Innate immune responses of preterm infants to coagulase-negative staphylococci

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Preface

The regulations of the University of Western Australia provide the option for candidates for the Degree of Doctor of Philosophy to present their thesis as a series of papers, including manuscripts that have been published in refereed journals, manuscripts that have been submitted for publication, but not yet accepted or manuscripts that could be submitted.

All manuscripts presented in this thesis are related to the innate immunity of preterm infants and their unique susceptibility to infection with coagulase-negative staphylococci. The papers have been presented in a logical format so to address the issues raised in the introductory chapters. Each paper is presented in its original form, but for the ease of reading, the formatting is uniform throughout the thesis. The general discussion integrates the findings of the studies as a whole and discusses how the results may influence further research and clinical practice relating to bacterial infection in preterm infants.

The candidate designed all of the presented studies in close consultation with the supervisors. The experimental work was performed in the laboratories of the School of Paediatrics and Child Health and the Division of Infectious Diseases, Children's Hospital Boston, Massachusetts, United States. The contribution to the individual manuscripts is presented in the following section.

Publications arising from this thesis

Chapter 2

Strunk T, Currie A, Simmer K, Richmond P, Levy O, Burgner D. Innate immunity in human newborn infants: prematurity means more than immaturity. (J Matern Fetal Neonatal Med 2011; 24(1):25-31)

Author contributions: TS performed the literature review and drafted the paper. AJC provided numerous discussions and suggestions towards further relevant literature and writing of the publication. KS, PR and OL provided intellectual input for the writing of the manuscript. DB supervised all aspects of work related to the paper.

Chapter 3

Strunk T, Richmond P, Currie A, Simmer K, Levy O, Burgner D. Neonatal immune responses to coagulase-negative staphylococci. (Curr Opin Infec Dis 2007; 20:370-5)

Author contributions: TS performed the literature review and drafted the paper. AJC, KS, PR and OL provided intellectual input for the writing of the manuscript. DB supervised all aspects of work related to the paper.

Chapter 4

Strunk T, Doherty D, Jacques A, Richmond P, Simmer K, Kohan R, Charles A and Burgner D. Chorioamnionitis is associated with reduced risk of late-onset septicemia in extremely preterm infants. (Manuscript under review at *Pediatrics*)

Author contributions: TS performed the review of placental histology, data collection and interpretation and drafted the paper. DB, PR, KS and AC helped TS design the study, and contributed to data analysis and interpretation. RK coordinated the retrieval of data from the neonatal database. AC conducted the histological analysis of placental specimens. DD and AJ performed the statistical analysis. All co-authors contributed to the writing of the paper. DB supervised all aspects of the study and its publication.

Chapter 5

Strunk T, Doherty D, Richmond P, Simmer K, Charles A, Levy O, Liyanage K, Smith T, Currie A, Burgner D. Reduced levels of antimicrobial proteins and peptides in human cord blood plasma. (Arch Dis Child, Fetal & Neonatal edition 2009; 94:230-31)

Author contributions: TS, AJC, DB and PR designed the study. TS performed most of the laboratory tests, did the statistical analysis and drafted the paper. DD provided assistance with the statistical analysis. KL and TS contributed to the laboratory work. AJC, PR, KS, OL and AC contributed data interpretation and to the writing of the paper. DB supervised and all aspects of the study and its publication.

Chapter 6

Strunk T, Prosser A, Riley K, Levy O, Philbin V, Simmer K, Doherty D, Charles A, Richmond P, Burgner D and Currie A. Human monocyte responsiveness to the commensal bacterium *Staphylococcus epidermidis* develops late in gestation. (Manuscript submitted to *Pediatric Research*)

Author contributions: TS designed the study, performed most of the laboratory work, did the statistical analysis and drafted the paper. VP provided mentorship with experimental techniques. AP contributed to the laboratory research. DB, AJC, PR and OL helped design the experiments and contributed to data analysis, interpretation and the writing of the paper. AC conducted the histologic analysis of placental specimens. KS contributed to the writing of the paper. DD helped with the statistical analysis. DB supervised all aspects of the study and its publication.

Chapter 7

Strunk T, Power MR, Burgner D, Currie A, Philbin V, Golenbock D, Otto M, Simmer K, Richmond P, Levy O. Staphylococcus epidermidis activates innate immunity via TLR2-independent and –dependent pathways. (PLoS One 2010; 5(4):e10111)

Author contributions: TS and MRP equally contributed to the laboratory work and the writing of the manuscript. DB, AJC, PR and OL helped design the experiments and with analysis and data interpretation. VP provided mentorship with experimental techniques. DG provided the cell lines and intellectual input. MO provided the strain of *S. epidermidis* and intellectual input. KS was involved

in the writing of the paper. OL supervised all aspects of the study and its publication.

Chapter 8

Strunk T, Richmond P, Prosser A, Burgner D, Currie A. Method of bacterial killing differentially affects the complexity of the human innate immune response to *Staphylococcus epidermidis* (Innate Immunity. 2010 Aug 31. [Epub ahead of print])

Author contributions: TS, AJC, DB and PR designed the study. TS performed most of the laboratory tests, did the statistical analysis and drafted the paper. AP contributed to the laboratory research. AJC assisted with data analysis, experimental design and writing of the manuscript. DB supervised all aspects of the study and its publication.

Summary

The incidence of premature birth continues to rise in developed countries worldwide. Advances in neonatal care have resulted in the improved survival of extremely premature infants and consequently to a growing population of infants with prolonged and profound susceptibility to invasive bacterial infection. Late-onset sepsis (LOS) with coagulase-negative staphylococci, of which *Staphylococcus epidermidis* (SE) is the most commonly isolated organism, is associated with significant neonatal morbidity, both directly and indirectly, largely due to adverse effects of inflammation. Therefore prevention and management LOS represents a key challenge to improving outcomes in preterm infants.

Despite the large numbers of infants who suffer LOS and the current lack of early diagnosis or effective preventative interventions, our understanding of the interaction of the neonatal host innate immune system with SE is very limited.

Exposure to chorioamnionitis constitutes another major risk factor for adverse long-term outcomes in this preterm population, but its potential effects on risks of LOS have not been studied. We hypothesised that exposure to chorioamnionitis influences the risk of LOS due to possible effects on postnatal innate immunity. The findings of our large retrospective cohort study clearly demonstrate that the presence of chorioamnionitis, while increasing the risk of early-onset sepsis, is independently associated with a significantly reduced incidence of LOS in high-risk preterm infants. Exposure to perinatal inflammation may accelerate immune maturation via transfer of bacterial ligands and/or maternal immune modulators across the placenta. These data may guide future immunomodulatory interventions to reduce the burden of LOS.

To characterise the innate immune response of extremely preterm infants, we investigated an emerging family of soluble innate immune molecules, antimicrobial proteins and peptides (APP), which, in addition to their direct antimicrobial activity also have important immunomodulatory properties. These important molecules have received little attention in preterm infants. We determined the cord blood levels of APPs and consistently found the lowest levels in the most premature infants, which may contribute to their decreased innate immune defences.

