neonates.

Interestingly, my data also provide some clues about why some infants develop these prematurity linked complications and I hypothesize that these complications emerge from an inappropriately precocious maturation in immune functions at early gestational ages in some infants. Although this is difficult to prove in humans, it may be interesting to pursue these questions using mouse models. One major caveat to this, however, is that there are major interspecies differences in the development of the immune system. For example, as I pointed out in
Chapter 4, constitutive expression of CD25 was not observed in mice. In fact, mice don’t even have iNKT cells until late after birth. Whereas, these cells are readily present and abundant early during gestation in humans. These differences across species limit our ability to test the relevance of findings using animal models and likely can only be studied in humans.

5.3 The contribution of the main findings to the literature

Past studies have affirmed that the development of the immune system happens in waves. The initial waves of development bring with them an immune repertoire that is restricted, and hence is able to respond to a lower number of invading pathogens\(^ {47} \). In line with these findings, we found that the innate immune cells from preterm cord blood are unable to match term cord blood cytokine profiles when stimulated with TLR4, 5 and RIG-I ligands. On the other hand, the same cells are at par with cells from full term infants when stimulated with TLR7/8. This suggests that the mechanisms downstream of TLR7/8 stimulation mature earlier in gestation.

Aside from innate immune cells, similar mechanisms are evident in innate-like immune cells. A lot has been established about the abundance of a unique subset of \( \gamma \delta \) T cells prior to birth\(^ {93} \). Similar to these cells, neonatal iNKT cells are most abundant during the third trimester of gestation. In spite of their abundance, they have a conserved phenotype where \( >90\% \) of them are CD\(^ {4}\text{pos} \). These iNKT cells are more likely to produce IL-4 upon stimulation and hence are more likely to propagate a T helper 2 type response during infections\(^ {206} \). Further studies are required to understand the basic mechanism of immune development during ontogeny.

The role of extrinsic factors on the restricted repertoire of the immune response has also been discussed occasionally in the literature. Clinically, the incidence of \textit{in utero} infections has been correlated with detrimental post-natal infections. Furthermore, animal models have shown
that these infections can dampen the immune response by tolerizing the immune system to a post-natal challenge (e.g. LPS)\textsuperscript{48}. In our study, we addressed these problems by comparing the innate cytokine production profile of preterm cord blood from subjects with or without chorioamnionitis. Given the low number of subjects enrolled in our comprehensive study, it was difficult to detect small effects of \textit{in utero} infections on the immune responses. Aside from intra-uterine infections, another prominent extrinsic factor that preterm neonates are exposed to is antenatal corticosteroids. Therapeutically, these drugs have been shown to enhance lung maturation after they have been injected into any mother expecting to deliver prematurely. The use of these corticosteroids has helped in dramatically reducing respiration related mortalities over the last 20 years\textsuperscript{10}. At the same time, the rate of infections has steadily risen in the same population. These observations have lead some researchers to question whether corticosteroids maybe dampen the immune response of premature babies during the neonatal period\textsuperscript{37}. However, like the responses with chorioamnionitis, we observe no effect of a recent administration of corticosteroids on \textit{ex vivo} immune responses. Rather, our results suggest that preterm cord blood responses were significantly attenuated due to developmental factors when compared to full-term responses.

To further characterize the functional capacity of the preterm immune system we studied neonatal monocytes, a major subset of innate immune cells in circulating blood. These cells play an important role from the initiation to the resolution of inflammation. As described earlier, there are 3 major subsets of monocytes in humans. Of these, both the CD14\textsuperscript{low}CD16\textsuperscript{pos} and CD14\textsuperscript{pos}CD16\textsuperscript{pos} subsets were abundant early in gestation (below 27-29 weeks), indicating immaturity in myelopoiesis. Interestingly, this latter subset of monocytes has also been shown to play a role in tissue remodelling and this may be relevant during embryonic/fetal life\textsuperscript{55}.  

