Preterm infant IFN-β responses to Group B streptococcus

This thesis is presented for the Honours degree in Biomedical Science at Murdoch University

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I declare this thesis is my own account of my research and contains
as its main content, work which has not been previously submitted
for any degree at any tertiary institution.

______________________________
Sherriannne Ng Qin Yin
Abstract

Group B streptococcus (GBS) is the leading cause of early onset sepsis in preterm infants. The production of IFN-β, a type I interferon (IFN), has been shown to be critical for host defense against GBS in murine studies. The release of GBS-DNA into the cytosol of mouse macrophages was found to induce IFN-β production. It is not known if infant or even adult human monocytes and macrophages produce IFN-β in response to GBS, and what role GBS-DNA plays in this process.

This study optimised a whole blood assay to determine if human monocyte subsets are capable of inducing IFN-β production to live GBS challenge as well as cytosolic GBS-DNA. The main findings were that (A) all adult blood monocyte subsets were capable of producing IFN-β to live GBS as well as cytosolic GBS-DNA, as determined by IP-10 expression, (B) the intermediate monocyte subset produced the highest amount of IP-10 to both live GBS and cytosolic GBS-DNA, and (C) the monocytes of a preterm infant had similar IP-10 responses to cytosolic GBS-DNA but much lower IP-10 responses to live GBS compared to adults. These findings indicate that human blood monocytes are capable of producing IFN-β-inducible cytokines in response to GBS, possibly through the release of cytosolic bacterial DNA. Deficiencies in the production of IFN-β in response to GBS, or its DNA, may contribute to the increased susceptibility of preterm infants to GBS infections.
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**Abbreviations**

AIM2 – Absent in melanoma 2

APC – Antigen-presenting cell

ASC – Apoptosis-associated speck-like protein containing a caspase activation and recruitment domain

BBW – Birth body weight

BFA – Brefeldin A

CARD – Caspase activation and recruitment domain

CFU – Colony forming units

CLR – C-type lectin receptor

CoNS – Coagulase-negative *Staphylococcus*

CpG DNA – CG dinucleotide rich deoxyribonucleic acid

DAI – DNA-dependent activator of interferon regulatory factors

DAMP – Danger/damage associated molecular pattern

DC – Dendritic cell

dsDNA – Double-stranded deoxyribonucleic acid

dsRNA – Double-stranded ribonucleic acid

ELISA – Enzyme-linked immunosorbent assay

EOS – Early-onset sepsis

FSC – Forward scatter

G-CSF – Granulocyte colony-stimulating factor

GA – Gestational age

GBS – Group B streptococcus

HIB – Heart infusion broth

HLA – Human leukocyte antigen
IFI16 – Interferon-inducible protein 16
IFN – Interferon
IFNAR – IFN-α/β receptor
Ig – Immunoglobulin
IL – Interleukin
IP-10 – Interferon-inducible protein 10
IRF – Interferon regulatory factor
JAK-STAT – Janus kinase-signal transducer and activator of transcription
LOS – Late-onset sepsis
LPS – Lipopolysaccharide
LRRFIP1 – Leucine-rich repeat in flightless I-interacting protein 1
LTA – Lipoteichoic acid
M-CSF – Macrophage colony-stimulating factor
MAPK – Mitogen-activated protein kinase
MDA5 – Melanoma differentiation-associated gene 5
mDC – Myeloid dendritic cell
MDP – Muramyl dipeptide
MFI – Median fluorescence intensity
MHC – Major histocompatibility complex
MNC – Mononuclear cell
MyD88 – Myeloid differentiation factor 88
NF-κB – Nuclear factor-kappa-B
NK – Natural killer
NLR – NOD-like receptor
NLRP3 – Nucleotide oligomerisation domain-like receptor family, pyrin domain containing 3
NOD – Nucleotide oligomerisation domain
PAMP – Pathogen-associated molecular pattern
PBS – Phosphate buffer saline
pDC – Plasmacytoid dendritic cell
PGN – Peptidoglycan
PRR – Pattern recognition receptor
PYD – Pyrin N-terminal homology domain
PYHIN – Pyrin and HIN-200 domain-containing protein
RBC – Red blood cell
RIG-I – Retinoic acid-induced gene I
RLR – Cytosolic RIG-I-like receptor
RNA Pol III – RNA polymerase III
RT – Room temperature
RT-PCR – Real-time polymerase chain reaction
SIRS – Systemic inflammatory response syndrome
SSC – Side scatter
ssRNA – Single-stranded ribonucleic acid
STING – Stimulator of interferon genes
TBK1 – TANK-binding kinase 1
Tc – T Cytotoxic cells
Th – T Helper cells
TLR – Toll-like receptor
TNF – Tumor necrosis factor
VLBW – Very low birth weight
Chapter 1  Literature Review

1.1  Introduction

Infection claims the lives of an estimated 4,000,000 infants (< 6 months of age) worldwide each year (PrabhuDas et al., 2011; UNICEF, 2009). Approximately 37% of this mortality is in neonates (birth to 1 month of age), representing more than four thousand deaths per day (PrabhuDas et al., 2011; Wynn and Levy, 2010). More than 50% of these deaths occur in extremely low birth weight infants (<1000g birth body weight, BBW) and extremely preterm infants (<28 weeks gestational age, GA), and are due to infections (Barton et al., 1999; Clapp, 2006; Levy, 2007; PrabhuDas et al., 2011; Russell, 2010).

Infections affecting preterm infants in the developed world are mainly due to bloodstream invasions by either nosocomially acquired Coagulase-negative Staphylococci (CoNS) or the vertically transmitted Group B Streptococcus (GBS, Streptococcus agalactiae)(Chhatwal and Graham, 2008; Edmond and Zaidi, 2010; Russell, 2010). Such bacterial infections maybe followed by a potentially deleterious systemic inflammatory response termed as neonatal sepsis, which causes 42% of all mortality occurring during the neonatal period, according to the World Health Organization (WHO) (Edmond and Zaidi, 2010; Rubarth, 2010). Additionally, preterm infants that survive sepsis face devastating sepsis-related morbidities including chronic lung disease, cerebral palsy and neurodevelopmental disabilities such as vision and hearing impairments (Schlapbach et al., 2011; Stoll et al., 2004; Wynn and Levy, 2010).
Despite the adverse repercussions of sepsis that preterm infants are challenged with, much remains to be understood about the mechanisms underlying their susceptibility to bacterial blood infections (Levy, 2007). There is evidence to suggest that immaturity of the neonatal immune system is a key factor contributing to infection vulnerability (Sadeghi et al., 2007). In particular, the innate immune system is important as it acts as a first-line defense against the multitude of bacterial species neonates are exposed to at birth (Hancock et al., 2012). A number of studies have shown that preterm infant innate immune cells present with both quantitative and qualitative defects including diminished phagocytosis function and impaired ability to differentiate and mature in response to bacterial components such as LPS (Levy, 2007; Strunk et al., 2004; Velilla et al., 2006). Furthermore, monocytes of very preterm infants (<30 weeks GA) demonstrate a significantly reduced ability to produce pro-inflammatory cytokines in response to live GBS and other bacterial-derived ligands compared to term infants (Currie et al., 2011; Levy, 2005). It is presumed that this functional impairment in inflammatory cytokine production is responsible for increased susceptibility to bacterial infections, especially that caused by GBS (Currie et al., 2011; Levy, 2007). Importantly, the type I interferons (IFN), including IFN-α and β, have been shown to be beneficial in mediating both innate and adaptive immune responses during bacterial infections (Monroe et al., 2010).

Several studies with both Gram-negative and Gram-positive bacteria have demonstrated the importance of type I IFNs for host defense during infections (Decker et al., 2005). In particular, recent studies using mouse models have demonstrated that type I IFNs play a critical role in the elimination of GBS
infection, where mice unable to produce IFN-β suffered unrestrained bacteraemia (Mancuso et al., 2007). The requirement for IFN-β during GBS infections is surprising, given the fact GBS is an extracellular bacterium and the production of type I IFN is normally associated with intracellular pathogens such as viruses (Charrel-Dennis et al., 2008). It is postulated that leakage of bacterial DNA from the phagolysosome into the cytosol triggers the intracellular production of type I IFNs, which then leads to the activation of important IFN-inducible genes (Charrel-Dennis et al., 2008; Trinchieri, 2010).

Several cytosolic and membrane DNA sensors that induce type I IFNs have recently been discovered. These include nucleic acid receptor LRRFIP1, RNA polymerase III, dsDNA sensor IFI16 and membrane bound TLR9 (Keating et al., 2011). Recognition of DNA by these sensors then leads to the activation of several signaling adaptors including RIG-I, STING, β-catenin and MyD88, which act through downstream mediators such as IRF3 and NF-κB to induce type I IFN production (Cavlar et al., 2012; Keating et al., 2011). These signaling pathways have predominantly been studied in mouse models, and to a much lesser extent in human adult cell types including monocytes (Ablasser et al., 2009; Keating et al., 2011; Unterholzner et al., 2010). It remains unknown if infant innate immune cells, especially those of preterm infants, are capable of producing type I IFN in response to bacterial DNA via these same pathways. The inability of the preterm infant innate immune system to recognize and respond to bacterial DNA may contribute to reduced type I IFN production, resulting in an increased risk of infection. This review will examine the importance of neonatal sepsis and the role of the neonatal immune system in response to bacterial infections, with a focus on the innate immune system of
preterm infants. It will also outline the function of type I IFNs in host defense against extracellular GBS and the associated pathways involved in cytosolic recognition of DNA.

1.2 Neonatal Infection and Mortality

The neonatal population contributes to 40% of mortality occurring worldwide in children under the age of five (WHO, The World Health Statistics, 2011). The leading causes of neonatal deaths are prematurity and infections (Edmond and Zaidi, 2010; PrabhuDas et al., 2011).

Neonates are exposed to a diverse range of microorganisms including bacteria, viruses and parasites after birth (Lawn et al., 2010; Rubarth, 2010). Importantly, bacterial infections occur at higher incidence (>80%) during this period, and are typically caused by nosocomially-acquired organisms (e.g. Staphylococcus aureus, Escherichia coli, Enterococcus spp. and CoNS) or vertically-transmitted invasive bacteria (i.e. GBS) (Henneke and Berner, 2006b; Russell, 2010; Stoll et al., 2002). These bacteria colonize neonates soon after birth and can then invade into the bloodstream to cause neonatal sepsis (Rubarth, 2010).

1.3 Neonatal Sepsis

Neonatal sepsis is caused by the invasion of bacteria into the bloodstream of newborns (Rubarth, 2010). Newborn infants are commonly classified according to their GA, where infants can fall into one of four categories: i) extremely preterm infants (<28 weeks gestation), ii) very preterm infants (<32 weeks
gestation), moderately preterm infants (<36 weeks gestation) or iv) term infants (>37 weeks gestation) (Currie et al., 2011; PrabhuDas et al., 2011). Alternatively and to a lesser extent, neonates can also be grouped according to their BBW, where very low birth weight (VLBW) infants (BBW, <1500g) are at increased risk of developing neonatal infections (Barton et al., 1999; Ng, 2004).

The incidence of sepsis has shown to be inversely correlated with GA and birth weight, resulting in very preterm infants showing increased risk of developing bacterial infections (Clapp, 2006; Stoll et al., 2002). The majority of sepsis occurs in preterm infants and is caused by commensal bacteria, particularly GBS, \textit{E.coli}, \textit{S. aureus} and \textit{S. epidermidis} (the predominant CoNS species), that are rarely pathogenic in healthy adults (Nizet and Klein, 2011; Russell, 2010). The colonization of neonates by bacteria usually occurs as a result of vertical transmission from the mother (during passage through the an infected birth canal or aspiration of infected amniotic fluid) or nosocomial acquisition (bacteria on intravenous medical devices or commensals on individuals who interact with neonates)(Liu and Nizet, 2004; Yeung and Kenny, 2009). The invasion of bacteria into the bloodstream is then termed bacteraemia, which can lead to a systemic inflammatory response that causes neonatal sepsis (Rubarth, 2010).

Neonatal sepsis can be classified as either early onset sepsis (EOS) or late onset sepsis (LOS) based on the timing of presentation (Robinson et al., 2008). EOS occurs within three days of birth and is predominantly caused by vertically-acquired GBS, whilst LOS arises more than three days up to thirty days post-
partum and is mainly due to nosocomially-acquired CoNS (Didier et al., 2012; Russell, 2010).

1.3.1. Early-onset Sepsis and GBS

EOS continues to be a foremost cause of newborn morbidity and mortality, especially in the preterm infant population (Polin, 2012). The incidence of EOS in the preterm and VLBW infant population occurs at a rate of 15-19 per 1000 live births, which is ten times higher than the incidence rate in term infants (Stoll et al., 2005). Approximately 10-30% of preterm and VLBW infants do not survive EOS, whilst survivors face devastating sepsis-related morbidities including neurodevelopmental impairments such as vision and hearing loss (Stoll et al., 2004; Wynn and Levy, 2010).

More than thirty years ago, GBS was identified as the main pathogen causing EOS in all newborn infants, regardless of GA (Henneke and Berner, 2006a). Since then, other commensals such as *E. coli* and *S. aureus* have also been implicated in early-onset disease due to improvements in preventing maternal transmission of GBS (Russell, 2010). However, despite advancements in screening and the use of maternal antibiotic prophylaxis, a UK based study indicated that GBS still accounts for more than 60% of culture-positive EOS (Johri et al., 2006; Russell, 2010).

GBS is a common colonizer of the gastrointestinal and genitourinary tract of healthy adults, although GBS colonization during pregnancy (affecting 10-40% of pregnant women) carries an increased risk of stillbirth, septic abortions and
premature delivery (Feuerschuelle et al., 2012; Koenig and Keenan, 2009). Colonization of the neonate by GBS typically occurs as a result of vertical transmission from the mother, where passage through the birth canal or aspiration of infected amniotic fluid exposes newborns to GBS (Liu and Nizet, 2004; Wynn and Levy, 2010). Several factors increase the risk of neonatal GBS infection and these include: i) maternal factors (prolonged rupture of membranes; intra-partum fever; GBS colonization during pregnancy, urinary tract infection and chorioamnionitis – inflammation of the amniotic fluid) and ii) neonatal factors (prematurity; low birth weight and resuscitation at birth) (Bersani et al., 2012; Gerdes, 2004; Klinger et al., 2010; Makhoul et al., 2009; Polin, 2012).

1.3.2. Late-onset Sepsis

LOS typically occurs when neonates interact with microbes from their environment or with commensals residing on individuals they come into direct contact with (Yeung and Kenny, 2009). With advances in neonatal healthcare, increasing numbers of preterm and VLBW infants are surviving early causes of mortality such as EOS (Stoll et al., 2002). The greater survival rates has led to an increased need for invasive monitoring, resulting in extended hospital stays and use of indwelling medical devices such as endotracheal intubation and intravascular catheterization, where bacterial biofilm formation can occur (Dimitriou et al., 2011; Polin and Randis, 2010; Stoll et al., 1996). These factors coupled with impaired immune defenses, such as compromised skin barrier functions and reduced normal endogenous microbial flora amounts, increases the risk of neonates developing LOS (Polin and Randis, 2010). GA and BBW of
are also inversely correlated with susceptibility to LOS and mortality (Stoll et al., 2002).

LOS is caused primarily by Gram-positive bacteria (~70%), particularly CoNS (68% of all Gram-positive infections) as well as S. aureus and Enterococcus spp. (Russell, 2010; Stoll et al., 2002). A lower incidence (~17%) of LOS is caused by Gram-negative pathogens such as Pseudomonas, Enterobacter, Serratia and Klebsiella (Stoll et al., 2002). It has been reported that neonates suffering late-onset Gram-negative sepsis have poorer prognosis including increased risk of death (Stoll et al., 2002; van der Ree et al., 2011). Recent studies have also found that preterm infants who develop LOS are at increased risk of neurodevelopmental disabilities including lower intelligence, poorer attention control as well as vision and hearing impairments (Stoll et al., 2004; van der Ree et al., 2011).

1.4 Inflammation and Sepsis

LOS and EOS occur as a result of the systemic inflammatory response to bacterial infections (Melvan et al., 2010). The systemic inflammatory response syndrome (SIRS) is characterized by the release of pro-inflammatory mediators, such as chemokines and cytokines, during infection or tissue injury (Adams-Chapman and Stoll, 2001; Castellheim et al., 2009). SIRS that occurs during a suspected or confirmed infection is then termed as sepsis (Castellheim et al., 2009). The failure to control SIRS can lead to severe sepsis or septic shock, and result in multiple organ dysfunction, severe hypotension and ultimately death of the neonate (Dollner et al., 2001; Marshall, 2008). Therefore, the
neonatal immune system is particularly important during sepsis to ensure release of pro-inflammatory cytokines during SIRS is tightly regulated.

1.5 Neonatal Immunity

The function of the neonatal immune system is to provide protection against the diverse range of microbes (bacteria, viruses and parasites) that newborns are exposed to following emergence from the sterile intra-uterine environment of the womb (Marodi, 2006b; Yoshio et al., 2004). The immune system can be divided into three basic levels: i) anatomical and physiological barriers; ii) innate immunity; and iii) adaptive immunity (Figure 1.1)(Turvey and Broide, 2010).