We then further characterised the innate immune responses of preterm infants to SE. Our results demonstrate that despite adequate capacity of several essential innate immune functions, including phagocytosis, bacterial killing, *Toll*-like receptor expression and activation of receptor-associated kinases, the expression of cytokines is significantly impaired in a gestational age-dependent manner. This indicates that the underlying deficiency lies in further downstream signalling pathways. Considering the incompletely defined interaction of SE with the innate immune system, we then investigated both the human (*in vitro*) and murine (*in vivo*) innate immune system and show that while in the murine model TLR2 is indispensible for innate immune recognition and clearance of SE, the human innate immune system is much more complex; in human SE interacts with TLR2-dependent and TLR2-independent pathways.

Finally, we describe that the complexity of the interaction of SE with the innate immune system is significantly affected by the method of bacterial inactivation. Both heat- and ethanol-inactivated SE engaged a limited number of innate immune pathways compared to live SE. This indicates that interpretation of data generated using purified bacterial ligands or inactivated bacteria should be interpreted with caution and future experiments need model the *in vivo* situation

by inclusion of live bacteria and whole blood. These findings therefore have ramifications for the field more generally, as *in vitro* stimulation of immune cells with inactivated bacteria is a widely used model to investigate human immune responses.

In conclusion, the data presented in this thesis significantly advance our understanding of innate immune function in preterm infants, particularly with respect to the most common LOS pathogen, SE. The data also provide a platform for future studies, including: (i) exploration of the consequences of exposure to chorioamnionitis on immune maturation and long-term risk of infection in affected infants, (ii) characterisation of innate immune maturation in extremely preterm infants, and (iii) further delineation of the immune mechanisms underlying the preterm infant's susceptibility to LOS with SE. Detailed characterisation of innate immune responsiveness in this population may allow for further risk stratification, targeted monitoring, prophylactic intervention and ultimately reduced incidence of LOS and improved outcomes of these uniquely susceptible infants.

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Abbreviations

APP, antimicrobial proteins and peptides

Bb, *Borrelia burgdorferi*

BPI, bactericidal/permeability-inducing protein

BSA, bovine serum albumin

BW, birth weight

CD, cluster of differentiation

CFU, colony-forming units

CI, confidence interval

CLD, chronic lung disease

CoNS, coagulase-negative staphylococci

CPAP, continuous positive airway pressure

CVL, central venous line

DC, dendritic cell

DMEM, Dulbecco's modified Eagle's medium

DMSO, dimethyl-sulphoxide

DNA, deoxyribonucleic acid

EKSE, ethanol-killed *Staphylococcus epidermidis*

ELBW, extremely low birth weight infant

ELISA, enzyme-linked immunosorbent assay

EOS, early-onset sepsis

ETN, erythema toxicum neonatorum

FACS, fluorescence-activated cell sorter

FCS, fetal calf serum

FSL-1, fibroblast-stimulating lipopeptide-1

GA, gestational age

GBA, group A streptococci

GBS, group B streptococci

HBD-2, human β-defensin-2

HCA, histologic chorioamnionitis

HEK, human embryonic kidney cells

HKSE, heat-killed *Staphylococcus epidermidis*

HNP1-3, human neutrophil defensins 1-3

HR, hazard ratio

IFN-γ, Interferon-gamma

IgG, immunoglobulin G

IL, Interleukin

IP-10, interferon-γ-inducible protein-10

IQR, inter-quartile range

LOS, late-onset sepsis

LPS, lipopolysaccharide

LSE, live *Staphylococcus epidermidis*

MBL, Mannose-binding lectin

MNC, mononuclear cells

NEC, necrotizing enterocolitis

NICU, neonatal intensive care unit

NLR, NOD-like receptor

NOD, nucleotide oligomerization domain protein

OD, optical density

OR, odds ratio

PAMP, pathogen associated molecular pattern

PBMC, peripheral mononuclear cells

PBS, phosphate-buffered saline

PCR, polymerase chain reaction

PG, peptidoglycan

PIA, polysaccharide intercellular adhesin

PPROM, premature preterm rupture of the membranes

PVL, periventricular leukomalacia

R, range

RbC, rabbit complement

RNA, ribonucleic acid

RPMI, Roswell Park Memorial Institute

RT-PCR, reverse transcription polymerase chain reaction

SA, *Staphylococcus aureus*

SE-S, soluble factor secreted by *Staphylococcus epidermidis*

SE, *Staphylococcus epidermidis*

sPLA2, secretory phospholipase A2

sTLR2, soluble TLR2

Th, T-helper

TLR, Toll-like receptor

TNF, tumor necrosis factor

TPN, total parenteral nutrition

TRF, time-resolved fluorometry assay

TSB, trypticase soy broth

VLBW, very low birth weight infant

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Chapter 1

General introduction

1.1 Premature birth

1.1.1 Epidemiology of premature birth

The extraordinary advances of perinatal care over the past two decades have resulted in significantly increased survival of premature neonates and the care of these infants remains one of the key challenges of neonatal medicine. The rate of premature birth has increased by 30% over the last two decades in developed countries worldwide, and recent reports suggest that currently 5-9% of all deliveries in Europe and Australia and almost 13% in the United States occur prematurely, equivalent to almost 22,000 premature babies each year in Australia alone [1, 2].

Premature delivery is defined as those that occur before 37 completed weeks of gestation. Premature birth is often a consequence of spontaneous onset of preterm labour, either with intact fetal membranes (40-45%) or following premature preterm rupture of the membranes (25-30%; PPROM: defined as rupture of membranes before 37 weeks and more than 1 hour before onset of labour). One third of preterm deliveries occur by induction of labour or caesarean section for maternal or fetal indication [2, 3]. The majority of preterm infants are born near term at 34-36 weeks gestational age (GA; 60-70%), 20% of preterm infants are delivered at 31-33 weeks GA (moderate prematurity), 15% at 28-30 weeks GA (severe prematurity) and only 5% of preterm births occur at less than 28 weeks GA (extreme prematurity) [2].

Several factors contribute to the overall increase in preterm births: i) rising numbers of medically indicated singleton preterm deliveries, ii) preterm delivery of multiple gestation pregnancies following assisted reproduction, iii) increased risk of premature delivery of singleton pregnancies after *in-vitro* fertilisation [4, 5]. However, the increased numbers of pregnancies following assisted reproduction only provide a partial explanation for the overall rise in preterm deliveries and in about half of the cases the underlying cause remains unknown [6, 7].

1.1.2 Significance of premature birth and health care costs

The rising incidence of premature birth and the significantly improved survival of the most premature infants is of great public health concern; premature infants combined account for approximately 75% of all perinatal morbidity and mortality as well as 50% of the long-term morbidity, causing substantial human and health care costs [8]. For example, the total expenditure required for the care of premature infants in the United States alone is estimated at US$ 26.2 billion per year [9]. The incidence and severity of complications of prematurity is inversely correlated with GA and birth weight and a substantial proportion of the total cost associated with premature birth is caused by the minority (approximately 5%) of infants born extremely prematurely at less than 28 weeks gestation [10]. Despite the improved survival, the overall morbidity in this uniquely vulnerable population remains high, and the most common complications of extreme prematurity include respiratory distress syndrome (93%), patent ductus arteriosus (46%), severe intraventricular haemorrhage (16%), necrotizing enterocolitis (13%) and late-onset sepsis (35%) [10].