119
Classically, the CD14\textsuperscript{low}CD16\textsuperscript{pos} subset has been considered to be more pro-inflammatory with enhanced antigen-presentation capacity\textsuperscript{79}. In contrast, a per cell analysis of preterm CD14\textsuperscript{pos} monocytes showed that they lacked the ability to secrete full-gestation term-like IL-1β protein upon stimulation. We found that the lack of IL-1β protein resulted from a deficiency of active caspase-1 in preterm monocytes. The distinct composition and functional capacity of preterm monocytes results in less diverse immune response that is capable of phagocytizing most common foreign entities, but is unable to mount a pro-inflammatory immune cascade required for the elimination of certain pathogens. Like the CD16\textsuperscript{pos} monocyte subset, iNKT cells are also abundant late in gestation. Unlike adult iNKT cells, neonatal iNKT cells are vastly CD4\textsuperscript{pos} and primarily produce IL-4, which is in line with previously described Th2 bias in the neonates\textsuperscript{206}. In spite of all their deficiencies, these cells do possess some unique characteristics. The surface expression of CD25 primes the neonatal iNKT cells to proliferate quickly without the need for an abundance of IL-2. The exact role of these cells in neonatal immunology has not been determined, but we can speculate that in an uneducated adaptive immune environment neonatal iNKT cells may play a compensatory role in the propagating the Th2 biased immune response.

Clinical observations of infections in preterm neonates have painted a complex picture, resulting in various hypothetical explanations behind the causes of these infections. In these studies, we showed that preterm neonates are unable to respond to pathogens because of an immaturity in certain pathways. At the same time, we assert that the immaturity in the immune system is not widespread. The abundance of highly proliferative iNKT cells; phagocytic CD16\textsuperscript{pos} monocytes; and the ability to produce abundant pro-inflammatory cytokines upon TLR7/8 stimulation, suggest distinct characteristics of the preterm immune system that may be therapeutically manipulated to reduce the risk of neonatal infections. Indeed, the use of TLR7/8
ligands as a vaccine adjuvant in neonates is already being considered. Furthermore, unpublished data from our lab suggests a role of neonatal iNKT cells in assisting rapid proliferation of naïve T cells from neonates (Sharma, unpublished data). Our studies have helped in partly uncovering the unique designs of the early-life and gestational immune system. However, a considerable amount research is yet to be conducted. We hope that these discoveries will pave the way for further studies looking at exploring and therapeutically exploiting the neonatal immune system, for the betterment of neonatal life.

5.4 Limitations and future directions

The use of human biological samples provides a powerful way to address mechanisms of diseases directly in humans. However, due to obvious limitations in working with vulnerable neonates as research subjects, I was often unable to obtain sufficient blood volume to pursue more detailed mechanistic investigations. Moreover, the neonatal samples that were examined in these studies were taken from prematurely born babies, a clinical scenario that can rarely be considered physiological. The possibility that some of the phenotypic differences would have resulted from interactions with microbes well associated with a premature birth cannot be entirely excluded. Alternatively, interactions with the mother’s own immune response against infection may also play a role in shaping the fetal/neonatal immune response. Well characterized microbial infections like ureaplasma, group B streptococcus or E. coli, can lead to chorioamnionitis, by infecting the fetus after transferring across the placental wall or by entering the amnionic sac via the urogenital tract. To try to estimate the contribution of these factors, we have analyzed the functional immune differences based on the mode of delivery, or in singleton vs twin deliveries. Vaginal deliveries may likely alter interactions with the microbiome at birth.
However, my studies in chapter 2 and chapter 4 do not show any effect of mode of delivery on cytokine production by CBMCs and the phenotype of iNKT cells, respectively. Along the same lines, twin deliveries are usually not associated with in utero infections. Consistent with a relatively negligible role of infection, I have not encountered significantly altered response between singletons and multiples, although these analyses were based on very small sample size. Lastly, the lack of tissue samples/body fluids from other parts of the body prevented me from confirming the unique phenotypes seen in cord blood immune cells. This could be tackled in future studies, by using surgical or post-mortem tissue sections or primary cell cultures.

Aside from the lack of available samples, the experiments done in this thesis were limited due to the lack of commercially available model systems to test/manipulate observations seen in human samples. For instance, iNKT cells are not detectable in mice until ~7 days after birth\textsuperscript{242}. In fact, preliminary studies done from our lab show that neonatal iNKT cells from C57BL/6 mice do not constitutively express CD25 and do not have enhanced capacity to proliferate when compared to their adult counterparts. In order to rectify this problem, in another preliminary experiment we analyzed the phenotype of iNKT cells in a humanized mouse model transplanted with human fetal thymus. As was the case with C57BL/6 mice, iNKT cells from the humanized mouse were not found in abundance and did not express CD25 constitutively. These preliminary findings point to the need for development of a model to further the study of neonatal iNKT cells. A more impressive but expensive method to study neonatal iNKT cells would be to analyze these cells in organisms like higher primates or established mammalian models. Alternatively, in vitro models to analyze the role of CD25 in neonatal iNKT cells could also be established.