Figure 1.1 Overview of the immune system (Turvey and Broide, 2010)
The skin and mucosa (respiratory, urogenital and intestinal) of neonates provide the anatomical and physiological barrier for protection against invading microorganisms (Wynn and Levy, 2010). The neonatal skin however is not completely developed after birth, and takes up to 2 weeks in term infants (\(>37\) weeks GA) and at least 8 weeks in very preterm infants (\(<32\) weeks GA) to become a fully functional protective barrier (Evans and Rutter, 1986; Kalia et al., 1998; Wynn and Levy, 2010). This leaves neonates, especially preterm infants, at increased risk of bacterial invasion due to barrier dysfunction (Wynn and Levy, 2010).

The two main arms of immunity (innate and adaptive) are thus critical to augment the protection (or lack thereof) offered by the skin and mucosa (Turvey and Broide, 2010; Wynn and Levy, 2010). Effective host immune defense against invading pathogens requires the coordination between both the innate and adaptive immune systems (Hancock et al., 2012). The innate immune system is comprised of specialized cells that initiate immediate response through broad recognition of conserved microbial structures (Lacy and Stow, 2011; Weigand et al., 2004), whilst the adaptive immune system consists of immune cells that activate highly specific long-lasting responses to pathogenic antigens (Shanker, 2010). Neonates display deficiencies in both the innate and adaptive arms of immunity, which is associated with impaired host defense against invading pathogens (Marodi, 2006a). This has led to the postulation that the immaturity of the neonatal immune system likely contributes to the increased susceptibility of neonates to bacterial infections (Sadeghi et al., 2007; Strunk et al., 2011).
The mechanisms underlying the immaturity of the neonatal immune system and its contribution to increased susceptibility of neonates to bacterial infections continues to remain unclear. Although there is emerging evidence that detail deficiencies in the immune system of neonates, there is little research providing insight into the immunity of preterm infants (Sharma et al., 2012), the population at highest risk of developing bacterial infections. The following section of this review will identify research on the immune responses of neonates to bacterial infections particularly GBS, with a focus on neonatal innate immunity, and where possible outline findings in preterm infants.

1.6 Neonatal Adaptive Immunity

The adaptive immune system is comprised of lymphocytes that provide highly specific long-lasting responses to pathogens (Shanker, 2010). These adaptive immune cells are responsible for the specific recognition of pathogens and long-term immunological memory (Castellheim et al., 2009; Shanker, 2010). The two main types of lymphocytes in the adaptive immune system are the T cells and B cells (Shanker, 2010). T cells can be subdivided into Helper or Cytotoxic T cells that are involved in the production of cytokines and induce apoptosis of infected host cells, whilst B cells secrete antibodies to mediate humoral immunity (Harty et al., 2000; Nakayamada et al., 2012; Weigand et al., 2004).

1.6.1. Neonatal Helper T Cells

T helper (Th) cells are important for the coordination of host immune defenses against pathogens (Akdis et al., 2012). They can differentiate into several
subsets including Th1, Th2, Th17, and T_{FH} (Akdis et al., 2012). Each subset is responsible for different functions and can produce a distinct set of cytokines, allowing the initiation and regulation of diverse host immune responses (Figure 1.2) (Akdis et al., 2012; Nakayamada et al., 2012). In neonates, the four main subsets are Th1, Th2, Th17 and T_{reg} (Zhu and Paul, 2008). Respectively, these subsets are important for host defense against intracellular pathogens; allergic inflammation; immunity against extracellular pathogens; and regulation of immune responses and lymphocyte homeostasis (Akdis et al., 2012; Zhu and Paul, 2008).

**Figure 1.2** Overview of differentiation pathways of CD4+ Th cell subsets (Akdis et al., 2012)

Fetal immunity is inclined toward Th2 anti-inflammatory cytokine polarization, as Th1 responses are harmful to the fetus and can cause pregnancy complications including spontaneous abortions (Koga et al., 2009). This Th2 preponderance
continues to persist during early life, increasing the susceptibility of newborns to infectious diseases that require Th1 immunity (Marodi, 2006a). A number of factors contribute to the Th2 skewed responses observed in newborns, and include defective antigen presentation and impaired cytokine production (Diesner et al., 2012).

Neonates, especially preterm infants, produce markedly lower levels of IL-12 compared to adults, which contributes to reduced differentiation of naïve CD4+ T cells into Th1 cells and bias towards Th2 cytokine responses (Diesner et al., 2012; La Pine et al., 2003; Langrish et al., 2002; Zhu and Paul, 2008). Since IL-12 is also known to promote IFN-γ production, diminished IL-12 production contributes to the significantly lower levels of IFN-γ produced by neonatal mononuclear cells compared to adults (Blanco-Quiros et al., 2000; La Pine et al., 2003; Langrish et al., 2002). The strikingly high amounts of IL-10 produced by neonates also act to antagonize Th1 responses by down-regulating MHC Class II expression on antigen-presenting cells (APC), thereby impairing antigen presentation that activates naïve T cells (Canaday et al., 2006; Kollmann et al., 2009; Marshall-Clarke et al., 2000; Roth et al., 1996). The impaired ability to mount sufficient Th1 cell responses contributes to increased susceptibility of preterm infants to bacterial infections including GBS (La Pine et al., 2003; Nussbaum and Sperandio, 2011).

Th cells of neonates also differ from adults because neonatal Th cells are not capable of proliferating in the presence of IL-7 (Fukui et al., 1997; Hassan and Reen, 1998; Marchant and Goldman, 2005). IL-7 is imperative for the survival, maintenance and expansion of naïve T cells in neonates (Correa-Rocha et al.,
2012; Marshall-Claire et al., 2000). Preterm infants (<37 weeks GA) have reduced IL-7 receptor expression and lower IL-7 plasma concentrations compared to term infants (>37 weeks GA) (Correa-Rocha et al., 2012). This IL-7 deficiency results in lower numbers of naïve T cells in preterm infants, thereby contributing to impaired capacity to mount adequate T cell responses to pathogens (Berrington et al., 2005; Correa-Rocha et al., 2012).

1.6.2. Neonatal Cytotoxic T Cells

T cytotoxic (Tc) cells primarily function to induce apoptosis of infected cells, but can also contribute to adaptive host defense against various pathogens including viruses and bacteria that cause intracellular infections (Harty et al., 2000). Following initial exposure to a microbe, specific pathogen-derived antigens are presented to CD8+ T cells (via MHC Class I molecules) by APCs in lymph nodes (Harty et al., 2000). This leads to the generation of effector CD8+ T cells that release lytic granules capable of destroying infected cells (Harty et al., 2000). Cord blood of neonates, particularly preterm infants, display lower levels of CD8+ T cells compared to adults (Correa-Rocha et al., 2012; Gasparoni et al., 2003). In addition, the production of IL-2 by CD8+ T cells is inversely correlated with GA (Gasparoni et al., 2003). Collectively, these impairments likely contribute to reduced immune capacity of preterm infants to respond to intracellular infections such as L. monocytogenes (Correa-Rocha et al., 2012; Marodi, 2006b).
Neonatal Antibodies

Neonates do not have a fully developed immune system at birth and rely on maternal antibodies for early immune protection against infectious agents (Hancock et al., 2012; Siegrist and Aspinall, 2009; Walker et al., 2011). At approximately 13 weeks of gestation, maternal immunoglobulin G (IgG) begins to be transported across the placental tissue into fetal blood (Palmeira et al., 2012; Saji et al., 1999; Simister and Story, 1997). The concentrations of fetal serum IgG continues to increase as the pregnancy progresses, with the majority of maternal IgG being acquired during the final four weeks of pregnancy (Malek et al., 1996; Palmeira et al., 2012; Saji et al., 1999). The IgG concentrations of full term infants (>37 weeks GA) normally exceed maternal IgG levels by birth (Palmeira et al., 2012; Saji et al., 1999).

Since IgG concentration in neonates is directly correlated to GA, many very preterm infants (<32 weeks GA) have markedly reduced serum IgG levels compared to full term infants (Palmeira et al., 2012; van den Berg et al., 2010). Furthermore, as transferred maternal IgG antibodies have a half-life of between 21 to 30 days, these infants are only protected for a short period of time following birth (Siegrist and Aspinall, 2009). Collectively, diminished concentrations of protective IgG antibodies and short immune protection provided by maternal IgG antibodies put preterm infants at higher risk of infections (Siegrist and Aspinall, 2009; Silveira Lessa et al., 2011).

There have been attempts made to decrease infection rates via the use of vaccines capable of inducing protective antibody responses to infectious agents.
such as GBS (Johri et al., 2006; Siegrist, 2007). However, these vaccine strategies have often been unsuccessful during early life due to several limitations including short-lived vaccine-induced antibody responses as well as maternal antibodies that inhibit vaccine efficacy (Siegrist, 2007). Importantly, although antibodies confer immune protection during early life, the use of intravenous immune globulin therapy has failed to improve the overall survival rate of neonates with sepsis (Weisman et al., 1992). Therefore, it is likely a deficient antibody response is not the primary factor contributing to susceptibility of neonates to infections (Palmeira et al., 2012; PrabhuDas et al., 2011).

1.7 Neonatal Innate Immunity

The neonatal innate immune system is critical for first-line host defense against invading microbial pathogens including bacteria and viruses (Marodi, 2006b). As the adaptive immune system is still relatively naïve at birth, newborns rely heavily on their innate immune system to prevent and control infections (Nussbaum and Sperandio, 2011).

The innate immune system is comprised of cells including eosinophils, mast cells, macrophages, dendritic cells (DC), natural killer (NK) cells, neutrophils and monocytes (Kenzel and Henneke, 2006; Turvey and Broide, 2010). Phagocytes such as monocytes and neutrophils facilitate the ingestion and clearance of cellular debris and pathogens including bacteria (Filius et al., 2011), whilst professional APCs such as DCs and macrophages coordinate both the innate and adaptive arms of immunity by presenting antigens to
adaptive immune cells (T and B cells) (Velilla et al., 2006; Willems et al., 2009). Innate immune cells are also responsible for the production of cytokines and chemokines, which are especially important for the mediation of inflammatory responses to invading pathogens (Markiewski et al., 2008). These three main functions (phagocytosis, antigen presentation and cytokine production) are mediated by pattern recognition receptors (PRR) (see section 1.7.4) that sense invading microbial pathogens such as GBS, resulting in the initiation of host innate immune defenses (Takeuchi and Akira, 2010).

1.7.1. Neutrophils

Neutrophils are highly motile and are the most abundant phagocytic cell type among circulating leukocytes in the blood (Marodi, 2006b; Segal, 2005). They have a much shorter lifespan (7-10 hours half-life) compared to monocytes (3 days half-life), and are important for the ingestion of microbes including GBS and E. coli during infections (Filias et al., 2011; Marodi, 2006b; Melvan et al., 2010).

The first neutrophils begin to appear approximately 5 weeks following conception, becoming the main cell type found in the fetal marrow space from 14 weeks to term gestation (>37 weeks GA) (Christensen and Sola-Visner, 2009). At birth, neutrophil concentrations in the blood exceed that of adults, but steadily decline to comparable amounts at around 2-3 days postpartum (Melvan et al., 2010). The increase in neutrophil numbers appears to be a partial compensation for its reduced phagocytic ability (Filias et al, 2011). By the 3rd postpartum day, neutrophils of term neonates begin to demonstrate phagocytic
activity levels similar to adults (Filias et al., 2011). Conversely, neutrophil storage pool numbers continue to remain lower in preterm infants compared to their term counterparts (Melvan et al., 2010).

Neutrophils are an important part of the phagocytic system and mediate innate immune defenses by facilitating the ingestion, killing and clearance of invading microorganisms as well as apoptotic cells and cellular debris (Filias et al., 2011). Interestingly, the phagocytic capacity of preterm infant (<37 weeks GA) neutrophils to phagocytose bacteria remains controversial (Currie et al., 2011; Filias et al., 2011). Although the studies by Currie et al. (2011) and Filias et al. (2011) both used cord blood from preterm infants and compared phagocytic abilities to term infants (>37 weeks GA), Filias et al. (2011) found preterm infant neutrophils had reduced capacity to phagocytose E.coli, whilst Currie et al. (2011) showed preterm infant neutrophils had a higher capacity to phagocytose GBS. It has been suggested that the size of the infecting bacteria as well as soluble factors in the serum may affect the phagocytic ability of neonatal neutrophils (Melvan et al., 2010).

1.7.2. Monocytes

Monocytes originate in the bone marrow and constitute approximately 10% of all circulating leukocytes in the blood (Yona and Jung, 2010). Monocytes differentiate from a common myeloid progenitor that granulocytes and neutrophils also share (Strauss-Ayali et al., 2007; Yona and Jung, 2010). Fetal monocytes begin to appear at 18 to 20 weeks of gestation, increasing to
approximately 3 – 7% by 30 weeks of gestation, and exceeding adult circulating monocyte concentrations by birth (Clapp, 2006).

Monocytes are involved in a range of host immune responses including phagocytosis, antigen presentation and cytokine production (Katsiari et al., 2010). However, monocytes of neonates, particularly preterm infants (<37 weeks GA), appear to have several functional impairments and are described below (Currie et al., 2011; Hallwirth et al., 2004; Velilla et al., 2006).

1.7.2.1. Phagocytosis

The phagocytic capacity of preterm infant monocytes remains controversial, with different studies showing preterm infant monocytes have both reduced (Strunk et al., 2004) and adequate capacity to phagocytose *E. coli* (Filiás et al., 2011). Notably however, a study by Currie *et al* has shown that monocytes of preterm infants (<37 weeks GA) can phagocytose GBS just as well as term infants and adults (Currie et al., 2011).

1.7.2.2. Antigen Presentation

Presentation of foreign antigens by APCs is critical to initiate innate immunity and induce long-lasting adaptive immune responses to microbes (Diesner et al., 2012; Hoebe et al., 2003; Velilla et al., 2006). Monocytes are an important source of APCs as they are capable of differentiating into DCs and macrophages, which express major histocompatibility class II (MHC-II) molecules (HLA in humans) involved in processing and presenting antigens to
CD4+ T cells (Canaday et al., 2006; Shi and Pamer, 2011; Velilla et al., 2006). Neonatal monocytes have shown reduced capacity to differentiate into DCs, which possibly contributes to impaired antigen presentation in neonates (Velilla et al., 2006). Furthermore, monocytes of preterm and VLBW infants have been shown to express lower levels of HLA-DR compared to term neonates (Hallwirth et al., 2004; Hallwirth et al., 2002). Since HLA-DR is essential for presentation of bacterial antigens to CD4+ T cells, reduced expression of HLA-DR may contribute to impaired T cell responses likely associated with increased susceptibility to infections (Hallwirth et al., 2004; Skrzczyńska et al., 2002).

### 1.7.2.3. Cytokine Production

Monocytes produce cytokines and chemokines that are involved in a range of biological processes including the activation and recruitment of other cells to sites of infection or injury (Lacy and Stow, 2011). Cytokines are particularly important for the mediation of inflammatory responses to invading pathogens (Markiewski et al., 2008). In neonates, monocytes represent an important source for the production of early cytokines such as TNF-α, IL-6 and IL-10 during microbial infections (Sohlberg et al., 2011). However, a study by Currie et al. has shown that in response to GBS, preterm infant monocytes are impaired in their ability to produce important pro-inflammatory cytokines such as TNF-α and IL-6 (Currie et al., 2011).

Overall, the diminished phagocytic activity; decreased antigen presentation capabilities and reduced pro-inflammatory cytokine production of preterm infant
monocytes likely contributes to their increased susceptibility to infections (Currie et al., 2011; Hallwirth et al., 2004; Velilla et al., 2006).

Human monocytes can be subdivided into three different subsets based on the levels of CD14 and CD16 expression (Strauss-Ayali et al., 2007). Differing in phenotype and function, the three subpopulations can be classified as: i) Classical monocytes (CD14+ CD16-); ii) Intermediate monocytes (CD14+ CD16+); and iii) Non-Classical or inflammatory monocytes (CD14- CD16+)(Strauss-Ayali et al., 2007; Wong et al., 2011). Classical monocytes (~85%) express high CD14 and low CD16 levels and are the most prevalent subtype in the blood circulation (Cros et al., 2010). In adults, they are involved in tissue repair and produce an extensive range of cytokines including G-CSF, IL-6 and IL-10 in response to bacterial lipopolysaccharides (LPS)(Wong et al., 2011). It is still unclear if neonatal classical monocytes display the same characteristics.

The remaining monocyte population is comprised of the intermediate (~5%) and non-classical (~7%) subsets (Cros et al., 2010). Intermediate monocytes have been proposed to be a transitional population between the classical and non-classical subpopulations (Wong et al., 2011). Its expansion precedes non-classical monocytes when patients were treated with IFN-γ and macrophage colony-stimulating factor (M-CSF), which induces proliferation and differentiation of mononuclear phagocytes (Weiner et al., 1994). Non-classical monocytes represent the most mature phenotype; displaying increased antigen capabilities, phagocytic functionality and ability to produce pro-inflammatory cytokines such as TNF-α and IL-1β (Sohlberg et al., 2011; Wong et al., 2011).
Increases in the proportion of CD14- CD16+ non-classical monocytes during bacterial sepsis and other inflammatory diseases have been reported by several studies (Sohlberg et al., 2011; Ziegler-Heitbrock, 2007). A study conducted on term neonates and small children have described significantly elevated levels of inflammatory monocytes during blood culture positive sepsis (Skrzeczynska et al., 2002). It is unknown if inflammatory monocyte responses will be similar in preterm infants during bacterial infections.