Importantly, and contrary to common perception, significant adverse outcomes of prematurity are not confined to those born at the limits of viability. The majority of preterm infants are born at 34-36 weeks GA (60-70% of all preterm infants) and they are physiologically and metabolically immature. As a consequence, late preterm infants have neonatal mortality and morbidity rates that are several times higher than those of term infants, contributing substantially to increased health care cost in this population [11, 12]. Complications of prematurity in late preterm infants include respiratory distress, hyperbilirubinemia, hypoglycemia, poor feeding, periventricular leukomalacia, and increased rates of rehospitalisation. Importantly the risk of early and late neonatal deaths due to invasive infections is significantly increased in these infants [13, 14, 15]. Furthermore, the heightened susceptibility to infection is not limited to the neonatal period, but persists into childhood and is associated with increased incidence of admission to hospitals and childhood mortality [15].

Some of the complications of prematurity, such as neurodevelopmental, behavioural and functional impairment, chronic respiratory conditions and growth restriction persist long-term and others, including increased risk of cardiovascular and metabolic disease, frequently become apparent only later in childhood or even adulthood. Therefore long-term surveillance is essential [16, 17]. However, with improvements in obstetric and neonatal care over the past two decades, not only the survival rates but also the outcomes of extremely premature infants have improved and most ex-preterm graduates of neonatal intensive care units (NICU) lead independent, productive and healthy lives as adults [18].

Premature birth still is incompletely understood and therefore, attempts to reverse the trend of increasing premature deliveries have largely failed [7]. In

order to reduce the incidence of premature deliveries and the associated adverse outcomes of extremely premature infants, a more comprehensive understanding of the relevant biological pathways is essential. The following sections will summarise the current understanding of the causes and consequences of prematurity and highlight how inflammation is central to both premature birth and to the resultant complications of prematurity itself.

1.1.3 Risk factors for premature birth

Spontaneous parturition at term is increasingly being appreciated as a physiological localised inflammatory process of the uterus [19, 20, 21, 22]. This inflammation is neither highly specific nor tightly regulated, but is a final common pathway that can be initiated prematurely by a variety of stimuli, the net result being premature labour and delivery [23]. Risk factors for the initiation of preterm labour include previous preterm delivery, multiple gestation pregnancy, infection of the mother or the fetus, maternal inflammatory conditions, placental ischemia or haemorrhage, low socioeconomic status, maternal African-American or Aboriginal ethnicity, maternal smoking and exposure to physical or psychological stressors [24, 25, 26, 27, 28]. Complex interactions between these contributing biological, psychological and socioeconomic factors often prevent a firm diagnosis being made as to the aetiology underlying the onset of preterm labour.

1.1.4 Infection and premature birth

Histopathological analyses of preterm placentae suggest asymptomatic bacterial infection of the membranes as one of the most common precursors of preterm labour [29]. Furthermore, PPROM is also strongly associated with the risk of ascending infection of the maternal and/or fetal membranes and the fetus and hence, differentiation of the initial trigger can be difficult [30]. Since the rate of infection of the placental membranes is twice that of the amniotic cavity, cultures of the amniotic fluid alone significantly underestimate the incidence of infection. In addition, routine bacteriological cultures of the amniotic fluid and/or placental membranes have low sensitivity for the detection of colonization or infection with anaerobic and difficult-to-culture organisms. Recent studies employing culture-independent, molecular microbiological techniques demonstrated the presence of bacteria in up to 75% of placentae from preterm infants [31, 32, 33]. *Ureaplasma* and *mycoplasma* species are the most commonly identified organisms and both typically are associated with clinically silent infection of the amniotic fluid and/or extraplacental membranes. However, a variety of other organisms have also been identified in samples of amniotic fluid and placental membranes [34]. *Ureaplasma* species and most other identified organisms generally are of low virulence and cause low-grade, chronic infection that remains clinically imperceptible until the resulting inflammation elicits the onset of premature labour [35]. Among women who subsequently deliver prematurely, mid-trimester levels of the proinflammatory interleukin (IL)-6 are elevated, indicating that clinically silent infection and inflammation often precede premature delivery by several weeks [36]. Despite the initiation of an histologically evident inflammatory response, *Ureaplasma* frequently are not detected by culture, but only by polymerase-chain reaction

(PCR) and the levels of bacterial deoxyribonucleic acid (DNA) detected in amniotic fluid correlate inversely with lower GA at birth [37]. The rate of intrauterine infection and histologic chorioamnionitis (HCA) increases with lower gestational age: at 21-24 weeks GA HCA is present in more than 60% of placentae compared to 10% at 35-36 weeks GA [38, 39, 40].

Infection of the maternal and/or fetal membranes (chorioamnionitis) activates the innate immune system and the subsequent release of pro-inflammatory cytokines, such as interleukin-(IL)1, IL8 and tumour necrosis factor (TNF), inducing the production of numerous downstream mediators. These mediators include matrix-degrading enzymes that result in PPROM and prostaglandins, which are potent stimulators of uterine contractility [24, 35]. As a result, the majority of women with PPROM experience spontaneous preterm labour within days of the rupture of their membranes [30]. Importantly, microbial invasion of the amniotic cavity and/or the extraplacental membranes and the resulting inflammation also initiate a fetal inflammatory response which is associated with significantly increased risk of both acute and long-term adverse outcomes of prematurity [41, 42, 43, 44]. Despite the clear association between intrauterine infection and preterm labour, the presence alone of microorganisms in the amniotic and/or extraplacental membranes is necessary but not sufficient to initiate preterm labour as demonstrated by the detection of bacteria with molecular methods in the membranes of up to 70% of women delivering by elective caesarean section at term [45].

1.1.5 Genetic factors

Several studies suggest that variations in the maternal immune responses to bacterial colonisation with exaggerated inflammatory response are central to the pathophysiology of preterm labour and delivery [23, 33]. Indeed, premature birth has a significant maternal genetic component; women whose grandmothers were born prematurely or whose sisters gave birth to premature infants are at significantly increased risk of giving birth prematurely to their own offspring [23, 46, 47]. However, the molecular basis of these observations has remained largely elusive and specific genetic determinants have not been identified. Gene-environment interactions that are associated with heightened risk of preterm delivery involve bacterial vaginosis and single nucleotide polymorphisms of the pro-inflammatory cytokines TNF, IL1β and IL6 [48, 49, 50, 51]. Furthermore, homozygosity for gene polymorphisms of principal receptors of the innate immune system, such as Toll-like receptor (TLR)2, the main receptor for recognition of Gram-positive bacteria, and mannose-binding lectin (MBL), which binds to and opsonises various microorganisms, have been associated with increased risk of preterm birth [52, 53]. In addition, a gene polymorphism of TLR4, the principal innate immune receptor for lipopolysaccharide from the cell membrane of Gram-negative bacteria, has been associated with the risk for preterm birth in some populations, but not in others [54, 55, 56]. Genetic variants of these and further molecules that are central to the initiation and regulation of the innate immune response are implicated in the variation of familial risk of premature delivery. These inherited variants not only determine the maternal immune response to vaginal and/or intrauterine infection, but crucially may also contribute to their preterm infant's susceptibility to invasive bacterial infections.