During my research, I attempted to establish a neonatal iNKT cell line using purified ex vivo iNKT (see chapter 4). I noticed that neonatal iNKT lose their constitutive CD25 expression and
the consequent lower threshold for proliferation once they are grown *in vitro* (data not shown).

Based on these preliminary studies, I would speculate the best time to study these cells is humans is immediately after their *ex vivo* purification. Unlike the iNKT cells, sheep models to study preterm innate immune cells under various clinical conditions have been the basis for the research done by Kramer, Jobe *et al*. Their findings on the impact of chorioamnionitis and the use of antenatal corticosteroids on innate immune cells have helped in formulating the objectives of chapter 2. Unlike the sheep models, no profound effect of chorioamnionitis and antenatal corticosteroids was noticed in our human samples. Animal models have been a key focal point in the discovery and testing of therapeutic immunological interventions. However, a better understanding of the differences between neonatal humans and neonatal animal models is an essential necessity.

Complex differences in immune cell phenotype exist with age, stimulation and inter-species variability. In recent years, these differences are being addressed by high throughput analysis of gene expression and DNA methylation. The Immunological Genome Project has already characterized the basal gene expression profile of several immune cells in the C57BL/6 mouse. Additionally, hundreds of studies looking at immune cell phenotype in adult humans have been published. In contrast, the study of neonatal immune cells using microarrays, methylation arrays and RNA sequencing technologies has been limited. Recent experiments from our lab have been focused on studying the phenotype of neonatal monocytes and T cells using these systems biology approaches. Preliminary data has shown that LPS-stimulated preterm monocytes have reduced levels of ribosomal machinery. The lack of ribosomal machinery can be hypothesized to cause a lack of translation of pro-inflammatory cytokines seen in chapters 2 and 3. In order to fully comprehend the mechanism behind this dry lab data, extensive wet lab
research needs to be conducted. Further studies, looking at the changes in high throughput biology in various cell types; under various stimulations; within different age groups will help in drawing an in-depth picture of the neonatal immune system.

Finally, the translation of data from *in vitro* and *in vivo* scenarios studied and suggested in this thesis has the potential to be clinically relevant. Throughout this thesis, we have observed clear evidence of a lack of immune response in the preterm. The lack of pro-inflammatory IL-1β and IL-6 production or the lack of caspase-1 activity are essential drawbacks in fighting infections. However, synthetic proteins injected into the body may lead to uncontrolled and possibly detrimental conditions. In conjunction, we also observed that the production of these cytokines is more robust after stimulation with more mature TLR ligands (e.g. R848 for TLR8). In accordance with this data, it would be more appropriate to use these TLR ligands as adjuvants to generate a controlled pro-inflammatory response. Similar to TLR8 stimulation, the stimulation of neonatal iNKT cells using α-GC during an infection may help in the replication of antigen specific T cells. However, as a precursor it is important to improve the understanding and the implications of the use of these stimulations using high throughput biology and animal models. I anticipate that neonatal infections resulting from immaturity of the immune system will be offset by harnessing the power of the more mature components of the neonatal immune system. The work described in this thesis has helped in shedding light on the deficiencies and the adequacies of a small part of the neonatal immune system. I hope this research will inspire future studies to better understand the immune system for the prospect of fighting neonatal infections.
Bibliography


26. Redline RW, Faye-Petersen O, Heller D, Qureshi F, Savell V, Vogler C, Society for Pediatric Pathology PSAFINC. Amniotic infection syndrome: nosology and


42. Zago CE, Silva S, Sanita PV, Barbugli PA, Dias CM, Lordello VB, Vergani CE. Dynamics of biofilm formation and the interaction between Candida albicans and methicillin-susceptible (MSSA) and -resistant Staphylococcus aureus (MRSA). *PloS one* 2015, **10**(4): e0123206.