1.7.3. Dendritic Cells

Dendritic cells are key APCs that are central for both innate and adaptive immune responses during microbial infections (Willems et al., 2009). DCs mature in response to pathogen stimulation, tissue damage and pro-inflammatory cytokines by a process mediated by MHC-II molecules (Jarrossay et al., 2001). Following maturation, DCs are involved in a range of immune responses including the production of regulatory cytokines and stimulation of naïve T cells (Jarrossay et al., 2001). Human DCs can be divided into two populations: i) myeloid DCs (mDC); and ii) plasmacytoid DCs (pDC)(Willems et al., 2009). Cord blood mDCs and pDCs have been described as being in a more immature state, with reduced MHC-II levels, compared to adult mDCs and pDCs (Willems et al., 2009). mDCs and pDCs differ in their phenotype, expression of Toll-like receptors (TLR)(see section 1.7.4.1) and chemokine, cytokine and type I IFN production (Piccioli et al., 2009). mDCs are a major source of IL-12p70 and express all TLRs apart from TLR7 and TLR9 (Jarrossay et al., 2001). However, cord blood mononuclear cell studies have found that neonates produce lower IL-12p70 and have impaired TLR functionality to
various stimuli including GBS and LPS compared to adults (Joyner et al., 2000; Kollmann et al., 2009). pDCs on the other hand express TLR7 and TLR9 and are mainly involved in the production of type I IFNs (Jarrossay et al., 2001).

1.7.3.1. **Plasmacytoid Dendritic Cells**

Plasmacytoid dendritic cells play a central role in linking the innate and adaptive arms of immunity by partaking in two essential activities (Guiducci et al., 2006). First, pDCs act as ‘professional IFN-α/β producing cells’ with a specialized ability to produce substantial amounts of type I IFN in response to a wide range of pathogens including parasites, viruses and bacteria (Kumagai et al., 2008; McKenna et al., 2005). Second, pDCs are capable of recruiting activated CD4+ and CD8+ T cells via the induction of chemokines as well as directly or indirectly activating other cell types (e.g. NK cells, B cells and monocytes) (Colonna et al., 2004; McKenna et al., 2005).

Several studies have been conducted on cord blood pDCs to determine their functionality in the human neonate. In two separate studies using whole blood stimulated with DNA rich in CG dinucleotides (CpG-DNA), IFN-α production by neonatal pDCs was shown to be either similar (Gold et al., 2006) or lower levels (De Wit et al., 2004) compared to adults. The discrepancy between these results may be due in part to the small sample size (n=3 per group) used by De Wit et al (2004), resulting in a larger variance between subjects. Importantly, both studies agree that neonatal pDCs demonstrate a diminished ability to mature and function in comparison to adult pDCs (De Wit et al., 2004; Gold et al., 2006).
A more recent study of neonatal pDCs has identified reduced translocation of IFN regulatory factor (IRF)-7 into the nucleus compared to adult pDCs (Danis et al., 2008). IRF-7 is an important component for the induction of type I IFN genes through the TLR7 and TLR9 pathways. Thus, as a result of this impairment in cord blood pDCs, neonates are limited in their ability to mount a sufficient type I IFN response to viruses (Danis et al., 2008).

Although many studies highlight the importance of pDCs in eliciting immune responses during viral infections, their role during bacterial infections is less well recognized. Unlike mDCs, pDCs are not equipped with several TLRs (i.e. TLR2, TLR4, TLR5 and TLR3) important for the recognition of bacterial products such as LPS and peptidoglycans (PGN) (Colonna et al., 2004). Importantly, a study using human mononuclear cells have found that pDCs are not capable of producing cytokines such as IFN-α, TNF-α and IL-8 in response to whole *E.coli* and GBS (Piccioli et al., 2009). This suggests that pDCs are likely to have a limited role during human bacterial infections (Piccioli et al., 2009).

1.7.4. Pathogen Recognition Receptors

Pattern recognition receptors are germ-line encoded receptors that sense endogenous danger signals (danger-associated molecular patterns, DAMP) and evolutionarily conserved microbial structures (pathogen-associated molecular patterns, PAMP) (Castellheim et al., 2009; Kumar et al., 2011; Medzhitov and Janeway, 1997). All microbes (pathogenic and commensal) express PAMPs, which are essential for their survival, and include flagellin and bacterial cell wall components such as LPS and PGN (Kumar et al., 2011; Medzhitov and
Janeway, 1997; Philpott and Girardin, 2004). The engagement of PRRs initiates intricate signaling pathways that culminate to induce inflammatory responses (via cytokines and chemokines) for pathogen clearance (Kumar et al., 2011).

1.7.4.1. **Toll-like Receptors**

*Toll* proteins were first discovered for their role in embryonic development in fruit flies, and subsequent susceptibility of the fruit fly *Drosophila melanogaster* to fungal and bacterial infections (Anderson et al., 1985; Lemaitre et al., 1996; Lemaitre et al., 1997; Philpott and Girardin, 2004). This later led to the identification of TLR4, the first human *Toll* homologue, and other members of the TLR family (Poltorak et al., 1998; Takeuchi et al., 1999). The TLR system is now recognized for the central role it plays in coordinating the primary innate, and subsequently adaptive, immune responses during infections in humans (Hertzog et al., 2003).

Human TLRs consists 10 family members localized on the cell surface (TLR1, 2, 4, 5, 6 and 11) or in intracellular vesicles such as endosomes (TLR3, 7, 8 and 9) (Kawai and Akira, 2009; Kumar et al., 2012). Cell surface TLRs primarily sense bacterial structural components, whilst endosomal TLRs typically recognize microbial nucleic acids (Hancock et al., 2012). Seven of these TLRs function primarily as sensors for bacterial molecules, making them important for the mediation of immune defenses during bacterial sepsis (Kumar et al., 2011; Tsujimoto et al., 2008). In particular, TLR2/6, TLR7/8 and TLR9 respectively recognize lipoproteins and lipoteichoic acid (LTA), ssRNA and dsDNA of GBS,
resulting in the production of pro-inflammatory cytokines including TNF-α, IL-1 and IFN-β (Henneke and Berner, 2006a; Mancuso et al., 2009).

Essentially, recognition of bacterial ligands by these TLRs initiates activation of adaptor protein myeloid differentiation factor 88 (MyD88), which triggers the recruitment of other adaptor molecules and activation of several kinases of the interleukin-1 receptor associated kinase family (Philpott and Girardin, 2004). This leads to translocation of transcription factor nuclear factor kappa B (NF-κB) to the nucleus and activation of mitogen-activated protein kinases (MAPK), which ultimately culminates to induce expression of pro-inflammatory cytokine genes (Kumar et al., 2012; Takeuchi and Akira, 2010; Trinchieri and Sher, 2007).

The neonatal TLR system is distinctly different from adults and has several functional impairments (Clapp, 2006; Marodi, 2006a). Neonatal cord blood plasma contains soluble factors such as adenosine that act to inhibit the activation of several TLRs and their heterodimers (TLR1/2, TLR2/6, TLR4 and TLR7), resulting in decreased production of TNF-α (Clapp, 2006; Diesner et al., 2012; Levy et al., 2004). This was confirmed with adult hematocytes, where addition of neonatal plasma resulted in reduced TNF-α production in response to TLR ligands such as LPS (Levy et al., 2004). Several studies have also reported lower expression and functionality of TLR4 in neonates, especially in preterm infants (Forster-Waldl et al., 2005; Levy et al., 2004; Sadeghi et al., 2007). As TLR4 expression is positively correlated to GA, very preterm infants (<32 weeks GA) produced significantly lower levels of TLR4-induced inflammatory cytokines (IL-6, IL-1β and TNF-α) compared to older neonates.
 (>30 weeks GA) (Forster-Waldl et al., 2005). It is likely that reduced expression of adaptor protein MyD88 contributes to impaired TLR4-mediated signaling in neonates (Levy et al., 2004; Sadeghi et al., 2007).

1.7.4.2. NOD-like Receptors

NOD-like receptors (NLRs) are a family of intracellular microbial sensors that consists of 23 members, which can be divided into two groups on the basis of function (Gille et al., 2009; Kumar et al., 2011).

The first group comprises of NLRs that induce the production of inflammatory cytokines, type I IFNs and anti-microbial peptides via activation of transcriptional factors such as NF-κB, MAPK and IRFs (Gille et al., 2009; Sabbah et al., 2009). Both NOD1 and NOD2 are members of this subpopulation, and are involved in initiating inflammatory responses to bacterial components such as PGN and muramyl dipeptide (MDP) of bacterial cell walls (Monroe et al., 2010; Takeuchi and Akira, 2010). NOD1 and NOD2 have been implicated in the recognition of E.coli, L. monocytogenes and S. aureus, which are associated with neonatal bacterial infections (Hotoura et al., 2012; Kumar et al., 2011). The function of NLRs during bacterial infections in preterm infants remains unclear.

The second group of NLRs mediates the assembly of inflammasomes via the activation of caspases such as caspase-1 (Hornung et al., 2009; Schroder and Tschopp, 2010). The formation of these multi-protein complexes can then
induce biologically active forms of inflammatory cytokines IL-1β and IL-18 as well as cell death (Martinon et al., 2009; Miao et al., 2010).

1.7.4.2.1. Inflammasomes

Several PRRs including NOD1, NOD2, NLRP3 and absent in melanoma 2 (AIM2) have been implicated in the formation of multi-protein complexes known as inflammasomes (Hornung et al., 2009; Khare et al., 2010). The formation of inflammasomes leads to the release of inflammatory caspases including caspase-1 (Khare et al., 2010). Caspase-1 is involved in two main inflammatory processes that mediate host immune responses to many intracellular pathogens including *F. tularensis* and *L. monocytogenes* (Fernandes-Alnemri et al., 2010; Rathinam et al., 2010). Firstly, it cleaves pro-IL-18 and pro-IL-1β to generate biologically active IL-18 and IL-1β (Hornung et al., 2009; Martinon et al., 2009). These pro-inflammatory cytokines are important for a range of immune responses including the induction of secondary pro-inflammatory mediators such as chemokines and cytokines including IFN-γ (Lamkanfi and Dixit, 2009). Secondly, activated caspase-1 is critical for pyroptosis, a pro-inflammatory programmed cell death that effectively limits intracellular bacterial growth (Fink and Cookson, 2005; Gille et al., 2009; Martinon et al., 2009).

Whilst most studies have reported the importance of inflammasomes for intracellular infections (Fernandes-Alnemri et al., 2010; Rathinam et al., 2010), a study by Costa *et al* (2012) has shown that inflammasomes are also critical for host defense against extracellular GBS in mouse DCs. The murine study found that the activation of NALP3 inflammasome was dependent on the
release of the pore-forming toxin β-hemolyin, but not CAMP factor (Costa et al., 2012). Importantly, they showed that all components of the NALP3 inflammasome including caspase-1 activation were essential for the secretion of pro-inflammatory IL-18 and IL-1β, and that this pathway was critical to control GBS growth in vivo (Costa et al., 2012). Additionally, it was found that mice lacking NALP3 inflammasome components were more susceptible to GBS infection (Costa et al., 2012). It remains unknown whether human preterm infants are capable of activating inflammasomes in response to GBS, and whether it contributes to their increased susceptibility to GBS infections.

1.7.4.2.1.1. AIM2 Inflammasome

AIM2 has recently been identified as a sensor for cytoplasmic DNA, capable of recognizing dsDNA of any size and origin but incapable of responding to ssDNA (Fernandes-Alnemri et al., 2009; Hornung et al., 2009). It is the most conserved member of the HIN-200 family of interferon response genes, and as the only member capable of binding to the adaptor molecule ASC, AIM2 is critical for the activation of caspase-1 but entirely dispensable for IFN-β induction (Burckstummer et al., 2009; Hornung et al., 2009; Vilaysane and Muruve, 2009). The activation of the AIM2 inflammasome is critical for host defense against intracellular infections caused by F. tularensis and L. monocytogenes, where it is responsible for IL-1β secretion as well as induction of a caspase-1-dependent cell death (Cervantes et al., 2008; Fernandes-Alnemri et al., 2010). Although the role of AIM2 is less established in extracellular infections, a study by Costa et al. (2012) found that AIM2 was dispensable for IL-1β secretion and
caspase-1 activation in mouse DC during extracellular GBS infections. The role of AIM2 during GBS infections in humans remains unclear.

1.7.4.3. RIG-I-like Receptors

RIG-I-like receptors (RLR) family members are a group of intracellular sensors for viral cytoplasmic dsRNA that include retinoic acid inducible gene-1 (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (Creagh and O'Neill, 2006). RLRs signal via NF-κB and IRF3 to induce the production of type I IFN (Creagh and O'Neill, 2006; Takeuchi and Akira, 2010). To date, most studies have identified RLRs as important for the sensing of viruses, whilst the role of RLRs for bacterial recognition remains unclear (Monroe et al., 2010). However, a study by Charrel-Dennis et al. (2008) has shown that GBS does not require RLRs for the induction of the type I IFNs.

Collectively, the engagement of these PRRs culminates to induce the production of pro-inflammatory cytokines important for host immune defense against invading pathogens. The role of these PRRs for the recognition of GBS is summarized in Table 1.1.
Table 1.1  Brief overview of PRRs for GBS

<table>
<thead>
<tr>
<th>Localisation</th>
<th>PAMP</th>
<th>PRR</th>
<th>Examples of cytokines induced</th>
</tr>
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<tbody>
<tr>
<td>Extracellular</td>
<td>PGN</td>
<td>TLR2</td>
<td>TNF-α, IL-1, IL-6, IL-12 and IFN-β</td>
</tr>
<tr>
<td></td>
<td>dsRNA</td>
<td>TLR3</td>
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<tr>
<td></td>
<td>Diacyl lipoprotein</td>
<td>TLR6</td>
<td></td>
</tr>
<tr>
<td>Endosomal</td>
<td>ssRNA</td>
<td>TLR7/8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dsDNA</td>
<td>TLR9</td>
<td></td>
</tr>
<tr>
<td>Cytosolic</td>
<td>MDP</td>
<td>NLR</td>
<td>IL-18 and IL-1β</td>
</tr>
<tr>
<td></td>
<td>DNA</td>
<td>Unidentified</td>
<td>IFN-β</td>
</tr>
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(Costa et al., 2012; Henneke and Berner, 2006a; Henneke et al., 2005; Mancuso et al., 2009; Talati et al., 2008)

1.8  Type I Interferons

IFNs were described more than half a century ago as a substance capable of inhibiting viral replication (Isaacs and Lindenmann, 1957). These cytokines can be classified as either type I IFNs or type II IFNs based on the cell-surface receptors expressed (Decker et al., 2005). Type II IFNs consist of a single member (IFN-γ), whilst several kinds of type I IFNs exists (IFN-α, IFN-β, IFN-ω, IFN-κ, IFN-ε)(Decker et al., 2005). All IFNs exert their effects via initiation of Janus kinase-signal transducer and activator of transcription (JAK-STAT) signal transduction, which culminates to induce hundreds of genes that direct various biological responses (Darnell et al., 1994; Platanias, 2005). The diverse functions of IFNs include mediating cytokine production, enhancing antigen presentation, and regulating cell survival, differentiation and proliferation (Boehm et al., 1997; Stark, 2007). Type I IFNs (mainly IFN-α and IFN-β) are
essential during viral and bacterial infections, where they potently regulate both the innate and adaptive arms of immunity (Monroe et al., 2010; Theofilopoulos et al., 2005; Trinchieri, 2010).

Almost all immune cells, especially monocytes, can produce type I IFNs constitutively at low levels or inducibly during viral and bacterial invasions (Hertzog et al., 2003; Li et al., 2011; Trinchieri, 2010). Type I IFNs exert their effects either directly (via IFNAR1/IFNAR2 heterodimeric receptor) or indirectly (via induction of cytokines and chemokines that regulate cell-mediated responses) (Hervas-Stubbs et al., 2011). The functions of type I IFNs extend to a range of cell types (Figure 1.3) and include enhancing antigen-presenting capacity of APCs, driving CD4+ and CD8+ T cell differentiation, amplifying antibody responses, facilitating recruitment of immune cells and regulating the production of cytokines such as IL-6, IL-12 and IL-18 (Bogdan et al., 2004; Brinkmann et al., 1993; Hervas-Stubbs et al., 2011; Le Bon et al., 2006; Tough et al., 1996).

Type I IFNs have long been recognized for their protective role during viral infections, where they are involved in activation of APCs, T- and B- cell responses and direct killing of viruses (Nagarajan, 2011). However, their roles in host defense during bacterial infections appear to be conflicting, where type I IFNs can be beneficial or detrimental depending on the pathogen species (Carrero et al., 2004; Nagarajan, 2011; Weigent et al., 1986). Importantly, type I IFNs are protective against extracellular pathogens such as S. pneumonia, E. coli and GBS (Mancuso et al., 2007; Nagarajan, 2011), which are common
causes of bacterial infections affecting newborns (Rubarth, 2010; Russell, 2010).

**Figure 1.3** Overview of direct type I IFN effects (Decker et al., 2005)

### 1.8.1 Importance in Extracellular Bacterial Infections

Until recently, studies have primarily focused on intracellular pathogens to delineate the role of type I IFNs during bacterial infections (Stetson and Medzhitov, 2006; Stockinger et al., 2004). The involvement of type I IFNs during extracellular bacterial infection however has been less recognized (Charrel-Dennis et al., 2008). It was initially hypothesized that extracellular bacteria could not induce type I IFN production, but various studies have proven otherwise (Charrel-Dennis et al., 2008; Gratz et al., 2011; Parker et al., 2011).