Although considerable progress has been made in our understanding of risk factors and mechanisms involved in premature delivery, this knowledge has so far not been translated into effective preventative interventions [57]. On the contrary, over the past 25-30 years, the incidence of premature birth has increased significantly and now up to 1 in every 6 neonates is delivered preterm, partly due to the increase in assisted reproduction [2, 58, 59, 60].

1.2 Infections in neonates

The neonatal period (from birth to day 28 of life) is the period with the highest incidence of childhood mortality. In fact, almost 40% of all childhood deaths occur during the neonatal period with the first postnatal week conferring the highest risk (75% of all neonatal deaths) [61]. Severe neonatal infections, most commonly sepsis, pneumonia, tetanus, omphalitis and diarrhoea, remain a leading cause of neonatal morbidity and mortality globally (36%) and recent data suggest that worldwide approximately 1.5 million newborn babies die of infection each year [62, 63]. In developed countries with low neonatal mortality, sepsis and pneumonia are the predominant invasive infections, responsible for approximately 20% of all neonatal deaths, whereas neonatal tetanus and severe diarrhoea are virtually non-existent [62].

1.2.1 Clinical signs of infection in neonates

Signs and symptoms of infection in neonates are non-specific and include respiratory distress, apnoea, lethargy or irritability, temperature instability, poor feeding and/or feeding intolerance, and jaundice [64, 65, 66]. These symptoms overlap with those caused by other common neonatal conditions, obscuring the

accurate diagnosis. Furthermore, the clinical presentation does not allow for differentiation between sepsis, pneumonia and/or meningitis, which complicates neonatal sepsis in up to 20% of EOS cases and up to 10% of LOS episodes, with variations in frequency dependent on the causative organism and the GA at birth and the postnatal age of the affected infant [66].

1.2.2 Early-onset sepsis

With regards to the postnatal age at presentation, neonatal infections are differentiated into early-onset sepsis (EOS, onset at less than 72 hours of age) and late-onset sepsis (LOS, onset at greater than 72 hours of age) [66, 67]. These definitions are clinically relevant as they signify distinct routes of infection, involve different causative organisms, likely have different host determinants of susceptibility, and can therefore provide guidance as to the initial empirical antimicrobial therapy [67].

Early-onset sepsis is the result of colonisation and invasive infection of the newborn before or during delivery with bacteria that reside in the birth canal or that have ascended into the amniotic cavity, and this frequently, but not exclusively, occurs following PPROM and/or chorioamnionitis [66]. The overall incidence of EOS has decreased over the past two decades, and currently affects an estimated 1% of all neonates [68]. Much of the decline in the incidence of EOS is due to the advent of universal screening of women in late pregnancy since the late 1990s and the use of peripartum antibiotic prophylaxis for those women vaginally colonised with Group B streptococci (GBS), Gram-positive bacteria, that previously was the leading cause of EOS among neonates of all gestational ages [69]. Gram-negative bacteria commonly

isolated in EOS include *Escherichia coli* and recent reports demonstrate an increased incidence of EOS with this organism in the era of widespread antibiotic GBS prophylaxis, particularly in preterm infants, where Gram-negative organisms now cause the majority of EOS, and *E. coli* alone is responsible for about 40% of all EOS episodes [70, 71, 72, 73]. Importantly, the institution of peripartum antibiotic prophylaxis has not reduced the incidence of EOS among preterm infants, and in this population it remains approximately twice that of term neonates [73, 74, 75, 76].

The outcomes of extremely preterm infants with EOS are universally worse compared to term infants, including significantly increased odds for intraventricular haemorrhage, bronchopulmonary dysplasia, severe retinopathy of prematurity, and a threefold excess risk of death or major neurologic morbidities [66, 73, 74]. In conclusion, EOS remains an important cause of serious illness in this population, particularly when caused by Gram-negative organisms, which are more frequently associated with septic shock, meningitis and death than EOS caused by other organisms [75].

1.2.3 Late-onset sepsis

Several studies have documented a significant increase in the incidence of LOS that parallels the overall decrease of EOS rates and this is largely related to the improved survival of preterm infants [68]. In contrast to the perinatal colonisation and infection in EOS, late-onset sepsis typically is a nosocomial infection with organisms that colonise the infant postnatally. Late-onset sepsis predominantly affects extremely preterm infants and in this population is several

times as common as EOS; LOS only occasionally occurs in late preterm infants, and is comparatively uncommon in full term neonates [67, 77, 78].

1.3 Infections in preterm infants

1.3.1 Epidemiology of late-onset sepsis

Invasive infections remain one of the essential challenges to further significant improvement of the long-term outcomes of extremely premature infants. With the rising survival of infants born extremely prematurely, the incidence of LOS has increased substantially and up to 50% of these infants will acquire at least one episode of LOS during their stay in the NICU. The incidence of LOS typically peaks in the second to third week of life, and the majority of infections occur during the first 6 weeks, but have been observed as late as several months of age in extremely preterm infants with prolonged hospitalization [79, 80]. The majority of infants acquire a single episode of LOS, but a recent study revealed that a significant proportion of infants have recurrent LOS: about 20% of infants had 2 episodes, 6% had 3 episodes and 2% had 4 or more episodes of LOS [71]. This underlines that the risk for invasive infection is persistent and even in moderately preterm infants this remains significantly elevated well into early childhood [15].

1.3.2 Risk factors for late-onset sepsis

The risk of acquiring one or more episodes of LOS is inversely related to GA (Figure 1.1) and to birth weight (BW), whereas maternal risk factors, in contrast to EOS, do not play a significant role [79]. Extremely preterm infants require

complex and invasive medical care for a prolonged period of time, frequently necessitating the use of central or peripheral venous catheters for the administration of parenteral nutrition and medications, endotracheal or nasal tubes for respiratory support, and gastric tubes for enteral nutrition. The significant risk of LOS associated with these plastic medical devices is well documented [81, 82]. Furthermore, parenteral nutrition independently adds to the risk of LOS, and the intravenous administration of lipid emulsion in particular is linked to impairment of immune functions [82, 83, 84]. Treatment with histamine type 2 receptor blockers and proton-pump inhibitors, through suppression of gastric acid production, contributes further to LOS risk [85, 86]. The intrinsic immunological risk factors predisposing preterm infants to LOS and their immune responses specific to CoNS will be discussed in detail in chapters 2 and 3, respectively.

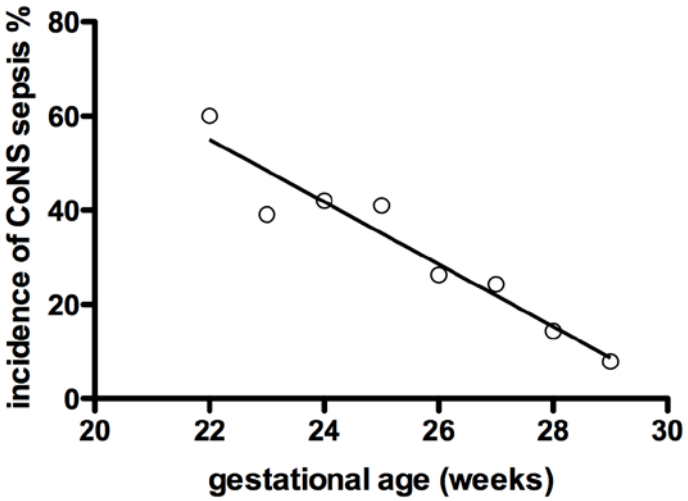

Figure 1.1 Incidence of late-onset sepsis with CoNS with gestational age.