Several recent studies using mouse models have demonstrated the critical role that type I IFNs (especially IFN-β) play during extracellular bacterial infections (Charrel-Dennis et al., 2008; Gratz et al., 2011; Parker et al., 2011). A study by
Gratz et al (2011) demonstrated that IFN-α/-β receptor (IFNAR)-deficient mice (IFNAR-/- mice) were considerably more susceptible to lethality by S. pyogenes, a human pathogen associated with cellulitis and streptococcal toxic shock syndrome, compared to wild-type control mice. Interestingly, the induction of type I IFNs by macrophages and DCs in this study were independent of TLRs (TLR2, 3, 4, 7 and 9) typically associated with recognition of bacterial products, but dependent on endosomal delivery of bacterial DNA and RNA (Gratz et al., 2011). These results suggest that although S. pyogenes is capable of evading recognition by important TLRs, it is likely that other sensors exist to respond to bacterial nucleic acids and trigger type I IFNs for host defense (Gratz et al., 2011).

A similar study conducted by Parker et al (2011) support these findings and gives evidence that type I IFNs are likewise protective during infections with S. pneumoniae, a foremost cause for bacterial pneumonia that affects neonates (Parker et al., 2011; Rubarth, 2010). It also showed that in the case of S. pneumoniae, expression of pneumolysin (pore-forming toxin) was essential for bacterial DNA to enter the cytosol and trigger type I IFN production (Parker et al., 2011). Importantly, there is evidence to suggest that live GBS induces type I IFN production via a similar mechanism (Charrel-Dennis et al., 2008).

A pioneering study by Mancuso et al demonstrated that type I IFNs were indispensable during GBS infections, where IFNAR-/- mice, incapable of producing IFN-β, suffered unrestrained bacteraemia (Mancuso et al., 2007). In another murine study, it was demonstrated that the induction of type I IFNs was dependent on both the phagocytosis and subsequent degradation of live GBS
in the phagolysosome as well as the release of pore-forming toxins (hemolysin and CAMP factor) by live GBS, which allows the release of bacterial components into the cytosol (Charrel-Dennis et al., 2008). Furthermore, only GBS-DNA that escaped the phagolysosomal compartment was capable of activating cytoplasmic receptors responsible for inducing IFN-β production (Charrel-Dennis et al., 2008).

Overall, these studies highlight the ability of extracellular pathogens to activate intracellular signaling of type I IFNs, which are protective during S. pyogenes, S. pneumoniae, and GBS infections (Parker et al., 2011). Importantly, during GBS infections, mouse models have demonstrated the need for phagolysosomal degradation by pore-forming toxins and subsequent release of GBS-DNA for the induction of type I IFNs, which are necessary for host defense during GBS infections (Charrel-Dennis et al., 2008; Mancuso et al., 2007). These mechanisms of type I IFN induction by GBS have yet to be studied in humans and impairment in these mechanisms may contribute to increase susceptibility of preterm infants to GBS infections.

1.8.2 Induction of Type I Interferons by Cytosolic DNA Sensors

It is well recognized that microbial nucleic acids such as DNA are capable of inducing type I IFN production, which is now recognized as vital for host defense during infections with extracellular GBS (Charrel-Dennis et al., 2008; Keating et al., 2011). It has been proposed that this recognition of nucleic acids is universal and does not discriminate between self and non-self DNA (Keating et al., 2011). Instead, it is an evolutionary mechanism to detect danger signals,
such as cytoplasmic DNA, rather than nucleic acids specifically associated with microbes (Keating et al., 2011).

The intracellular sensors and signaling components involved in recognizing cytoplasmic DNA and inducing type I IFNs have recently been elucidated (Cavlar et al., 2012). There are several pathways involved in type I IFN production following recognition of DNA in the cytosol, which are described below and illustrated in (Figure 1.4) (Keating et al., 2011).

1. **Interferon-inducible protein 16 (IFI16)**

IFI16 shares the same pyrin and HIN-200 domain (PYHIN)-containing protein family as AIM2, a cytoplasmic dsDNA sensor capable of initiating inflammasome formation (see section 1.7.7.2.1.1) (Keating et al., 2011). IFI16 mediates IFN-β induction by recognizing dsDNA, especially DNA of longer lengths (>70 base pairs) (Unterholzner et al., 2010). The recognition of dsDNA by IFI16 results in the direct recruitment of the signaling adaptor stimulator of IFN genes (STING), and subsequent activation of a TANK binding kinase 1 and interferon regulatory factor 3 (TBK1-IRF3) signaling axis that leads to the induction of IFN-β (Burdette et al., 2011; Unterholzner et al., 2010). It has been demonstrated that GBS-DNA triggers a TBK1-IRF3 pathway for IFN-β production (Charrel-Dennis et al., 2008), however it is unclear if IFI16 is the sensor responsible for GBS-DNA recognition.
2. **RNA Polymerase III (RNA Pol III)**

RNA Pol III is capable of sensing dsDNA, especially dsDNA rich in AT dinucleotides known as poly(dA:dT), and transcribing it into dsRNA (Ablasser et al., 2009; Chiu et al., 2009). The transcription of dsDNA into dsRNA leads to the activation of an RNA sensor, RIG-I, which signals through the downstream mitochondrial antiviral signaling protein (MAVS). This pathway ultimately culminates to induce type I IFN production (Ablasser et al., 2009; Chiu et al., 2009). It is unknown if GBS is capable of inducing type I IFN production via this pathway.

3. **Leucine-rich repeat in flightless I-interacting protein 1 (LRRFIP1)**

LRRFIP1 is capable of recognizing dsRNA and dsDNA and initiates type I IFN production via a highly specific pathway dependent on β-catenin (Keating et al., 2011). Unlike the aforementioned type I IFN signaling pathways, LRRFIP1 does not directly mediate the activation of IRF3 or NF-κB (Keating et al., 2011). Instead, it functions by facilitating the phosphorylation and translocation of the signaling molecule β-catenin into the nucleus (Keating et al., 2011; Yang et al., 2010). β-catenin subsequently complexes with IRF3 to initiate the production of type I IFNs (Cavlar et al., 2012; Yang et al., 2010). It is unknown if LRRFIP1 can sense GBS-DNA and induce type I IFN production via this pathway.

4. **DNA-dependent activator of IFN-regulatory factors (DAI)**

DAI was discovered for its ability to recognize poly(dA:dT) and it induces IFN-β production via a TBK1-IRF3 dependent pathway following dsDNA recognition (Takaoka et al., 2007; Trinchieri, 2010). It has been
demonstrated that GBS-induced type I IFN production is independent of DAI (Charrel-Dennis et al., 2008).

Additionally, although NLRs and RLRs have also been implicated in the sensing of DNA, it has been determined in a mouse model that these PRRs are not required for type I IFN production in response to GBS-DNA (Charrel-Dennis et al., 2008). The functionality of these pathways in response to GBS-DNA remains to be studied in human preterm infants.

**Several pathways induce Type I IFN production**

![Diagram of intracellular DNA sensors and pathways](image)

**Figure 1.4** Overview of intracellular DNA sensors (blue and grey boxes) and pathways involved in type I IFN induction. Adapted from (Keating et al., 2011).
1.9 Conclusion

The past few years have seen the discovery of new cytosolic sensors and pathways that lead to the induction of type I IFNs, and increasing evidence that suggest type I IFNs have critical roles in host defense against extracellular bacterial infections, including GBS. However, the function of type I IFNs and the mechanisms that induce these cytokines have remain unstudied in the human preterm infant, a population highly susceptible to infections caused by extracellular GBS. Therefore, the purpose of this study was to determine if type I IFNs, specifically IFN-β, are induced in human monocytes during GBS infection and if preterm infant monocytes are also capable of this response. In addition, to determine if cytosolic GBS-DNA is capable of inducing IFN-β production in human monocytes. By comparing the expression of IFN-β in monocytes of preterm infants to term infants and adults, it was expected that insight into the role of type I IFNs for host defense against GBS infections would be gained.

1.10 Aims

1) To determine if human blood monocyte subsets can detect live GBS and induce IFN-β production
2) To determine if cytosolic GBS-DNA is capable of stimulating the production of IFN-β in preterm infant, term infant and adult monocytes
3) To determine if GBS-DNA leaks out equally into cytosol of monocytes in preterm infants, term infants and adults
1.11 Hypotheses

1) That the inflammatory monocyte subset (CD14- CD16+) will be responsible for the majority of the induction of IFN-β in response to GBS infection

2) That preterm infant monocytes do not respond to cytosolic GBS-DNA in terms of IFN-β production

3) That there will be significantly lower levels of GBS-DNA being leaked out of preterm infant monocytes compared to term infant and adult monocytes
Chapter 2 Materials and Methods

2.1 Study Population

Cord blood samples were collected as part of the RE-SPIN study approved by the King Edward Memorial Hospital Ethics Committee. Adult samples were collected from healthy adult donors partaking in an on-going blood biobank approved by the Princess Margaret Hospital Ethics Committee. Written informed consent was obtained from all parents/guardians and participants.

2.2 Materials and Reagents

2.2.1 Bacterial strain

The serotype III human isolate of *Streptococcus agalactiae* strain COH-1 (ATCC® BAA-1176™, USA), originally isolated from a newborn infant with sepsis, was used in this study. This strain was chosen as it was used in published studies to investigate innate immune responses to GBS (Deshmukh et al., 2011; Kenzel et al., 2009; Mancuso et al., 2007).

2.2.2 Stimulants

**CLO97 (TLR7/8 ligand)**

Lyophilized CLO97 (InvivoGen, USA) was reconstituted with sterile endotoxin-free water (InvivoGen, USA). The stock solution (1 mg/mL) was aliquoted and stored at -20°C. The final concentration used was 1 µg/mL.
2.2.3 Flow cytometry

2.2.3.1 Antibodies

See Table 2.1 for panel of antibodies used for flow cytometry.

2.2.3.2 Buffers and reagents

**Brefeldin A (BFA) solution**

1000X BFA solution (eBioscience, USA) was stored at 4°C and diluted 1:50 in 0.9% (w/v) sodium chloride for irrigation (Baxter, USA). The final concentration used was 6 µg/mL.

**Phosphate buffer saline (PBS)**

1X PBS (Gibco of Life Technologies, USA) was stored at RT and used as neat.

**Distilled water**

Sterile water for irrigation (Baxter, USA) was used at RT.

**Flow cytometry buffer**

1X PBS containing 2% (v/v) fetal calf serum (Sigma-Aldrich, USA), 2% (w/v) bovine serum albumin (Sigma-Aldrich, USA) and 0.01% (w/v) sodium azide (NaN₃)(Sigma-Aldrich, USA) was prepared and stored at 4°C.

**Cell lysing solution**

10X FACS lysing solution (BD Biosciences, USA) was stored at RT and used at 1:10 dilution in distilled water.
Cell permeabilization solution

10X FACS permeabilizing solution 2 (BD Biosciences, USA) was stored at RT and used at 1:10 dilution in distilled water.

Cell stabilizing fixative solution

3X Stabilizing fixative (BD Biosciences, USA) was stored at RT and used at 1:3 dilution in distilled water.

2.2.4 Cell viability assay

WST-1 cell proliferation reagent

WST-1 (Roche, USA) was stored at -20°C and used neat.

2.2.5 DNA extractions from live GBS

Ethanol

100% Ethanol (Scot Scientific, Perth, Western Australia) was stored at RT and used neat or diluted 7:10 in distilled water to make 70% ethanol.

Nuclease free water

Water (Sigma-Aldrich, USA) was stored at RT and used neat.

Lysozyme from chicken egg white

Lyophilized lysozyme (Sigma-Aldrich, USA) was reconstituted with 1X PBS. The stock solution (10 mg/mL) was aliquoted and stored at -20°C. The final concentration used was 1 mg/mL.
**Proteinase K solution**

Proteinase K (Qiagen, USA) was stored at RT and used at a final concentration of 2 mg/mL.

**Isolate II genomic DNA kit**

The Isolate II genomic DNA kit (Bioline, London, UK) includes:

- Isolate II genomic DNA spin columns
- Collection tubes
- Buffers: buffer G1, buffer G2, wash buffer GW1, wash buffer GW2 and elution buffer G
- Proteinase K (not used)

Buffer G3 was prepared by adding 6 mL of buffer G1 to 1.5 mL of buffer G2 and mixed well. All buffers were stored at RT and used neat.

**SureClean solution**

SureClean solution (Bioline, London, UK) was stored at RT in dark and used neat.

2.2.6 Agarose gel electrophoresis

2.2.6.1 Agarose gel reagents

**TAE Buffer**

10X TAE buffer (Invitrogen, USA) was diluted 1:10 in milli-Q water (Merck, Germany) and used at RT.
Agarose

Agarose powder (Amresco, USA) was stored at RT.

2.2.6.2 Electrophoresis reagents and buffers

The following items were purchased from Invitrogen, USA:

- SYBR® Safe DNA Gel Stain
- 10X BlueJuice™ Gel Loading Buffer
- TrackIt™ 1kb+ DNA Ladder
- E-Gel® Low Range Quantitative DNA Ladder

All reagents and buffers were stored at RT and used neat.

2.2.7 Cell transfections and treatments

LyoVec

Lyophilized LyoVec (InvivoGen, USA) was reconstituted with sterile endotoxin-free water (InvivoGen, USA). The stock solution (160 μg/mL) was stored at 4°C and used at a final concentration of 2 μg/mL.

Poly(dA:dT)/LyoVec complexes

Lyophilized poly(dA:dT)/LyoVec (InvivoGen, USA) was reconstituted with sterile endotoxin-free water (InvivoGen, USA). The stock solution (25 μg/mL) was stored at -20°C in aliquots and used at a final concentration of 1 μg/mL.
Chloroquine
Powdered chloroquine (Sigma-Aldrich, USA) was reconstituted with nuclease free water. The stock solution (50 mg/mL) was stored at RT in dark and used at a final concentration of 2.6 µg/mL.

DNase 1
DNase 1 (Sigma-Aldrich, USA) was kindly provided by Dr. Manori Amarasekera (Childhood Allergy and Immunology Research, Perth, Australia). The aliquot (1 mg/mL) was stored at -20°C and used at a final concentration of 0.1 mg/mL.

RNase A solution
RNase A solution (Sigma-Aldrich, USA) was stored at RT and diluted 1:20 with nuclease free water. The final concentration used was 0.1 mg/mL.

2.2.8 Staining for confocal microscopy

Syto® 9 Green fluorescent nucleic acid stain
Syto9 (Invitrogen, USA) was diluted with nuclease free water and used at a final concentration of 2 mg/mL.

Hoechst 33342
Hoechst 33342 (Invitrogen, USA) was used diluted 1:1000 with sterile saline.
Table 2.1 Antibodies used for flow cytometry. Intracellular markers are highlighted in green.

<table>
<thead>
<tr>
<th>Flow Cytometry Panels</th>
<th>Surface and intracellular staining</th>
<th>Isotype control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PerCP-Cy5.5</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>Mouse Anti-Human CD14</td>
<td>Mouse IgG1, κ</td>
</tr>
<tr>
<td>Clone</td>
<td>M5E2</td>
<td>MOPC-21</td>
</tr>
<tr>
<td>Supplier</td>
<td>BD Biosciences, USA</td>
<td>BD Biosciences, USA</td>
</tr>
<tr>
<td><strong>APC-H7</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>Mouse Anti-Human CD16</td>
<td>Mouse IgG1, κ</td>
</tr>
<tr>
<td>Clone</td>
<td>3G8</td>
<td>MOPC-21</td>
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<td>BD Biosciences, USA</td>
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<tr>
<td><strong>APC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>Mouse Anti-Human HLA-DR</td>
<td>Mouse IgG2a, κ</td>
</tr>
<tr>
<td>Clone</td>
<td>G46-6</td>
<td>G155-178</td>
</tr>
<tr>
<td>Supplier</td>
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<td>BD Biosciences, USA</td>
</tr>
<tr>
<td><strong>PE</strong></td>
<td></td>
<td></td>
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<td>Specificity</td>
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<td>Mouse IgG2a, κ</td>
</tr>
<tr>
<td>Clone</td>
<td>GD4/D6/G2</td>
<td>G155-178</td>
</tr>
<tr>
<td>Supplier</td>
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<td>BD Biosciences, USA</td>
</tr>
<tr>
<td><strong>FITC</strong></td>
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<tr>
<td>Specificity</td>
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<td>Mouse IgG1</td>
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<tr>
<td>Clone</td>
<td>MMHB-3</td>
<td>MOPC-31C</td>
</tr>
<tr>
<td>Supplier</td>
<td>PBL Interferon Source, USA</td>
<td>BD Biosciences, USA</td>
</tr>
</tbody>
</table>

PerCP, peridinin chlorophyll protein; APC, allophycocyanin; PE, phycoerythrin; FITC, fluorescein isothiocyanate
2.3 Methods

2.3.1 Collection of blood samples
Cord blood was collected immediately after delivery from cord vessels and placental surface cleaned with an alcohol swab to avoid maternal contamination. Peripheral blood was obtained from healthy adults by peripheral venipuncture. All blood samples were collected into pre-heparinized vacutainer tubes (BD Biosciences, USA).

2.3.2 Preparation of live GBS
*S. agalactiae* was grown on 5% citrated horse blood agar plates (PathWest Laboratory Medicine, Perth, Western Australia) overnight at 37°C (5% CO₂). Bacterial colonies were removed from plates, inoculated in sterile heart infusion broth (HIB) (Oxoid, UK) prepared from distilled water and grown overnight shaking at 37°C. The overnight culture was diluted in HIB and grown to mid-logarithmic phase (OD<sub>600</sub> = 0.5).

Live GBS were counted on a Helber counting chamber (ProSciTech, Queensland, Australia) using a light microscope, diluted to desired concentrations with 1X PBS, and used for stimulation experiments. For further quantification of bacteria, an aliquot of live GBS culture was serially diluted and plated in duplicate on blood agar plates. Following incubation at 37°C (5% CO₂) for 24 h, the number of colony forming units (CFU) were counted and averaged for each dilution with the results expressed as CFU/mL.
2.3.3 Stimulation of whole blood with live GBS

All incubations were at 37°C (5% CO₂) in a 96-well round-bottom polypropylene plate. 50 µL of fresh adult blood was either left unstimulated or respectively stimulated with live GBS and CLO97 (1 µg/mL) for both kinetic and dose experiments. Adult whole blood was used to determine a “competent” cytokine response. For kinetic experiments, live GBS (10⁶ CFU/mL) was incubated with whole blood for 30 min, 1 h, 2 h and 4 h. For dose experiments, whole blood was stimulated with single log increments of live GBS (10³ to 10⁷ CFU/mL) for 2 h. Under optimal conditions, whole blood was stimulated for 2 h with 10⁵ CFU/mL of live GBS. The term “optimal” refers to maximal cytokine production with minimal cytotoxicity. BFA (6 µg/mL) was added for the final 4 h of all reactions to enable intracellular cytokine detection.

2.3.4 Surface and Intracellular Staining

All incubations were done at RT in the dark. Cells were stained with surface markers or appropriate isotype controls (see Table 2.1) for 15 min and transferred to a round-bottom 96-well polystyrene plate. Cells were washed twice using 1X lysing solution for 10 min and pelleted (340g, 4 min, 10°C) before permeabilization. A single wash-and-spin was done as previously described with 1X permeabilizing solution before cells were washed once with flow buffer. Cells were resuspended in flow buffer and incubated for 30 min with the respective intracellular marker or appropriate isotype control (see Table 2.1). The cells were then washed twice with flow buffer before being fixed with 1X stabilizing fixative solution and stored in dark at 4°C until flow cytometric analysis.
2.3.5 Flow Cytometric Analysis

Cells were acquired using a BD FACSCanto™ II flow cytometer (BD Biosciences, USA) and analysed with the FlowJo software program (v9.3.1, Tree Star, USA). The inclusion gate for cellular events was determined through the exclusion of red blood cells, platelets and non-cellular events by granularity (side scatter, SSC) and size (forward scatter, FSC) using area properties (Figure 3.1A). From cellular events, doublet cells were excluded based on forward scatter height (FSC-H) and area (FSC-A) to obtain singlet cells (Figure 3.1B). The population of interest was then identified based on side scatter area (SSC-A) and expression of HLA-DR (Figure 3.1C), a phenotypic marker for monocytes and DCs. Following this, monocytes were gated into CD14+ CD16-, CD14+ CD16+ and CD14- CD16+ subsets based on their surface expression pattern of CD14 and CD16 (Figure 3.1D). A threshold gate of 1.5% was set on unstimulated cells and used to distinguish cells positive for IP-10 expression for each monocyte subset. The degree of IP-10 expression was determined using median fluorescence intensity (MFI).

2.3.6 Viability Assay

100 µL of fresh whole blood was added to a 96-well round-bottom polypropylene plate and incubated with live GBS (10^6 CFU/mL) for varying stimulation times (30 min, 1 h, 2 h and 4 h) at 37°C (5% CO₂). WST-1 was added to wells, incubated for 1 h at 37°C (5% CO₂) then mixed with 150 µL of PBS. Plate was centrifuged (244g, 3 min) and supernatant was transferred to a 96-well flat-bottomed polystyrene plate (eBioscience, USA). Absorbance was measured using a Multiskan FC microplate photometer (Thermo Scientific, USA) at 420-480 nm. The following formula was used to obtain cell viability:
This assay was kindly performed by Emma de Jong.

2.3.7 DNA extractions from live GBS

Live GBS culture was grown to mid-log phase as previously described in section 2.3.2. DNA extractions were conducted based on previously published methods (Stetson and Medzhitov, 2006). 5 mL of bacterial culture was pelleted (3220g, 5 min, 25°C), resuspended in 450 µL 1X PBS and lysed by freeze-thawing two times in liquid nitrogen. Bacterial lysates were then incubated with lysozyme (1 mg/mL) for 30 min at 37°C, and sonicated for a total of 2 min in 30 s intervals (resting on ice during intervals). After sonication, the bacterial suspension was incubated with proteinase K (2 mg/mL) for 1.5 h at 56°C. Subsequently, GBS-DNA was extracted using reagents from the Isolate II genomic DNA kit according to manufacturer’s instructions (Bioline, London, UK).

2.3.8 DNA quality determination

Genomic DNA quality and concentration was determined spectrophotometrically using a NanoDrop 2000™ (Thermo Scientific, USA). Nuclease free water was used as blank. The absorbance ratio OD_{260}/OD_{280} was used as an indicator for RNA (OD_{260}/OD_{280} > 2) and protein (OD_{260}/OD_{280} < 1.8) contamination (Teare et al., 1997). The integrity of the extracted DNA was also visually assessed using gel electrophoresis. All reagents and buffers used were
from Invitrogen (USA) unless otherwise stated. The extracted DNA was electrophoresed in a 1% agarose gel (Amresco, USA) with 1X TAE buffer for 30 min (100V, 400mA). The gel was then stained with SYBR safe DNA gel stain and visualized using a Gel Doc 2000 (Bio-Rad, CA, USA). 10X blue juice loading dye was used to track DNA in samples, whilst 1Kb+ DNA ladder and E-Gel low range quantitative DNA ladder were used to determine size and weight of extracted DNA.

2.3.9 Concentration genomic DNA

To concentrate GBS-DNA extracts, an equal volume of eluted DNA and SureClean solution (Bioline, London, UK) were mixed by vortexing. The suspension was incubated for 10 min at RT then pelleted (3220g, 20 min, 25°C). The supernatant was carefully removed and the pellet was resuspended in 70% ethanol (twice original DNA elution volume). Following this, the resuspension was mixed by vortexing for 10 s and pelleted (3220g, 10 min, 25°C) before the supernatant was carefully removed. The pellet was air-dried to remove residue ethanol then resuspended in nuclease free water to a final DNA concentration of 40 µg/mL.

2.3.10 Preparation of GBS-DNA/LyoVec complexes

GBS extracts (40 µg/mL) were left untreated or treated separately either with DNase 1 (0.1 mg/mL) or RNase A (0.1 mg/mL) for 45 min at 37°C (5% CO₂). GBS-DNA extracts were then complexed with LyoVec at a ratio of 2 µg LyoVec to 1 µg DNA for 15 min at RT. The concentration of untreated GBS-DNA/LyoVec complexes used was 0.1 µg/mL, 0.5 µg/mL and 1 µg/mL. DNase 1
and RNase A digested GBS-DNA/LyoVec complexes were used at a final concentration of 1 µg/mL.

2.3.11 Transfection and treatment of cells

50 µL of whole blood was pre-treated with chloroquine (2.6 µg/mL) for 30 min at 37°C (5% CO₂) and stimulated with GBS-DNA/LyoVec complexes. Separately, cells were left untreated or stimulated with previously described GBS-DNA/LyoVec complexes (see section 2.3.10), GBS-DNA extracts only (1 µg/mL), LyoVec only (2 µg/mL), poly(dA:dT)/LyoVec (1 µg/mL), CLO97 (1 µg/mL) and live GBS (10⁵ CFU/mL). All stimulations were for 2 h at 37°C (5% CO₂) with a further 4 h incubation with BFA (6 µg/mL).

2.3.12 Syto9 staining of cells

GBS-DNA extracts only (1 µg/mL), LyoVec only (2 µg/mL), GBS-DNA/LyoVec (1 µg/mL), live GBS (10⁵ CFU/mL) and unstimulated cells were incubated for 30 min at RT in the dark with Syto 9 (0.006 mg/mL) then used for stimulations as described in section 2.3.11. Flow cytometric analysis was done as described in section 2.3.5 using a 1.5% threshold set on unstimulated monocytes.

2.3.13 Cytocentrifuging, staining and mounting of cells for confocal microscopy

35 µL of cell suspension (see in section 2.3.11 and 2.3.4) was centrifuged onto SuperFrost® Plus slides (Thermo Fisher Scientific, USA) at 221g for 20 min using a cytospin centrifuge (Hettich Rotafix 32) then air dried. Cytocentrifuged cells were circled with a liquid blocker pen (Ted Pella Inc., USA), then stained with 50 µL Hoechst 33342 (1:1000 dilution) (Invitrogen, USA) for 30 min in the
dark followed by a single wash with sterile saline for 10 min. Cover slips were mounted onto the slides using an in-house low fade mounting media and sealed with nail polish. Slides were stored at -20°C until ready for imaging using a Nikon A1Si Confocal Microscope (Centre for Microscopy, Characterization and Analysis, UWA, Perth, Australia) and high resolution x60 oil immersion objective. NIS element software was used to analyze data. Dr. Ruth Thornton kindly helped with all confocal microscopy imaging.

2.3.14 Statistical Analysis

Associate Professor Brad Zhang was consulted for all statistical analyses performed in this thesis. Prism 5 (GraphPad Software, USA) was used to perform statistical analyses. Outliers were determined using a Grubbs’ Test and excluded from data sets. A Wilcoxon signed rank test (non-parametric, paired t-test) was used to compare all matched data sets. P-values of less than 0.05 were considered as statistically significant.
Chapter 3  Assessing IFN-β responses of blood monocyte subsets during GBS infection

3.1 Introduction

Human monocytes can be divided into three subpopulations based on their surface expression of CD14 and CD16 (Zawada et al., 2011). These subsets are classified as: i) classical (CD14+ CD16-); ii) intermediate (CD14+ CD16+); and iii) inflammatory (CD14- CD16+) monocytes. In healthy adults, the majority of blood monocytes are classical (~85%), with a much lower proportion of the intermediate (~6%) and inflammatory (~9%) subsets (Wong et al., 2011). Importantly, inflammatory monocyte numbers are markedly increased during bacterial sepsis in neonates, children and adults (Fingerle et al., 1993; Skrzeczynska et al., 2002). Inflammatory monocytes are also known to be a major source of pro-inflammatory cytokines such as TNF and IL-12p70 (Belge et al., 2002; Sohlberg et al., 2011). However, it is not known if inflammatory monocytes are responsible for IFN-β production in humans, especially during GBS infection.

Therefore, this chapter investigates the production of IFN-β by human blood monocyte subsets challenged with live GBS. Healthy adult whole blood was used to examine the kinetics and responses to varying live GBS doses and to optimise conditions for subsequent infant studies.
3.2 Results

Since this was the first study in humans using whole blood to analyse intracellular IFN-β production by blood monocyte subsets to live GBS, it was imperative to optimise monocyte subset-specific staining for IFN-β production using flow cytometry as well as stimulation conditions with GBS.

3.2.1 Optimisation of intracellular IFN-β and IP-10 staining by flow cytometry

To induce IFN-β expression in monocytes in all experiments, whole blood from healthy adults was stimulated with CLO97, a TLR7/8 ligand known to induce IFN-β in monocytes (Levy et al., 2004; Monroe et al., 2010) (see section 2.3.3). IFN-β-induced protein IP-10 was included as an alternative read-out for IFN-β expression (Thomas et al., 2006). BFA was added to allow intracellular accumulation of cytokines for detection by flow cytometry (O'Mahony et al., 1998). Monocyte subpopulations were discriminated based on the surface expression of HLA-DR, CD14 and CD16 (Skrzeczynska et al., 2002) as described in Figure 3.1. The mean proportions of each subset measured in all adult donors with respect to total monocyte frequency were as follows: 87.36±1.98% classical monocytes, 5.28%±1.40% intermediate monocytes and 7.35%±0.85% for inflammatory monocytes (mean±SEM for 6 adult donors). Adult monocyte populations were therefore similar to published values (Wong et al., 2011).

The percentage of monocytes expressing FITC-labeled anti-IFN-β (FITC-anti-IFN-β) or PE-labeled anti-IP-10 (PE-anti-IP-10) antibodies after stimulation was determined based on a 1.5% threshold set on matched antibody isotype
controls (see Table 2.1 and Figure 3.1). An antibody titration curve for FITC-anti-IFN-β and PE-anti-IP-10 antibodies was created for each monocyte subset (Figure 3.2).

As seen in Figure 3.2A-B, a high percentage of stimulated cells (>80%) from all monocyte subsets appeared to express IFN-β (MFI). The level of expression was shown to increase with increasing concentrations of FITC-anti-IFN-β antibody used. As expected, matched isotype controls (identifies non-specific binding of antibodies to Fc receptors) (Pala et al., 2000) had minimal FITC-anti-IFN-β fluorescence at each concentration used. Surprisingly, a greater proportion of unstimulated cells (>98%) appeared to express IFN-β (MFI) compared to stimulated cells (Figure 3.2A-B and Figure 3.3A). It was therefore not possible to detect CLO97-dependent stimulation of IFN-β expression using the FITC-anti-IFN-β antibody.

For each monocyte subset, IP-10 expression (MFI) increased with increasing anti-IP-10 antibody concentration used (Figure 3.2C). The majority of monocytes from the classical (CD14+ CD16-, >85%), intermediate (CD14+ CD16+, >90%) and inflammatory (CD14- CD16+, >50%) subsets expressed IP-10 at concentrations of PE-anti-IP-10 antibody above 0.5 µg/mL (Figure 3.2D). In contrast to the findings with FITC-anti-IFN-β antibody, a significantly higher signal-to-noise ratio was observed between unstimulated and stimulated cells using the PE-anti-IP-10 antibody (Figure 3.3B). Therefore, IP-10 was used as a surrogate marker to measure intracellular IFN-β production for all subsequent experiments.
Figure 3.1  Gating strategy for flow cytometric analysis of intracellular IFN-β or IP-10 expression

Dot plots show the gating strategy to identify monocyte subsets. (A) Inclusion gate for cellular events (R1) and exclusion of RBCs, platelets, and non-cellular events based on granularity (SSC-A) and size (FSC-A). (B) Singlet selection (R2) by forward scatter height (FSC-H) and area (FSC-A) properties to avoid cellular doublets. (C) Identification of monocytes and DCs (R3) based on SSC-A and expression of phenotypic marker HLA-DR. (D) Determination of CD14+ CD16- (D1), CD14+ CD16+ (D2), and CD14- CD16+ (D3).
(D2) and CD14- CD16+ (D3) monocyte subsets using CD14 and CD16 markers. (E-G) Histogram plots of CLO97 stimulated cells (orange histogram) for each monocyte subset. A 1.5% marker was set on isotype controls (blue histogram) to determine percentage (%) of IP-10-positive cells. Dot and histogram plots shown are representative a single healthy adult donor.
Monocytes from the classical (diamond), intermediate (square) and inflammatory (triangle) subsets were left unstimulated (outlined shapes) or stimulated (solid shapes) with CLO97. Matched isotype controls were used for each antibody concentration. (A-B) Monocytes were stained with increasing concentrations of FITC-anti-IFN-β antibody. Figures show the level of IFN-β staining detected (MFI)(A) and proportion (%) of monocytes positive for staining (B). (C-D) Increasing concentrations of PE-anti-IP-10 antibody were used to stain monocytes. Figures show the level of IP-10 expressed (MFI)(C) and proportion (%) of monocytes expressing IP-10 (D). Data from a single donor shown.
Figure 3.3 High background staining of IFN-β but not IP-10 in unstimulated monocytes.

Flow cytometry histograms depict percentage (%) of cells cytokine-positive based on a 1.5% threshold marker set on appropriate isotype controls (orange histogram). (A) >95% of unstimulated (black line) and stimulated (blue tinted) cells were positive for IFN-β expression. (B) Percentages of IP-10-positive cells were significantly lower in unstimulated (black line) compared to stimulated (blue tinted) cells for each monocyte subset. Data from a single donor shown.
3.2.2 Assessing the relationship between live GBS viability and chain lengths

Live GBS survives as a single coccus or as chains of cocci in varying lengths (Chhatwal and Graham, 2008). It was expected that sonication would break GBS chains into individual cocci, allowing better control of inoculum size used for stimulations (and estimation of GBS-DNA load for experiments in Chapter 4). As maintaining bacterial viability was also a priority, GBS viability was assessed in relation to chain length. Live GBS was sonicated for increasing lengths of time (0, 3, 6 and 9 minutes) and quantified using a Helber counting chamber. At 0 minutes, the majority of live GBS were in chains instead of individual cocci (based on the number of chains and cocci at the start and end of sonication, average chain length was estimated to be 5 cocci/chain). Chains were successfully dispersed into individual cocci by 9 minutes of sonication (Figure 3.4A), however GBS viability (based on CFU counts following 24-hour growth) was severely impaired by more than 3 minutes of sonication (Figure 3.4B). It was therefore decided to use intact GBS chains without sonication for all stimulation experiments.
Figure 3.4  Effect of sonication on GBS viability and chain lengths

Live GBS (10^8 CFU/mL) was sonicated for increasing lengths of time (0 to 9 minutes).  
(A) Cocci (circle) and chain (square) numbers (cells/mL) for each time point calculated using a Helber counting chamber.  (B) Changes in GBS viability (%), determined from colony counts, with increasing sonication time. Data shows a single count on a Helber counting chamber.
3.2.3 Optimisation of whole blood culture conditions with live GBS

Culture conditions were optimised for challenge time, live GBS dose and cell viability for maximal IP-10 expression. IP-10 expression was used as an indirect measure of intracellular IFN-β production based on optimised staining conditions determined above (see section 3.2.1).