Incidence of late-onset sepsis with coagulase-negative staphylococci (CoNS) according to GA from 2001-2007 at King Edward Memorial Hospital, Perth, Western Australia (n=838).

1.3.3 Diagnosis of late onset-sepsis

Clinical signs and symptoms of LOS in preterm infants are non-specific and resemble those of infection in more mature infants. Typical signs and symptoms include increased bradycardia and/or apnoea, increased oxygen requirement, increased requirements for ventilatory support, gastrointestinal impairment, temperature instability and lethargy [65, 87]. Since these clinical indicators are unreliable, laboratory tests are required to support the diagnosis of LOS. Common laboratory findings include an abnormal white cell count (46%), metabolic acidosis (11%) and hyperglycemia (10%), but the positive predictive value of these parameters is low [88].

A generally accepted definition of LOS requires the combination of the presence of three or more clinical signs of sepsis and a blood culture positive with a single microorganism [80, 89]. Although blood cultures represent the gold standard for the diagnosis of blood stream infection, in neonates and particularly in extremely low birth weight infants (ELBW), they are difficult to obtain, obtainable blood volumes are small and a substantial number of blood cultures are negative or contaminated [89]. Some investigators suggest the addition of raised C-reactive protein (albeit with varying cut-off levels) and, in case of CoNS LOS, two positive blood cultures with this organism obtained from different sites, although this is not practical in ELBW infants and is therefore not universally accepted standard practice. Numerous inflammatory mediators, including but not limited to procalcitonin, IL6 and IL8, cluster of differentiation (CD)64, *Toll*-like receptors (TLR) and interferon-γ-inducible protein (IP)-10 have been suggested as adjunct tools for early diagnosis of LOS in neonates, but none has yet gained widespread acceptance in routine practice [90, 91, 92].

1.3.4 Microorganisms causing late-onset sepsis

Late-onset sepsis in preterm infants is predominantly caused by Gram-positive organisms (45-77% of cases) and the most commonly isolated group of Gram-positive bacteria are coagulase-negative staphylococci (CoNS; about 50% of all LOS) [65, 68, 80, 93]. Coagulase-negative staphylococci (mainly comprised of *Staphylococcus epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *S. lugdunensis*, *S. schleiferi*, etc.), normally innocuous commensals of the skin and mucous membranes, were initially not regarded as true pathogens but as contaminants of blood cultures [94]. However, over the last two decades, CoNS have increasingly gained recognition as the most common group of organisms causing nosocomial sepsis in particularly vulnerable populations, such as patients taking immunosuppressive medication, critically ill patients in intensive care units, and extremely preterm infants [95, 96].

In contrast, Gram-negative bacteria, including *E. coli*, *Klebsiella*, *Enterobacter* and *Pseudomonas* species, are found in less than 20% of blood cultures, but these organisms are associated with substantially higher morbidity and mortality than LOS with Gram-positive bacteria [71, 97]. Fungi and yeasts are isolated in a minority of LOS cases (approximately 3-12% of cases, depending on GA, BW and study centre), but they are difficult to treat and generally convey the poorest prognosis of all LOS organisms, both in terms of survival and long-term outcomes [98, 99, 100].

1.3.5 Late-onset sepsis and neonatal outcomes

Late-onset sepsis carries a significantly increased risk of both morbidity and mortality, significantly prolongs hospitalisation and therefore increases health care cost [65, 101, 102]. For preterm infants who survived respiratory distress syndrome and other causes of early mortality, LOS now has become a frequent cause of late mortality [65], and the mortality rates directly attributed to LOS (death within 72 hours of onset of LOS) vary from 1.8% for CoNS to 22.6% for *Pseudomonas* species [97]. While the direct mortality of CoNS LOS is lower than for LOS with Gram-negative organisms or Candida, CoNS infections are three to four times as common [65].

In addition to the mortality directly caused by LOS, common adverse long-term outcomes of prematurity, such as chronic lung disease, periventricular leukomalacia, neurodevelopmental impairment and cerebral palsy are believed to be worsened by the inflammation induced by postnatal infections [42, 99]. The link between sepsis, brain injury and adverse long-term outcomes was first proposed by Leviton *et al.* and some of the mechanisms involved have since been identified [103]. Infection and the ensuing inflammation initiate the release of numerous inflammatory mediators, such as cytokines, reactive oxygen species and free radicals, all of which have neurotoxic effects, causing direct injury to premyelinating oligodendrocytes, cells essential for normal brain development [104, 105]. As a result, preterm infants who acquire LOS are at significantly increased risk for abnormalities of brain white matter as detected on magnetic resonance imaging, and more importantly, on functional neurodevelopmental testing in early childhood [99, 106, 107]. Furthermore, LOS with any organism significantly prolongs hospitalization and increases the cost

of medical care, even without taking the long-term care costs for infants with neurodevelopmental delay into account [98].

1.3.6 Summary

The increasing incidence of premature births and the remarkable achievements in perinatal care has resulted in improved long-term survival of rising numbers of extremely preterm infants. Perinatal and postnatal infections are the most common cause for generalised inflammation which is at the core of many common adverse outcomes of extreme prematurity. Therefore, a more comprehensive understanding of the biological pathways underlying the preterm infants' unique susceptibility to invasive infection is urgently required to allow for the development of new diagnostic and preventive strategies.

The following chapters provide a detailed review of the development of the innate immune system of the preterm infant in general (**Chapter 2**) and with a particular focus on those innate immune responses related to LOS with coagulase-negative staphylococci (**Chapter 3**).

Chapter 2

Innate immunity in human newborn infants: prematurity means more than immaturity

2.1 Abstract

Neonates, particularly those born prematurely, are exquisitely vulnerable to life-threatening infections. This increased susceptibility to infection is maintained into childhood. Despite the considerable human and economic cost of infection-related neonatal morbidity and mortality, the mechanisms underlying this heightened susceptibility are only partly understood. It is increasingly recognised that innate immune responses are key to the protection against infection early in life and emerging data suggest that such responses are deficient in the newborn and especially in preterm infants. Here we review the current understanding of the maturation of the innate immune response in human neonates highlighting the clinical relevance and possible avenues for therapeutic intervention.

2.2 Introduction

Birth constitutes probably the most important change of environment of our lifetime - the transition from a ‘sterile’ intrauterine existence through the colonised birth canal into largely peaceful co-existence with a myriad of microbes, both commensals and potentially invasive pathogens. This new environment holds two seemingly contradictory challenges for the newborn; (a)

recognition and efficient elimination of potentially invasive pathogens and (b) controlled co-existence and tolerance of commensals on skin and mucosal surfaces.