3.2.3.1 Challenge time

A kinetic experiment was performed using a live GBS dose of $10^6$ CFU/mL. This initial dose was chosen based on a pilot experiment with a healthy adult donor (data not shown). Whole blood from healthy adult donors (n=6) was stimulated as described in section 2.3.3, with TLR7/8 ligand CLO97 as a positive control (see section 3.2.1). No significant changes in the proportions of each monocyte subset after GBS stimulation were observed in comparison to unstimulated blood with mean±SEM of 75.45%±1.29% for classical, 8.11%±1.64% for intermediate and 16.44%±2.48% for the inflammatory subset.

Overall, IP-10 was produced rapidly by all monocyte subsets (with an MFI >2000) although only a small proportion of these monocytes (~10%) were producing IP-10 (Figure 3.5). Both the percentage of monocytes producing IP-10 and amount of IP-10 produced increased with time, peaking at 2 hours in ~20% of monocytes at an MFI of >3000. However, IP-10 expression induced by GBS was much lower than that by CLO97 stimulation, where 60-80% of monocytes expressed IP-10 and MFIs were >8000 for the classical and intermediate monocyte subsets (inflammatory monocytes produced similar levels of IP-10 to both live GBS and CLO97). Two hours was chosen as the stimulation time for all subsequent stimulation experiments.
Figure 3.5  The effect of GBS stimulation time on IP-10 expression by monocyte subsets

Adult whole blood was stimulated with live GBS (10^6 CFU/mL) for 30 minutes, 1 hour, 2 hours and 4 hours. Graphs show IP-10 expression of cells from classical (A-B), intermediate (C-D) and inflammatory (E-F) monocyte subsets. (A, C and E) Level of IP-10 expression shown as MFI. (B, D and F) Percentage (%) of cells expressing IP-10 over time. Data show mean±SEM (n=6).
3.2.3.2 GBS dose-response

A dose-response experiment was used to determine the optimal concentration of live GBS (low cell cytotoxicity and maximal IP-10 expression) for stimulation. Single log increments of live GBS (10³ to 10⁷ CFU/mL) were used to stimulate whole blood from adult donors (n=6) (see section 2.3.3). IP-10 expression levels peaked at a dose of 10⁵ CFU/mL live GBS in classical (MFI of >2000 in 19.1%) and intermediate (MFI of >4000 in 20.9%) monocytes (Figure 3.6A-D). Significant expression of IP-10 could be detected at a dose of 10⁵ CFU/mL for all subsets (MFI of >2000). Classical monocytes from a single donor caused an unexpected increase in IP-10 produced at 10⁷ CFU/mL live GBS (From MFI of 1417±257.7 to 1790±863.8; Figure 3.6A). A Grubbs' test did not quantify the value as a significant outlier (p>0.05). Inflammatory monocytes appeared to produce higher levels of IP-10 (MFI of 2930±733.8) at a GBS dose of 10⁶ CFU/mL but this was not significantly higher (p=0.4375) than the response induced at 10⁵ CFU/mL live GBS (MFI of 2348±423)(Figure 3.6E). As expected, fewer monocytes from each subset (<10%) produced IP-10 in response to low (10³ – 10⁴ CFU/mL) as well as extremely high (10⁷ CFU/mL) live GBS doses (Figure 3.6B, D and F). Across all monocyte subsets, the highest proportion (~20%) of cells expressing IP-10 was induced by 10⁵ CFU/mL live GBS, and so this dose was used for all subsequent stimulation experiments.
Figure 3.6  Dose response analysis of IP-10 expression by monocyte subsets

Adult whole blood was stimulated with increasing concentrations of live GBS (10^3 - 10^7 CFU/mL). Figures show IP-10 expression in classical (A-B), intermediate (C-D) and inflammatory (E-F) monocyte subsets. (A, C and E) Level of IP-10 expressed (MFI) by monocyte subsets. (B, D and F) Percentage (%) of cells expressing IP-10 from each monocyte subset. Data show mean±SEM (n=6). Wilcoxon signed rank test (non-parametric, paired t-test), *p<0.05, NS=non-significant.
3.2.3.3 Cell viability

Cell viability was assessed in relation to GBS challenge time using a WST-1 cell viability assay (see section 2.3.6), whereby formazan dye is formed when reduced by cell membrane reductases in viable cells (Berridge et al., 2005). To assess cell death, whole blood from adults (n=3) were stimulated with live GBS (10^6 CFU/mL) as part of the kinetic experiment (Figure 3.5). Viability of cells remained at nearly 100% during the first 2 hours of culture, followed by a non-significant drop (20%) at 4 hours (Figure 3.7).

![Figure 3.7 Effect of GBS stimulation on cell viability](image)

Percentage viability of cells stimulated with live GBS (10^6 CFU/mL) for 30 minutes, 1 hour, 2 hours and 4 hours are shown. Data show mean±SEM (n=3). Wilcoxon signed rank test (non-parametric; paired t-test), NS=non-significant.
3.2.4 Similar proportions of all monocytes subsets express IP-10 to live GBS

The difference in expression of IP-10 between the monocyte subsets was compared under conditions of optimal stimulation time and dose. Similar proportions of monocytes from the classical (CD14+ CD16-), intermediate (CD14+ CD16+) and inflammatory (CD14- CD16+) subsets produced IP-10 (17.2%, 21.0% and 22.1% respectively; Figure 3.8A). A Wilcoxon signed rank test confirmed that the percentage of IP-10-positive cells was not statistically different between the monocyte subsets (p>0.05).

3.2.5 Intermediate monocytes express the highest levels of IP-10 to live GBS

Similar levels of IP-10 were expressed by both classical (CD14+ CD16-) and inflammatory (CD14- CD16+) monocytes (MFIs of 2037±339.8 and 2348±423 respectively; p>0.05)(Figure 3.8B). Surprisingly, intermediate (CD14+ CD16+) monocytes produced approximately double the amount of IP-10 (MFI of 4715±1175) compared to classical and inflammatory monocytes. A Wilcoxon signed ranked test confirmed IP-10 production by intermediate monocytes was significantly higher compared to both classical and inflammatory monocytes to $10^5$ CFU/mL of live GBS (p=0.0313).
Figure 3.8  Comparison of IP-10 responses of monocyte subsets to live GBS

Whole blood from healthy adult donors was left unstimulated, or stimulated under optimal conditions (10^5 CFU/mL live GBS for 2 hours). The level of IP-10 expression determined from MFI (A) and percentage of monocytes expressing IP-10 (B) across the classical (CD14+ CD16-), intermediate (CD14+ CD16+) and inflammatory (CD14-CD16+) monocyte subsets. Data show mean±SEM (n=6). Wilcoxon signed rank test (non-parametric, paired t-test) was used to compare between two monocyte subset groups, *p<0.05.
3.3 Discussion

IFN-β is critical for the control and clearance of experimental GBS blood infection in mice (Mancuso et al., 2007), but it is unknown if human blood monocytes can produce IFN-β during GBS infection. A whole blood assay was optimised to investigate monocyte subset IFN-β responses to live GBS, either directly or through the production of IP-10. The main findings were: (i) GBS induced IFN-β production in monocytes but this could only be determined indirectly using IP-10 expression; (ii) all monocyte subsets are capable of producing IP-10; and (iii) intermediate monocytes are the main producers of IP-10. Overall, these findings suggest that different levels of IFN-β-induced IP-10 are produced by all monocyte subsets following live GBS challenge.

3.3.1 GBS induces IP-10 production in monocytes

This study found that all monocyte subsets were capable of producing IFN-β to live GBS stimulation, although IFN-β responses could only be determined using IP-10 as a surrogate. This was because unstimulated monocytes stained with anti-IFN-β antibodies had high levels of FITC fluorescence, which made it difficult to distinguish a positive signal over background noise. A series of optimisation experiments that included use of human AB serum (pre-block Fc receptors responsible for non-specific binding) as well as extra washing steps (remove excess antibodies) were conducted (data not shown). However, these steps failed to reduce FITC background fluorescence of unstimulated monocytes. Therefore, because anti-IP-10 stained monocytes had a higher signal-to-noise ratio compared to anti-IFN-β stained monocytes, IP-10 was chosen as an indirect measure of IFN-β production. Of note, although IFN-β could not be directly measured in this study, a previous study has assessed
IFN-β expression in human Tenon’s fibroblasts by flow cytometry (Chang et al., 2001). In addition, although RT-PCR would have provided a more sensitive and precise method of directly assessing cytokine production in real time (Valasek and Repa, 2005), and has been successfully used by studies to assess IFN-β production (Aksoy et al., 2007; Li et al., 2011), we did not use RT-PCR for this study as it would require pre-sorting of monocytes into subsets by flow cytometry (Frankenberger et al., 1996). This may result in non-specific activation of monocytes associated with the use of CD14 and CD16 antibodies for cell-sorting (Frankenberger et al., 1996). Therefore, flow cytometry was used to directly assess cytokine expression at the single-cell level to avoid artificial changes to monocytes, allowing culture conditions to closely model in vivo GBS infections.

The data also showed that IP-10 was rapidly produced in response to GBS challenge. By 30 minutes of GBS challenge, high levels of IP-10 were already produced, although this was only in a small proportion of monocytes. Importantly, most monocytes produced maximal IP-10 expression by 2 hours of live GBS stimulation. The rapid production of IP-10 suggests that IFN-β was likely responsible for the induction of IP-10, which was previously demonstrated by a published study (Thomas et al., 2006). This result was also consistent with other studies in myeloid cells that have also reported rapid IFN-β induction to bacterial stimuli such as LPS (Aksoy et al., 2007; Li et al., 2011). Additionally, the rapid production of IP-10 also suggests that IFN-γ did not contribute to IP-10 responses to GBS. This is supported by a previous study that showed IFN-γ were produced following 18 hours of culture with GBS in mononuclear cells (Joyner et al., 2000). Collectively, these results suggest that IP-10 can be used
as a surrogate to measure IFN-β production. It would have been useful if IFN-γ antibodies were also used to confirm that IFN-γ did not contribute to IP-10 expression. Additionally, a direct measure of IFN-β using RT-PCR or ELISA would also be helpful.

Finally, the results also showed that IP-10 responses were dependent on the dose of live GBS used. IP-10 expression increased with live GBS dose ($10^3 - 10^5$ CFU/mL), peaking at $10^5$ CFU/mL, before declining at higher doses (above $10^6$ CFU/mL). We attempted to determine whether lower proportions of IP-10 producing monocytes were due to early apoptosis or necrosis in response to GBS by using an Annexin V and Propidium Iodide kit (data not shown)(Miao et al., 2011). However, contamination from RBCs made it difficult to analyse cell viability by flow cytometry. Therefore, a WST-1 cell viability assay was performed instead, and showed that overall cell viability remained high in response to $10^6$ CFU/mL. A limitation of this assay however was that the result obtained was representative of all viable white blood cells and not of monocytes only.

Although overall cell viability remained high at $10^6$ CFU/mL, the reduced proportion of monocytes producing IP-10 at higher GBS concentrations ($10^7$ CFU/mL) could have still been due to cell death. A previous study found that high numbers of extracellular bacteria (E. coli and K. pneumoniae) in human monocytes result in a non-apoptotic form of cell death (Webster et al., 2010), whereby cell death is likely due to pyroptosis, a death process associated with antimicrobial responses to pathogens during inflammation (Martinon et al., 2009; Webster et al., 2010). Likewise, it is possible that high concentrations of
GBS in human monocytes may have also triggered pyroptosis. Pyroptosis is dependent on the activation of an inflammasome as well as the release of caspase-1 (Hornung et al., 2009; Martinon et al., 2009). With AIM2 recently identified as a sensor for cytosolic bacterial dsDNA capable of activating an inflammasome (Hornung et al., 2009; Rathinam et al., 2010) and the release of GBS dsDNA into cytosol of mouse macrophages shown (Charrel-Dennis et al., 2008), it is possible that higher GBS loads resulted in higher concentrations of dsDNA being released into the cytosol of monocytes to trigger pyroptosis. Separately, a study showed that high concentrations of cytoplasmic dsDNA also results in a DNA-induced cell death (Roberts et al., 2009).

Overall, these results suggest that the lower proportion of monocytes producing IP-10 at higher live GBS doses may have been due to pyroptosis. Therefore, highlights the importance of controlling bacterial inoculum size since it may affect the amount of dsDNA infecting cells (Charrel-Dennis et al., 2008; Rathinam et al., 2010; Webster et al., 2010).

In relation to this study, since live GBS survive as cocci or chains of varying lengths (Chhatwal and Graham, 2008), and it is unknown which is the infectious unit, it was expected that dispersing chains into cocci via sonication would better control numbers of live GBS used for stimulations. However, it was found that breakage of live GBS chains severely compromised the viability of live GBS. Comparison to published studies found that viability of other bacterial species (E. coli and K. pneumoniae) were also impaired following sonication (Joyce et al., 2011; Joyce et al., 2003). Whilst Gram-positive cocci are known to be more resistant to the effects of sonication due to the thick peptidoglycan cell
wall (Foladori et al., 2007; Joyce et al., 2011), this was not true for GBS. Since there was no sonication time where live GBS was viable and surviving as individual cocci, GBS was enumerated prior to all subsequent experiments to ensure infecting live GBS numbers remained consistent. Taken together, these results show that production of IFN-β during GBS infections is likely dependent on GBS bacterial load, with higher GBS burden possibly resulting in pyroptosis of monocytes. However, further studies would be needed to address the relationship between higher bacterial load, lower cytokine production and pyroptic cell death during GBS infections in humans.

### 3.3.2 Similar proportions of each monocyte subset produce IP-10 in response to live GBS

This was the first study comparing IP-10 production of monocyte subsets to live GBS. Flow cytometric analysis showed that all monocyte subsets were capable of producing IP-10 in response to live GBS. Interestingly, all monocyte subsets had similar proportion of cells producing IP-10 to live GBS, suggesting that all monocyte subsets had a similar capacity to respond to live GBS. This finding was in contrast to other studies that found inflammatory monocytes had a higher capacity to respond to microbial stimulation due to higher expression of TLR2 (Belge et al., 2002; Sohlberg et al., 2011). Since TLR2 is more highly expressed in the inflammatory monocyte subset (Belge et al., 2002; Sohlberg et al., 2011), and LTA of GBS has been shown to engage TLR2 (Henneke et al., 2005), it does not appear that GBS LTA nor TLR2 plays a significant role in terms of inducing IP-10 production. This is because despite different expression of TLR2 for each monocyte subset, all monocyte subsets responded equally to live GBS in terms of IP-10 production. Overall, these results show that all
monocyte subsets have similar capacity to respond to GBS in terms of IP-10 production. Further investigation into the expression and activation of surface receptors of each monocyte subset would provide a better understanding of how each subset responds to live GBS in terms of IFN-β production.

3.3.3 Intermediate monocytes produce the highest amount of IP-10

In terms of the amount of IP-10 produced, intermediate (CD14+ CD16+) monocytes were found to be the main producer of IP-10 in response to live GBS challenge. The markedly higher production of IP-10 in intermediate monocytes was statistically different compared to both classical and inflammatory monocytes (p<0.05). This was in contrast to other studies that showed inflammatory monocytes were mainly responsible for pro-inflammatory cytokine production to bacterial components such as PGN and LPS (Belge et al., 2002; Sohlberg et al., 2011). A possible reason for this difference may be because intermediate monocytes are poorly characterized (Zawada et al., 2011), with previous studies mostly disregarding this subset or classifying them into the same subset as inflammatory monocytes (Blumenstein et al., 1997; Fingerle et al., 1993; Nockher and Scherberich, 1998). A recent study however has described intermediate monocytes, which express low CD14 and high CD16 markers, as a transitional population between classical and inflammatory monocytes (Wong et al., 2011). Collectively, these results suggest that intermediate monocytes instead of inflammatory monocytes are likely to be responsible for the majority of IFN-β produced during GBS infections. In addition to CD14 and CD16 markers, future studies could include the use of other markers such as HLA-DR and CCR5, which are also differentially...
expressed by monocyte subsets (Wong et al., 2011; Zawada et al., 2011), and that would help in better classification of monocyte subsets.