Despite recent advances in neonatal intensive care, infections remain an important cause of morbidity and mortality in neonates. The incidence of invasive bacterial infection is largely dependent on gestational age (GA) and birth weight (BW) and affects a significant minority of newborn infants, with the extremely preterm infants (≤28 wk GA) being particularly susceptible to nosocomial infections [65, 79]. Although these infants constitute only ~1.0% of all births in developed countries, they contribute disproportionately to neonatal morbidity and mortality (~50%), with infection-related complications being the leading cause of adverse outcomes [1, 108]. The rising incidence of preterm birth, coupled with improved survival, puts increasing numbers of vulnerable infants at prolonged risk of infection. Late-onset septicaemia (that occurring after 72 hours of age) in this high-risk population significantly adds to the economic burden associated with premature birth [102].

The heightened risk of bacterial infection in preterm infants has a number of determinants: Firstly, extremely preterm infants require prolonged intensive care, including mechanical ventilation, parenteral feeding and intravenous access, which breach physical barriers against infection and facilitate invasion by nosocomial pathogens [79]. Secondly, neonates are rapidly colonised by microbes in the neonatal intensive care unit (NICU) environment [109]; the increased susceptibility of the preterm neonate renders usually innocuous microbes potentially pathogenic. Thirdly, many elements of the acquired immune system function less well in the neonate, with gestational and postnatal

age amongst the most important determinants of immune function [110]. Under these circumstances, neonates rely primarily on the innate immune system for protection against invasive infections [111]. Increasing evidence suggests that infection-driven inflammation also contributes to the pathogenesis of common adverse outcomes of prematurity, including chronic lung disease and neurodevelopmental impairment [106, 107, 112, 113, 114]. The development of innate immune function in neonates and the mechanisms underpinning its relative impairment in preterm infants are poorly understood.

Here we focus on the current data concerning the maturation of the innate immune system in newborn infants with particular reference to their increased risk of invasive bacterial infection. We highlight some recent advances in the understanding of the neonatal innate immune system and discuss therapeutic developments aimed at reducing the burden of infection in newborns.

2.3 Maturation of the innate immune system in neonates

The innate immune system is an evolutionarily ancient, non-antigen specific host defence system that provides immediate protection against invasive microorganisms, but, unlike adaptive immune responses, does not result in long-lasting immunity. It consists of two effector arms: (a) a multitude of soluble proteins and peptides (including antimicrobial proteins and peptides and complement) that bind to, opsonise or directly destroy microorganisms and (b) professional phagocytes such as neutrophil granulocytes and monocytes. These cells are capable of processing pathogens and presenting antigenic peptides to the adaptive immune system, acting as the critical link between non-specific innate defences and the antigen-specific adaptive immunity.

2.3.1 Soluble factors

While it is beyond the scope of this review to discuss the extensive literature on all soluble factors of the innate immune system, we will focus on those which are most clinically relevant to the newborn infant; immunoglobulins, complement, antimicrobial proteins and peptides (APPs) and mannose-binding lectin (MBL).

2.3.2 Immunoglobulins and complement

Placentally transmitted immunoglobulins play an important role in first line defence against invading pathogens. Transfer of significant amounts of immunoglobulin G (IgG) occurs largely after 32 weeks of gestation and as a result plasma levels of maternal immunoglobulins are low in preterm infants at birth [115]. Levels of IgG decline rapidly during the first week of life before the onset of autologous production of immunoglobulins over the following weeks [88, 115]. In addition to significant hypogammaglobulinemia, the levels and functional capacity of components of the complement system are commonly decreased in newborns, particularly in preterm infants [116, 117, 118]. The combined deficiency of immunoglobulins and complement factors result in reduced efficiency of opsonisation and contribute to infectious susceptibility [115]. However, the significance of immunoglobulins for host defence is context dependent and differs between common neonatal pathogens. For example, levels of placentally transferred antibodies are inversely correlated with the risk for neonatal group B streptococcal sepsis [119, 120], whereas no such association could be demonstrated for invasive infection with coagulase-negative staphylococci [117]. Indeed, trials of specific polyclonal anti-

staphylococcal antibodies to prevent neonatal LOS have been disappointing [121, 122].

2.3.3 Antimicrobial proteins and peptides (APP)

Human leukocytes secrete numerous oxygen-independent cationic poly/peptides in large quantities (micrograms) that bind to and destroy microorganisms in the extracellular space. Most of these soluble innate immune factors are produced by primary immune cells, such as neutrophils and monocytes, but platelets, hepatocytes and numerous epithelial/mucosal cells have recently also been shown to synthesise significant amounts of APPs, providing a first line of defence at skin and mucosal surfaces [123].

Antimicrobial proteins and peptides are detectable in early gestation and the levels generally show a positive correlation with GA. Recently, we have described GA-dependent maturation of the cord blood levels of bactericidal/permeability-increasing protein (BPI), α-defensins (also known as HNP1-3), LL37, phospholipase A2a and calprotectin [124]. Furthermore expression of human β-defensin-2 (HBD-2) in preterm lung increases with GA and HBD-2 levels are increased during lung infection [125, 126]. Significant levels of APPs have also been detected in secretions from fetal tissues such as vernix caseosa and meconium, where they not only provide a first line of defence against microbial invasion, but may also influence initial colonization [127, 128]. In addition to their critical bactericidal properties, APPs also interact with other key aspects of the innate immune response, including the *Toll*-like receptor (TLR) and related pathways. Recognition of pathogen-associated molecular patterns (PAMP) by TLRs induces the expression and/or release of

APPs and *vice versa*, APPs can enhance the immune response via activation of TLRs or TLR-related pathways [123]. Such immunomodulatory functions of APPs may be as important for host defence as their direct antimicrobial activity [123].

2.3.4 Mannose-binding lectin

Mannose-binding lectin (MBL) is a circulating pattern-recognition receptor produced by hepatocytes that binds to and opsonises various microorganisms. In addition, MBL is central to a third complement-activating pathway that is distinct from the classical and alternative complement activation mechanisms [129]. MBL also interacts with the TLR pathway by augmenting TLR2 and TLR6 signalling inside the phagosome [130]. Functional genetic variants of MBL are extremely common; approximately 5 % of Caucasians are heterozygous for these variants and 1% are functionally MBL deficient [131]. These variants have been associated with increased risk of serious bacterial infections in critically ill adults, although the impact on survival is controversial [131, 132, 133, 134]. Polymorphisms conferring reduced MBL protein expression are also associated with increased risk of premature birth [53]. In addition to genetic determinants of MBL levels, preterm infants are relatively MBL deficient *per se*, with serum levels rising with gestational and postnatal age [135, 136]. Furthermore reduced plasma levels of MBL, particularly those less than 0.5 µg/ml (normal adult range = 1-5 µg/ml) in infants of <28 wk GA and/or a BW of <1000g, significantly increase the risk of early- and late-onset neonatal sepsis as well as neonatal pneumonia and also prolong the required duration of antibiotic therapy [135, 137, 138].

The combined effect of GA-dependent deficiency in complement, APPs and MBL are likely to contribute to the observed susceptibility of preterm infants to invasive bacterial infection.