3.3.4 Conclusion

IP-10 is rapidly produced by human monocytes in response to live GBS chains in a dose-dependent manner. Even though the production of IFN-β could not be directly detected, it is likely that this IP-10 is IFN-β derived given the rapid response. Whilst all monocyte subsets were capable of responding to live GBS in terms of IP-10 expression, intermediate monocytes expressed nearly twice as much IP-10 as the classical and inflammatory monocytes. Further studies are needed to confirm the release of IFN-β and clarify the roles of each monocyte subset in terms of cytokine production during actual bacterial infection.
Chapter 4 Investigating IFN-β responses to cytosolic GBS dsDNA

4.1 Introduction

It has been recently recognized that nucleic acids such as DNA and RNA from extracellular bacteria are capable of inducing IFN-β production by cells of the innate immune system (Charrel-Dennis et al., 2008; Gratz et al., 2011; Mancuso et al., 2009; Parker et al., 2011). Importantly, GBS-DNA in the cytosol of mouse macrophages has been found to be a potent ligand for IFN-β production (Charrel-Dennis et al., 2008). The murine study showed that during GBS infection, mouse macrophages phagocytose live GBS into their phagolysosomes, where they release pore-forming toxins that alter the phagolysosomal membrane integrity (Charrel-Dennis et al., 2008). Subsequently, phagolysosomal enzymes like chymotrypsin degrades GBS into bacterial products including dsDNA and RNA, which are then released into the cytosol of the phagocytosing mouse macrophages. The presence of GBS-DNA in the cytosol then potently induces IFN-β production in a manner that is not yet fully understood (Charrel-Dennis et al., 2008). It is not known if GBS-DNA in the cytosol of human monocytes would also induce IFN-β production, and whether neonatal monocytes would respond similarly to those in adults.

This chapter therefore investigates the production of IFN-β-induced IP-10 by human monocytes in response to cytosolic GBS-DNA, and whether these IP-10 responses are impaired in monocytes of an extremely preterm infant (28 weeks GA) in comparison to adult monocytes.
4.2 Results

4.2.1 Optimisation of GBS-DNA extraction

It was essential for the DNA-based experiments in this chapter to isolate GBS dsDNA in a relatively large quantity and with high purity and integrity. The objective was thus to compare yield and purity of DNA following extractions from GBS using two commercially available genomic DNA extraction kits. DNA was purified using the Qiagen DNA Mini Kit (Qiagen) and Bioline Isolate II Genomic DNA Kit (Bioline) according to manufacturer’s instructions. In order to determine whether additional elutions would affect purity and concentration of extracted DNA, three elutions with nuclease free water were performed for Qiagen extractions (Figure 4.1). In contrast, only a single elution with Elution Buffer G was used for the Bioline kit. As seen in Figure 4.1A, DNA yield was approximately double using Bioline (>4000 ng) compared to Qiagen (<2200 ng). Spectrophotometric analysis of GBS-DNA extracts (see section 2.3.8) showed that apart from the first elution of Qiagen, pure DNA (defined from OD260/OD280) was obtained for both the final two elutions of the Qiagen protocol and for first extraction with the Bioline kit. Gel electrophoresis was then performed to confirm that the extracted DNA was not degraded (see section 2.3.8). Consistent with results found in Figure 4.1A-B, a single brighter band was observed for GBS-DNA extracted using Bioline compared to all Qiagen extractions, indicating higher concentrations of whole genomic DNA (Figure 4.1C). All subsequent DNA extractions were performed using the Bioline protocol as the yield was higher.
Figure 4.1 Extraction of GBS-DNA from GBS lysates using 2 commercial kits

Genomic DNA was extracted from GBS lysates using Qiagen DNA Mini Kit in three elutions (a-c) and Bioline Isolate II Genomic DNA Kit in a single elution (d). (A) The DNA yield (ng) from Qiagen after each elution (a-c) and Bioline (d). (B) DNA purity was spectrophotometrically analyzed using NanoDrop technology. (C) Integrity of DNA was visually assessed using electrophoresis on a 1% agarose gel in TAE.
4.2.2 Optimisation of GBS-DNA dose for maximal IP-10 expression

To determine the concentration of cytosolic GBS-DNA required for maximal IP-10 expression by human monocytes, a dose-response experiment was performed. Whole blood from a single adult donor was stimulated with increasing concentrations of GBS-DNA, or with 5 µg/mL of poly(dA:dT) based on a published study (Unterholzner et al., 2010). Poly(dA:dT) is a synthetic dsDNA known to induce IFN-β by cytosolic sensors (Ablasser et al., 2009; Chiu et al., 2009), and was thus used as a positive control. Both GBS-DNA and poly(dA:dT) were complexed with a fixed amount of LyoVec (recommended by the manufacturer), a cationic lipid-based molecule that transfects DNA into the cytosol of cells (Charrel-Dennis et al., 2008). The production of IP-10 was dependent on the stimulating dose of GBS-DNA complexed with LyoVec (Figure 4.2). The highest amount of IP-10 produced and proportion of positive classical, intermediate and inflammatory monocytes was at the 1 µg/mL GBS-DNA dose (MFIs of 228, 245 and 207 and proportion of positive monocytes of 9.5%, 28.2% and 28.9% respectively). The amount of IP-10 produced (for all subsets) and percentage of positive monocytes remained low (MFI of <150 in <5% of cells) for all other GBS-DNA concentrations used (0.5, 5 and 10 µg/mL). Poly(dA:dT) also produced similar levels of IP-10 with MFI of 150 in <10% for all 3 monocyte subsets.
Figure 4.2 Dose-response of IP-10 production by adult monocytes subsets to cytosolic GBS-DNA

Adult whole blood was stimulated with increasing doses of GBS-DNA (0.5, 1, 2.5, 5 and 10 µg/mL)(solid shapes) or poly(dA:dT)(5 µg/mL)(outlined shapes). Both GBS-DNA and poly(dA:dT) were complexed with (2 µg/ml) LyoVec prior to transfections. Amount (MFI) of IP-10 expressed (A) and percentage of monocytes expressing IP-10 (B) from the classical (blue diamond), intermediate (green square) and inflammatory (pink triangle) subsets. (Data shown from a pilot experiment using a single adult donor).
4.2.3 Requirement of cytosolic delivery of GBS-DNA for IP-10 induction

To confirm that the human monocyte response to GBS-DNA was due to cytosolic delivery, and not to extracellular DNA receptor activation, adult whole blood (n=3-8 donors) was stimulated with varying doses of GBS-DNA/LyoVec (i.e. cytosolic GBS-DNA) up to 1 µg/mL, or with an extracellular GBS-DNA extract at 1 µg/mL. LyoVec alone (2 µg/mL), or Poly(dA:dT) complexed with LyoVec (1 µg/mL), were included as negative and positive controls, respectively. Increasing concentrations of GBS-DNA/LyoVec were tested up to the optimal dose from Figure 4.2 to allow for potential differences in sensitivity to DNA concentration between donors (see section 4.2.2).

Although monocytes stimulated with GBS-DNA/LyoVec produced higher amounts of IP-10 (MFI of 890.1±232.8) compared to cells stimulated with GBS-DNA extracts only (MFI of 576.6±54.4) (Figure 4.3A), the Wilcoxon signed rank test found no statistical difference (p>0.05) between the two groups. Consistent with a previous experiment (see section 4.2.2), higher levels of IP-10 were produced at 1 µg/mL of GBS-DNA/LyoVec compared to lower doses (MFI of ~450-500). Notably, comparable amounts of IP-10 were produced to both GBS-DNA/LyoVec and poly(dA:dT)/LyoVec when used at a concentration of 1 µg/mL (MFI of ~900-950). Interestingly, the highest proportion of IP-10-positive monocytes (3.3%) was at the lowest GBS-DNA/LyoVec dose (0.1 µg/mL), but this was not significantly higher (p>0.05) than the proportion of IP-10-positive cells to other stimulants used (~2%; Figure 4.3B). Notably, GBS-DNA/LyoVec stimulated monocytes produced significantly higher levels of IP-10 (p<0.05) compared to LyoVec only stimulation.
Adult whole blood was left unstimulated, or stimulated with increasing concentrations of GBS-DNA complexed with LyoVec (0.1, 0.5 and 1 µg/mL) and GBS-DNA extracts only (1 µg/mL). LyoVec only (2 µg/mL) and poly(dA:dT) complexed with LyoVec (1 µg/mL) were used as controls. Amount (MFI) of IP-10 expressed (A) and proportion of monocytes expressing IP-10 (B). Data show mean±SEM (n=3-8). Wilcoxon signed rank test (non-parametric, paired t-test) *p<0.05.
4.2.4 Role of cytosolic GBS-DNA and endosomal TLRs for IP-10 production

To confirm the role of intact cytosolic dsDNA in IP-10 production, GBS-DNA extracts were first treated with DNase 1 – to digest dsDNA – or with RNase A – to cleave ssRNA – before complexing with LyoVec. Monocytes stimulated with GBS-DNA/LyoVec produced comparable levels of IP-10 as poly(dA:dT)/LyoVec (MFI of ~900-950; Figure 4.4A). IP-10 production was higher compared to monocytes treated with DNase 1 or RNase A digested GBS-DNA extracts (MFI of 629.6±71.8 and 618.1±77.8 respectively). Across all treatments, similar proportions of monocytes produced IP-10 (~2%; Figure 4.4B).

Separately, monocytes were also pre-treated with chloroquine to determine whether endosomal TLRs were involved in GBS-DNA recognition. Chloroquine is a 4-aminoquinoline drug that inhibits endosomal acidification and hence the activity of endosomal TLRs including the CpG DNA receptor, TLR9 (Ewaschuk et al., 2007). Although a higher proportion of chloroquine-treated monocytes (4.1%) produced IP-10 compared to untreated monocytes (2.1%), both the proportion of monocytes and amount of IP-10 produced (MFI of 714±152.5) were not statistically different (p>0.05) between chloroquine-treated and untreated monocytes.
Figure 4.4 Induction of IP-10 by cytosolic dsDNA is not due to endosomal TLRs or RNA

GBS-DNA (1 µg/mL) was left untreated or treated with DNase 1 (0.1 µg/mL) or RNase A (0.1 µg/mL) then complexed with LyoVec (2 µg/mL) before being used to stimulate adult blood. Separately, healthy adult blood was pre-treated with chloroquine (2.6 µg/mL) for 30 min prior to stimulation. Poly(dA:dT)/LyoVec complexes (1 µg/mL) were used as a positive control. IP-10 expressed (MFI) by monocytes (A) and proportion of monocytes expressing IP-10 (B). Data show mean±SEM (n=7-8).
4.2.5 Monocyte subset IP-10 responses to cytosolic GBS-DNA and live GBS

IP-10 responses of classical, intermediate and inflammatory monocytes to GBS-DNA/LyoVec, live GBS, poly(dA:dT) and CLO97 were compared. CLO97 induced higher IP-10 expression (MFI of >3000 in >30% of monocytes) compared to all other stimulants (Figure 4.5). For all subsets, comparable levels of IP-10 were produced in response to both poly(dA:dT) and the optimised GBS-DNA/LyoVec dose (1 µg/mL)(MFI of ~800 in <5%), although these were lower compared to live GBS (MFI of >1000 in >18%). Each subset had a higher proportion of IP-10-positive monocytes when stimulated with live GBS (17.0%, 33.3% and 27.0% respectively; Figure 4.5A) compared to all doses of GBS-DNA/LyoVec (<5% for all monocyte subsets). The amount of IP-10 produced by each subset was also higher to live GBS (MFI of 1314±164.4, 2726±635.4 and 1045±79.8 respectively; Figure 4.5B) compared to all doses of GBS-DNA/LyoVec (MFI of ~1000, ~1200 and ~550 respectively). Notably, intermediate monocytes produced the highest amount of IP-10 to both GBS-DNA/LyoVec and live GBS.
Whole adult blood was treated with GBS-DNA/LyoVec (0.1, 0.5 and 1 µg/mL), live GBS (10^5 CFU/mL), poly(dA:dT)/LyoVec (1 µg/mL) and CLO97 (1 µg/mL). Proportion (%) of monocytes expressing IP-10 (A) and amount of IP-10 produced (B) by the classical (CD14+ CD16-)(blue), intermediate (CD14+ CD16+)(green) and inflammatory (CD14- CD16+)(pink) monocyte subsets. Data show mean ± SEM (n=3-8).
4.2.6 IP-10 responses of monocytes from an extremely preterm infant to GBS (live GBS and cytosolic GBS-DNA)

To investigate whether monocytes of extremely preterm infants (28 weeks GA) were capable of producing IP-10 in response to both cytosolic GBS-DNA and live GBS, whole cord blood was stimulated with GBS-DNA/LyoVec (0.1, 0.5 and 1 µg/mL) and live GBS ($10^5$ CFU/mL) (see section 2.3.11). It was originally hoped that 5-8 preterm infants would be studied, but due to the time taken to optimise the whole blood model and the challenges in recruiting such small infants, only a single infant was available for study.

Overall, the studied infant was capable of producing IP-10 to both cytosolic GBS-DNA and live GBS, since the responses were higher compared to unstimulated monocytes. The infant had similar proportions of IP-10-positive monocytes as the adults in response to GBS-DNA/LyoVec (<5%) (Figure 4.6A). However, the amount of IP-10 produced was only comparable to adults at lower doses of GBS-DNA/LyoVec (0.1 and 0.5 µg/mL) (MFI of <600) (Figure 4.6B). Interestingly, visibly lower levels of IP-10 were produced by monocytes of the infant (MFI of 361) compared to adults at the optimised GBS-DNA/LyoVec dose (1 µg/mL) (MFI of 890.1±232.8). In addition, IP-10 responses to live GBS were visibly lower (MFI of 364 in 4.4% of monocytes) compared to adults (MFI of 1212±127.1 in ~20% of monocytes; Figure 4.6). All monocyte subsets of the studied infant were able to produce IP-10 to all stimulants used (MFI of <400 in ~5-10% of cells), although responses were lower compared to adults (MFI of ~1000-3000 in ~20-35%; Figure 4.7 and Figure 4.8). Statistical tests could not be performed as only one cord blood sample was obtained.
Figure 4.6  IP-10 expression by very preterm infant and adult monocytes to cytosolic GBS-DNA and live GBS

Cord blood from a preterm infant (28 weeks GA) and peripheral blood from healthy adults were stimulated with GBS-DNA/LyoVec (0.1, 0.5 and 1 µg/mL) and live GBS (10^5 CFU/mL). Proportion of all monocytes producing IP-10 (A) and amount (MFI) of IP-10 produced (B) are shown. Data show mean ± SEM (n=3-8 adults).
Figure 4.7  IP-10 expression in preterm infant and adult monocyte subsets

Fresh cord blood from a very preterm infant (28 weeks GA) and peripheral blood of healthy adults were stimulated with GBS DNA/LyoVec (1 µg/mL), LyoVec only (2 µg/mL) and live GBS (10⁵ CFU/mL). Amount (MFI) of IP-10 expressed by monocytes from the intermediate (A), classical (B) and inflammatory (C) subsets. Data shown are mean ± SEM for n=3-8 adults and values from a single preterm infant.
Figure 4.8 Proportion of preterm infant and adult monocytes expressing IP-10

Fresh cord blood from a very preterm infant (28 weeks GA) and peripheral blood of healthy adults were stimulated with GBS-DNA/LyoVec (1 µg/mL), LyoVec only (2 µg/mL) and live GBS (10^5 CFU/mL). Proportions of monocytes expressing IP-10 from the intermediate (A), classical (B) and inflammatory (C) subsets. Data shown are mean ± SEM for n=3-8 adults and values from a single preterm infant.
4.2.7 Determining amount of GBS-DNA within cytosol of host monocytes

To assess the amount of GBS-DNA present within the cytosol of cells after GBS challenge, DNA was labeled with Syto9 as described in section 2.3.12. According to flow cytometric analysis, DNA from live GBS was found within a much higher proportion of monocytes (~42%) compared to GBS-DNA/LyoVec (~0.2%) or GBS-DNA extracts (~1.7%) only (Figure 4.9).

In parallel, confocal microscopy was used to visually assess the uptake of GBS-DNA/LyoVec and live GBS in cells. Hoechst 33342 (Invitrogen, USA) was used to stain cell nuclei blue. GBS-DNA/LyoVec complexes were found within the cell cytoplasm, although only with the counterstain used (Hoechst 33342) and not Syto9 (Figure 4.10A). Morphologically, the cell appeared to be a monocyte although fluorescent-labeling of monocyte markers such as CD14 and CD16 will be needed to confirm this. In contrast to Figure 4.10A, large numbers of live GBS cocci and chains with Syto9-tagged-DNA were found within the cytoplasm of the cell (Figure 4.10B).
Proportion of cells expressing Syto 9 are shown. GBS-DNA/LyoVec (1 µg/mL), LyoVec only (2 µg/mL) and live GBS (10^5 CFU/mL) were stained with Syto 9 to label DNA prior to monocyte stimulations. A 1.5% threshold marker was set on unstimulated cells stained with Syto 9. Data show mean±SEM (n=3). Wilcoxon signed rank test, NS=non-significant.
Figure 4.10  GBS-DNA and live GBS in host cell cytoplasm

Confocal microscopy (x60) of cells stimulated with GBS-DNA/LyoVec complexes and live GBS (10⁵ CFU/mL). (A) GBS-DNA/LyoVec complexes and host DNA were stained with Hoechst 33342 (blue). (B) DNA within live GBS cocci and chains were stained with Syto9 dye (green).
4.3 Discussion

Cytosolic GBS-DNA in mouse macrophages was found to induce intracellular IFN-β production (Charrel-Dennis et al., 2008). However, it is unknown whether cytosolic GBS-DNA would induce IFN-β-induced IP-10 production in human monocytes, and whether these responses are impaired in extremely preterm infant (<30 weeks GA) monocytes. A whole blood assay was therefore used to determine whether GBS-DNA in the cytosol of human monocytes would induce IP-10 production. The main findings included: (i) GBS-DNA in the cytosol of host monocytes induces the production of IP-10; (ii) IP-10 is mainly produced by the intermediate monocyte subset; and (iii) extremely preterm infant monocytes produce lower levels of IP-10 compared to adults.