2.3.5 Neutrophil granulocyte phagocytosis, intracellular killing, generation of reactive oxygen species

Preterm and growth restricted infants have a limited neutrophil precursor pool, often resulting in neutropenia, which is thought to increase the risk for bacterial infections [139, 140, 141]. Investigations of phagocytic capacity of human neonatal neutrophils have yielded inconsistent results [141]. However investigations using the most physiologically relevant sample, fresh whole blood, have demonstrated significantly impaired phagocytosis by preterm neutrophils [142]. This probably results from deficient opsonisation by complement and immunoglobulins, as supplementation with adult serum or immunoglobulins restores phagocytic capacity *in vitro* [141]. Intracellular killing of microorganisms by the respiratory burst of preterm neutrophils is markedly reduced compared to term neonatal and adult cells [143, 144]. Interestingly, this persists for at least the first two months of life and correlates better with clinical illness than with postnatal age [145]. In contrast, generation of superoxide (O_2^-) by term neutrophils may actually be increased compared to adults, particularly in infants delivered by spontaneous birth compared to caesarean section [146, 147]. In addition to these deficiencies of neonatal neutrophil function, impaired formation of neutrophil extracellular traps, lattices of DNA, chromatin and antibacterial proteins that mediate killing of microorganisms, has recently been

described [148]. The impaired neutrophil trap formation is associated with a previously unrecognised deficiency of extracellular bacterial killing [148].

Several clinical studies have trialled hematopoietic growth factors (G-CSF and GM-CSF) for LOS reduction in preterm infants and have found that incidence of LOS remains unchanged despite augmentation of circulating neutrophil counts and function [149, 150, 151].

2.3.6 Monocyte phagocytosis and intracellular killing

Monocytes and macrophages are key effector cells of the innate immune responses with important phagocytic and cytokine producing capacities. They also provide a critical link to the adaptive immune system by presenting antigens of phagocytosed and processed microorganisms to T-cells. Impaired phagocytosis has been suggested as a possible mechanism underlying the increased susceptibility of preterm and term neonates to infection. However, the bacterial phagocytic and intracellular killing capacity of term cord blood monocytes is comparable to that of adults [152] and in preterm infants, the monocyte activity towards bacteria has been reported as similar or even higher than in adult monocytes [153](Strunk T, manuscript submitted).

Although neonatal monocytes appear to have largely intact ability to phagocytose and kill bacteria intracellularly, their capacity to amplify the signal and activate adaptive immune cells by cytokine production and upregulation of HLA-DR expression is significantly reduced (discussed below) [153, 154], suggestive of a developmentally impaired innate-adaptive immunity interaction. Furthermore, dendritic cells (DC), another key antigen-presenting cell type, are

functionally impaired in second trimester fetuses; the ability to recognise, take up, process and present antigens to the adaptive immune system improves with GA, but is immature at term compared to adult DCs [155].

2.3.7 Expression of pattern recognition receptors

Human TLRs, a family of 10 transmembrane and intracellular signalling proteins, are ancient innate immune receptors crucial for the recognition of PAMPs. TLRs are expressed by primary immune cells and indeed by most human tissues [156]. Each TLR interacts primarily with a different PAMP, thereby providing recognition of a wide variety of pathogens [157]. For example, TLR2 predominantly recognises Gram-positive bacteria, whereas TLR4 is critical for detection of lipopolysaccharide (LPS), the integral endotoxin membrane component of the Gram-negative bacterial cell wall [157].

Expression of TLR2 and TLR4 has been detected in intestinal tissue of human fetuses as early as 18 weeks gestation [158]. Basal expression of TLRs in term neonates is comparable to that of adults at both mRNA and protein level [90, 159]. Furthermore, we have recently demonstrated similar basal expression of TLRs 2, 4 and 9 at mRNA and protein level in cord blood mononuclear cells of preterm infants, term neonates and adults (Strunk T, unpublished data). In contrast, there have been conflicting reports of an inverse relationship of TLR4 expression with GA and an association of reduced TLR4 expression with impaired cytokine responses upon stimulation with LPS [160].

Despite the normal expression of TLRs on neonatal monocytes, there are substantial differences between neonates and adults in the magnitude of

activation of the TLR pathway by agonists. For example, production of tumor necrosis factor (TNF)-alpha by neonatal monocytes is markedly reduced compared to adult monocytes following exposure to TLR-ligands [161]. The level of cytokine response is related to reduced expression of the TLR-related adaptor protein MyD88 [162]. Similarly, impaired phosphorylation of the TLR-associated protein kinases p38 and extracellular regulated kinase 1/2 associated with low cytokine responses were observed in preterm infants [163]. Therefore, functional integrity of the receptor and associated signalling molecules appear to be more relevant for cellular activation than TLR expression levels *per se*.

Preliminary and as yet unreplicated genetic studies support this concept in the context of perinatal outcomes. A non-synonymous TLR4 polymorphism (TLR4-Asp299Gly) and two TLR2 polymorphisms have been associated with a higher risk of preterm birth, suggesting that altered function of the variant receptor protein may lead to increased rates of (subclinical) maternal and/or fetal infections that trigger inflammatory processes or result in aberrant immune responses contributing to preterm labour and delivery birth [52, 164].

2.3.8 Cytokine responses

Fetal cytokine responses are generally biased towards T-helper (Th)2 cytokines and this is suggested to be an evolutionary mechanism aimed at reducing deleterious inflammation *in utero* that may result in preterm birth and pregnancy loss [165]. Most published investigations to date have demonstrated reduced cytokine producing capacity of preterm infants' monocytes compared to those of term neonates and adults [160, 163, 166, 167]. In contrast, cytokine levels

produced by monocytes from term neonates are not uniformly reduced. For example, levels of Interleukin-(IL)6, a key early cytokine in the neonatal response to infection may be similar to or even greater than adult levels [168, 169, 170]. This GA-dependent pattern of cytokine responses has been described for *in vitro* responses to neonatal pathogens and some commensals [77, 171, 172] and also for purified strong activators of monocytes, such as LPS, bacterial lipopeptides and interferon-gamma (IFN-γ) [173]. The postnatal maturation of mononuclear cell cytokine production in response to LPS or IFN-γ throughout infancy has been assessed longitudinally in only one study, demonstrating a gradual increase in cytokine producing capacity over the first few years of life [174].

Tight regulation of the inflammatory response appears to be of evolutionary importance for the *in utero* survival of the fetus, an advantage that may outweigh the adverse effects of reduced neonatal immune responses to commensals and pathogens. However, very early in gestation, i.e. in infants born before 30 weeks GA, the down-regulation of the innate immune response is significantly greater than in term infants and contributes to the greatly increased infection rates in this highly vulnerable population. Interestingly, the neonatal innate immune responses to pathogens and TLR-ligands are modulated by a proteinaceous factor in early human breast milk, most likely reflecting physiologic maturation of neonatal innate immune responses [175].

2.4 Where are the functional defects of the innate immune system in preterm infants?

The limited evidence suggests that the GA-dependent maturation of the neonatal innate immune response potentially impacts on a wide array of innate immune functions rather than resulting in a single defect. It seems likely that uptake and killing of many pathogens in preterm infants is comparable to term infants, but that immune signalling and the link between innate and adaptive immunity may be impaired. The widespread functional impairments may be the result of GA-dependent regulation of the sensitivity and activity of central common innate immune signalling pathways. However, the hypothesis of altered sensitivity of multiple innate immune pathways has not been addressed systematically in the neonatal population. Emerging technologies increasingly allow simultaneous analyses of multiple small volume samples. Integrated analysis of gene expression profiles, surface marker expression, and cell-specific kinase activity and cytokine production are likely to reveal both the extent of the immunological deficiencies in preterm infants, but also assist in their biological localisation, thereby opening up specific and novel avenues for therapeutic interventions.