4.3.1 GBS-DNA in the cytosol of host monocytes induces IP-10 production

It is unknown whether GBS-DNA in the cytosol of human monocytes would induce the production of IP-10. This study found that adult monocytes were capable of producing IP-10 in response to GBS-DNA in the cytosol. Of note, a much lower IP-10 response was observed compared to live GBS stimulations, which is discussed in more detail in section 4.3.2 below. Importantly, several experiments were conducted to ensure that the IP-10 responses observed were indeed due to cytosolic GBS-DNA.

First, stimulation of monocytes with LyoVec only was used as a negative control. Although baseline IP-10 production to LyoVec was surprising, a much earlier study found that liposomal reagents such as lipofectamine could induce
IFN-stimulated genes in the absence of DNA (Li et al., 1998). Importantly, IP-10 induction by LyoVec was significantly lower compared to cytosolic GBS-DNA.

Second, stimulation of monocytes with extracellular GBS-DNA extracts confirmed that surface receptors were not activated, and was consistent with published studies (Charrel-Dennis et al., 2008; Takeuchi and Akira, 2010). It should be noted that whilst GBS-DNA extracts were spectrophotometrically determined to be pure DNA (Teare et al., 1997), there is a possibility that contamination by other nucleic acids (dsRNA or ssRNA) occurred. Although it is not known whether GBS dsRNA induces IP-10 production, GBS ssRNA has been shown to induce IFN-β production via TLR7 in mouse dendritic cells (Mancuso et al., 2009). Therefore, activation of endocytic nucleic acid sensing TLRs (i.e. TLR3, TLR7/8 and TLR9) (Takeuchi and Akira, 2010) may have contributed to the IP-10 expression observed. Importantly, cytosolic GBS-DNA induced a higher IP-10 production compared to extracellular GBS-DNA (GBS-DNA found outside of the host cell cytoplasm).

Third, among nucleic-acid sensing TLRs, only TLR9 has been shown to recognize DNA within the cytoplasm (Stetson and Medzhitov, 2006). In order to exclude a role for TLR9 in the sensing of cytosolic GBS-DNA, cells were treated with chloroquine, an antimalarial drug known to block TLR9 activity (Yasuda et al., 2008). Since IP-10 production remained comparable to untreated monocytes, this suggests TLR9 was not involved in the recognition of GBS-DNA in the cytosol under the conditions used in this study. It is important to note that the role of TLR9 cannot be entirely excluded, as chloroquine concentrations used may not have successfully inhibited TLR9 activity. As CpG-
oligonucleotides are known to induce IFN-β production by TLR9, chloroquine concentrations could be optimized using a dose-response experiment to provide further conclusions on the role of TLR9 in recognizing cytosolic GBS-DNA.

Finally and most importantly, the production of IP-10 to cytosolic GBS-DNA was comparable to poly(dA:dT), a ligand known to stimulate several cytosolic DNA sensors capable of inducing IFN-β production (Keating et al., 2011). Collectively, these results suggest that human monocytes are capable of producing IP-10 to GBS-DNA in the cytosol by a pathway independent of TLR9, which is consistent with a previously published mouse study (Charrel-Dennis et al., 2008). It would be insightful to examine in detail whether recently elucidated cytosolic DNA sensors (Keating et al., 2011) are involved in the yet to be identified pathway in human monocytes.

This study also found that IP-10 production in monocytes was dependent on the concentration of extracted GBS-DNA used. Importantly, both GBS-DNA and poly(dA:dT) induced similar levels of IP-10 production when used at the same concentration. This likely suggests that IP-10 production was independent of the DNA sequence, but dependent on the concentration of DNA used. The data was consistent with other studies that found induction of IFN-β responses were independent of the DNA sequence used (Unterholzner et al., 2010), but that DNA-induced cell death was dependent on DNA concentrations (Roberts et al., 2009). Whilst it appears lower IP-10 responses at higher DNA doses maybe due to DNA-induced cell death, it is important to note that only a single donor was used. A larger sample size would therefore be useful to confirm whether IP-
10 responses are indeed dependent on the concentration of DNA. Taken together, these results suggest that IP-10 responses are not influenced by the DNA sequence but are likely dependent on the concentration of DNA used.

In contrast to published findings (Charrel-Dennis et al., 2008), this study found that transfection of GBS-DNA extracts digested with DNase 1 did not abolish IP-10 production. Although this suggests that intact cytosolic GBS-DNA maybe redundant for IP-10 production, it is also possible that the DNase 1 concentration used may not have effectively digested all GBS dsDNA. A previous study has shown that the length of DNA influences IFN-β responses (Unterholzner et al., 2010). It is therefore possible that the DNase 1 fragmented oligonucleotides were still long enough in length to induce IP-10 production. A gel electrophoresis should be performed following digestions to assess whether GBS dsDNA is effectively removed. Cumulatively, these results suggest that GBS-DNA in the cytosol of host monocytes contributes to IP-10 production although further studies with more optimal conditions should be performed.

4.3.2 IP-10 is mainly produced by the intermediate monocyte subset

Having identified that extracted GBS-DNA in the cytosol of host monocytes (cytosolic GBS-DNA) induces IP-10 production, the next step was to investigate IP-10 responses of the three monocyte subsets (classical, intermediate and inflammatory). It was found that all monocyte subsets were capable of responding to cytosolic GBS-DNA in terms of IP-10 production. Consistent with an earlier study using live GBS stimulations (see section 3.3.2 and 3.3.3), the intermediate monocyte subset was responsible for the highest amount of IP-10 being produced to cytosolic GBS-DNA. It is still unknown whether different
levels of cytoplasmic DNA sensors exist within each monocyte subset. In addition, similar to an earlier observation, each monocyte subset produced comparable amounts of IP-10 to both cytosolic GBS-DNA and poly(dA:dT) when used at the same concentration. It would be interesting to determine whether IP-10 responses of monocyte subsets would be affected by the concentrations of GBS-DNA or poly(dA:dT) used. Overall these results suggest that all monocyte subsets are capable of producing IP-10 to cytosolic GBS-DNA, although intermediate monocytes produce the highest amount of IP-10.

This study also found that live GBS induced a visibly higher IP-10 response compared to cytosolic GBS-DNA, with each monocyte subset producing higher levels of IP-10 to live GBS compared to cytosolic GBS-DNA. Additionally, more monocytes were capable of responding to live GBS instead of cytosolic GBS-DNA in terms of IP-10 responses. These results suggest that monocytes (all subsets) cannot respond as efficiently to cytosolic GBS-DNA as they do live GBS. A reason for this maybe because, even though GBS-DNA was successfully transfected into the cytosol of cells, it was at a much lower concentration compared to live GBS (cocci and chains). It is also possible that apart from cytosolic GBS-DNA, other mechanisms such as the uptake and degradation of live GBS are important for the regulation of IP-10 responses. A murine study has shown that phagocytosis and subsequent degradation of live GBS in the phagolysosomes were critical for IFN-β responses to live GBS in mouse macrophages (Charrel-Dennis et al., 2008). Therefore, a similar requirement may exist in human monocytes in terms of IP-10 responses to GBS. An investigation of whether uptake and degradation of live GBS is essential for IFN-β responses in human monocytes is warranted.
4.3.3 Extremely preterm infant monocytes have lower IP-10 responses compared to adult monocytes

Previous research from our lab has shown that monocytes of very preterm infants (<30 weeks GA) are significantly impaired in their capacity to produce pro-inflammatory cytokines such as TNF and IL-6 to live GBS (Currie et al., 2011). Consistent with this finding (Currie et al., 2011), this study found that monocytes from one extremely preterm infant (28 weeks GA) produced visibly lower amounts of IP-10 compared to adults to live GBS. This may have likewise also been due to the lower proportion of monocytes producing IP-10 to live GBS. In contrast, IP-10 responses to cytosolic GBS-DNA did not appear to differ between the very preterm infant and adults. Of note, adults produced slightly higher amounts of IP-10 to the highest dose of GBS-DNA used. However, a statistical difference could not be tested as only a single preterm cord blood sample was collected. There are several possible explanations for the visibly lower IP-10 responses of neonatal monocytes to live GBS compared to adults. First, very preterm infant monocytes have impaired capacity to produce IP-10 in response to live GBS. Second, uptake and degradation mechanisms found to be important for IFN-β responses to live GBS (Charrel-Dennis et al., 2008) maybe impaired in neonatal monocytes. Since phagocytosis of live GBS was shown to be critical for IFN-β production, impaired phagocytic functions of neonatal monocytes may have contributed to the lower IP-10 responses observed. However, a study by Currie et al. has shown that lower cytokine responses were not related to impaired phagocytic functions of preterm infant monocytes (Currie et al., 2011). It is important to note that heat-killed GBS and not live GBS were used to determine phagocytic capacity of preterm infants in that study (Currie et al., 2011). Overall these
results suggest that whilst very preterm infant monocytes appear to be deficient in their capacity to produce IP-10, a larger sample size would be needed to confirm the findings. As uptake and degradation mechanisms appear to be fundamental for IFN-β responses to live GBS (Charrel-Dennis et al., 2008), it is imperative that additional research (see Chapter 5) is done to elucidate the role of these mechanisms and whether they are fully functional in preterm and term infants.

4.3.4 Conclusion

Human monocytes are capable of responding to cytosolic GBS-DNA in terms of IP-10 production, although to a lower extent compared to live GBS. All monocyte subsets are capable of producing IP-10 to cytosolic GBS-DNA, although intermediate monocytes produce the highest amount of IP-10. Monocytes of a very preterm infant appeared to have impaired IP-10 responses to live GBS compared to adult monocytes, although a larger sample size would be required to confirm this. Further studies investigating the role of uptake and degradation for IFN-β responses to live GBS is warranted.
Chapter 5 Conclusions and Future Studies

5.1 Conclusions

GBS is a leading cause of EOS in newborn infants, particularly those born very premature. The deficient monocyte-driven pro-inflammatory cytokine responses of extremely preterm infants to various bacterial stimuli, including GBS, may contribute to their increased susceptibility to EOS. The type I IFN, IFN-β, has been shown to be critical for host defense against GBS infections in mouse macrophages and in vivo using a GBS sepsis model (Mancuso et al., 2007). The production of IFN-β in mouse macrophages was subsequently found to be induced by cytosolic GBS-DNA (Charrel-Dennis et al., 2008). It remains unknown whether human monocytes, from adults or infants, are also capable of inducing IFN-β in response to GBS or cytosolic GBS-DNA.

This study found that all human adult blood monocyte subsets were capable of responding to both live GBS and cytosolic GBS-DNA in terms of IP-10 production. Contrary to our first hypothesis, that the inflammatory monocyte would be the main subset involved in IFN-β production, the intermediate monocyte subset produced the highest amount of IP-10 to both live GBS and cytosolic GBS-DNA. However, the IP-10 responses induced by cytosolic GBS-DNA were much smaller in comparison to those by live GBS. This was possibly due to inefficient delivery of GBS-DNA into the cytosol of host monocytes by LyoVec.

In contrast to our second hypothesis, that preterm infant monocytes would not respond to cytosolic GBS-DNA, the monocytes of an extremely preterm infant
were capable of producing a similar IP-10 response to cytosolic GBS-DNA as adult monocytes. Although this suggests preterm infant monocytes can respond to cytosolic GBS-DNA in terms of IP-10 production, it may also be because transfection conditions were not optimal for induction of IP-10 in adult monocytes. In contrast to the cytosolic DNA result, preterm infant monocytes produced visibly lower levels of IP-10 compared to adults in response to live GBS. It is possible uptake and degradation of GBS was not as efficient in the preterm infant monocytes compared to adults. However, a larger sample size (n>8) would be required to determine whether these results are truly representative of the preterm infant population.

Taken together, these results show that both live GBS and cytosolic GBS-DNA are capable of inducing IP-10 responses in adult monocyte subsets, and that the intermediate monocyte subset is responsible for most of the IP-10 produced. The lower IP-10 responses to cytosolic GBS-DNA but not live GBS suggests that either uptake and degradation of live GBS results in more effective release of cytosolic DNA and induction of IP-10 or that GBS-DNA was not efficiently transfected into the cytosol of host monocytes using the experimental LyoVec system, and was thus not effective at inducing IP-10. In addition, the data also shows that monocytes of an extremely preterm infant are impaired in the ability to produce IP-10 in response to live GBS but possibly not cytosolic GBS-DNA. However, these results may differ if transfection of GBS-DNA in host monocytes were more effective.

Finally, there were a number of limitations to this study. Firstly, the high background fluorescence of anti-IFN-β stained monocytes made it difficult to
detect a positive signal for IFN-β production, even with CLO97 stimulation. For this reason, IP-10 had to be used as a surrogate to indirectly measure intracellular IFN-β production. Secondly, the purity of GBS-DNA extracts and the effectiveness of DNase 1 and RNase A treatment of GBS-DNA extracts was not confirmed. Agarose gel electrophoresis could allow for the presence of RNA-contaminated or fragmented GBS-DNA to be identified. Thirdly, the concentration of chloroquine used in the inhibitor studies was not optimized specifically for whole blood and may not have been effective in blocking TLR9. This means that TLR9 may be involved in the recognition of cytosolic GBS-DNA in terms of IP-10 production. Of note, the concentration used in this study was based on previously published values using mouse macrophages (Charrel-Dennis et al., 2008). Fourthly, only one preterm blood sample was available to compare IP-10 responses of preterm infant monocytes to adult monocytes. The results obtained may thus not have been a true representation of preterm infant monocyte IFN-β / IP-10 responses. Further studies would therefore have to be conducted using a larger sample size (n>8) in order to determine whether preterm infant monocytes have impaired monocyte IFN-β and IP-10 responses to live GBS, and if so whether these are impaired to cytosolic GBS-DNA as well.

5.2 Future Studies

In addition to optimised GBS challenge culture conditions and a larger sample size for future studies, further research can be undertaken in the following areas:
5.2.1 Investigating the role of the AIM2 inflammasome during GBS infection

The lower IP-10 responses observed in response to higher concentrations of both cytosolic GBS-DNA and poly(dA:dT) found in our study (see section 4.3.1) may have been associated with monocyte pyroptosis. Studies using mouse macrophages have found that AIM2 responds to cytosolic DNA from extracellular \textit{S. pneumoniae} and that the formation of an AIM2 inflammasome in response to poly(dA:dT) could induce pyroptosis (Koppe et al., 2012; Rathinam et al., 2010). It is therefore possible that AIM2 may also be capable of sensing cytosolic GBS-DNA and activating an inflammasome to induce pyroptosis of monocytes. A better understanding of the role of AIM2 during GBS infections would thus help determine whether these mechanisms are important for recognition and initiation of host defense against GBS.

5.2.2 Role of uptake and degradation mechanisms in terms of IFN-\(\beta\) production

Although we initially set out to assess if GBS-DNA would leak out equally into the cytosol of preterm infants, term infants and adults, this could not be accomplished due to time constraints. However, it is clear that understanding bacterial uptake and degradation mechanisms involved in the induction of IFN-\(\beta\) to GBS is critical. This is especially relevant as previous murine studies have found IFN-\(\beta\) production in mouse macrophages and dendritic cells required phagocytosis and degradation of GBS in the phagolysosomes (Charrel-Dennis et al., 2008; Mancuso et al., 2009). Therefore, it is possible that a similar requirement exists in terms of IFN-\(\beta\) production by human monocytes to GBS. To investigate this, the Click-it EdU kit (Invitrogen, USA) can be used, where a
specially-modified nucleotide can be incorporated into the DNA of live GBS to be fluorescently labeled. The fate of the GBS-DNA can then be followed using fluorescent confocal microscopy once it has been phagocytosed by monocytes. The amount of incorporated cytosolic GBS-DNA could then be measured using a densitometry. In contrast to GBS-DNA/LyoVec experiments, this method would allow a more accurate measure of the amount of GBS-DNA released from GBS during infections.

Separately, the role of phagocytosis, phagolysosomes and phagolysosomal enzyme degradation can also be investigated by respectively using cytochalasin D, bafilomycin A1 and chymostatin. These reagents respectively inhibit phagocytosis (via inhibition of actin polymerization), the formation of phagolysosomes (via inhibition of endosomal acidification) and the activity of phagolysosomal enzymes (e.g. serine and cysteine proteinases).

5.2.3 Identifying the expression of cytoplasmic DNA sensors within human monocytes responsible for IFN-β induction

The production of IP-10 by human monocytes to cytosolic GBS-DNA and poly(dA:dT) (section 4.3.2) suggests that cytosolic DNA sensors likely contributed to the induction of IP-10. Several cytosolic DNA sensors that regulate type I IFN production have been identified and include DAI and LRRFIP1 (see section 1.8.2) (Keating et al., 2011), however it remains unknown whether these DNA sensors exists in the cytosol of human monocytes. Identification of these cytosolic DNA sensors in humans will increase our understanding of how the innate immune system is involved in detecting and
initiating type I IFN responses to invading pathogens, including extracellular bacteria.

Collectively, these studies will help elucidate the mechanisms involved in IFN-β production during GBS infections in humans. Determining whether these mechanisms are functional in the human neonate would contribute to a better understanding of neonatal innate immunity to GBS infections.
Chapter 6  References


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