2.5 Potential therapeutic avenues

Previous trials with biological therapeutics, including intravenous immunoglobulins, anti-staphylococcal antibodies and haematological growth factors, have failed to demonstrate clinically relevant improvement of sepsis rates and their routine therapeutic use is not recommended [121, 122, 150, 176, 177, 178]. A more detailed understanding of both the key protective and

pathogenic pathways and the effects of GA on innate immunity will allow the development of new preventative and therapeutic strategies for neonatal infection. Recently, new avenues to augment innate immune responses have emerged, some of which have shown promising results in animal models or early human studies. For example, levels of BPI are low in human newborns and thought to contribute to infectious disease susceptibility [124, 179]. Furthermore, the availability and excellent safety profile of recombinant human BPI (rBPI) has allowed the administration of rBPI in severe meningococcal disease where it resulted in a trend towards better outcome [180]. There are as yet no further published trials in humans.

The relative deficiency in TLR responses in neonates has led to interest in augmenting the innate immune responses by use of specific TLR-agonists. Such an approach in a murine model of neonatal polymicrobial sepsis lead to increased peak levels, but abbreviated duration of the early systemic inflammatory response, reduced bacteraemia and improved survival [181]. Another promising application for TLR-agonists is their use as vaccine adjuvants, potentially rendering vaccines more effective in the neonatal population by overcoming the bias against T-helper (Th)1-responses [111, 182], an approach that has successfully been trialled in adult non-human primates [183].

Bacterial colonization of the preterm intestinal tract differs significantly from that of healthy term neonates, largely because of the intensive care required, including widespread use of antibiotics, parenteral nutrition and early colonization with nosocomial microorganisms. The short- and long-term effects of this alteration of the intestinal flora are yet unknown, but current evidence suggests that it contributes to adverse neonatal outcomes such as necrotising

enterocolitis (NEC) and late-onset sepsis (LOS) [184, 185]. Modulation of the intestinal immunity by administration of probiotics has been used for prevention and treatment of childhood allergies [186, 187]. Interestingly, the early administration of probiotics with enteral nutritidon in extremely preterm infants appears to be well tolerated, safe and results in decreased incidence of NEC and neonatal mortality [188]. However, the effect of probiotics on the incidence of LOS in preterm infants has not yet been convincingly demonstrated [189].

2.6 Future perspectives

Infectious diseases remain one of the major challenges in neonatal medicine, particularly in the increasing number of surviving extremely preterm infants, who remain at great risk of invasive infections for a prolonged period of time. Therefore, new approaches for early identification of at-risk infants that allow for preventative and/or prophylactic intervention are urgently needed. Future studies should aim to dissect the key immune pathways and identify the critical innate immune defects, hopefully facilitating the exploration of new avenues of targeted prevention to augment the ability of the innate immune system to appropriately respond to microbial invasion.

Chapter 3

Neonatal immune responses to coagulase-negative staphylococci

3.1 Abstract

Coagulase-negative staphylococci (CoNS) have emerged as the most common nosocomial pathogen in neonatal intensive care units worldwide. Our understanding of the interactions between CoNS and the immune system is incomplete, especially in the newborn. Here, we review the current knowledge on the human immune response to CoNS with particular emphasis on the neonatal innate immune system.

There are very limited data on innate immune responses to CoNS in neonates. Levels of serum proteins, including transplacental anti-CoNS immunoglobulin and complement, correlate with gestational age and this relative deficiency in preterm infants contributes to their sub-optimal opsonisation and impaired bacterial killing of CoNS. In adults, CoNS elicit significant cytokine responses *in vitro*, which are likely mediated in part via *Toll*-like receptors (TLR), including TLR2, but these pathways have not been characterised in the high-risk neonatal population.

The susceptibility of human preterm neonates to CoNS relates in part to immaturity of the neonatal immune response. Strategies to reduce the burden of CoNS infections require a thorough understanding of host-pathogen

interactions, particularly the engagement of CoNS by the neonatal innate immune system.

3.2 Introduction

The group of *coagulase-negative staphylococci (*CoNS*, e.g., Staphylococcus epidermidis, S. haemolyticus, S. saprophyticus, S. lugdunensis, S. schleiferi etc.)* are primarily ubiquitous colonisers of healthy skin and mucosal surfaces, and generally have low pathogenic potential [94]. However, CoNS are now the most commonly isolated group of organisms in the microbiology laboratory and have become increasingly important over the past two decades as nosocomial pathogens causing significant morbidity, mortality and health care costs worldwide [65, 80, 95, 190, 191, 192].

In adults, CoNS infections usually affect patients with indwelling plastic devices and the immunocompromised, such as those with advanced HIV/AIDS, malignant diseases or following immunosuppression [96]. Advances in neonatal intensive care have led to a growing population of children surviving even extreme prematurity and the most vulnerable groups for CoNS infections are the increasing numbers of significantly preterm infants (<32 weeks gestation) [193, 194]. Of note, premature infants account for more than 70% of consequent neonatal and infantile severe illness and death, much of which is related to infection and its sequelae [193, 195]. A large number of extremely low birth weight infants (ELBW, <1000g) have at least one episode of invasive bacterial infection [71, 196, 197]. Despite the greatly increased susceptibility of preterm infants to CoNS, the underlying immunological mechanisms have received scant attention.

Although much of the scientific effort has focused on early–onset sepsis (EOS, occurring within 72 h after birth), and GBS in particular, late-onset sepsis (LOS, onset after 72 h) is approximately 10 times more common in preterm infants [71, 88, 198]. CoNS have emerged as the most prevalent and important neonatal pathogens, responsible for around 50% of all episodes of LOS in neonatal intensive care units around the world. CoNS infections affect up to one half of very low birth weight (VLBW) infants, whereas term infants are rarely affected [80, 87, 199, 200].

Despite the marked improvement of neonatal intensive care, there is considerable morbidity, mortality and prolonged hospitalisation attributable to CoNS infection [71, 80]. CoNS sepsis has been estimated to prolong the length of hospital stay, depending on birth weight, by 4-19 days, causing significantly increased health care costs [102, 201]. Markedly increased risks for the development of bronchopulmonary dysplasia and cerebral palsy in preterm infants affected by CoNS sepsis have been reported from small studies and clearly warrant further investigation [113, 202]. Furthermore, one study found an association of delta-toxin producing CoNS with necrotising enterocolitis [203]. Thus, reducing the burden of CoNS would reduce not only morbidity and mortality but also significant secondary complications and health care cost.

3.3 Clinical features of CoNS infections in preterm infants

In preterm infants, CoNS infections most commonly occur in the third week of life and manifest with the typical, but non-specific signs of infections in neonates, of which the commonest are increased frequency of apnoea, worsening respiratory status, lethargy, temperature instability and

gastrointestinal disorders [65, 204]. Almost all CoNS are isolated from blood culture, whereas infections of the CNS, heart or other organs occur infrequently [80, 203]. Recently, it has been suggested that *erythema toxicum neonatorum*, affecting 50-70% of newborns, reflects an innate immune response to CoNS that have penetrated via hair shafts into the dermis. The phenomenon is probably mediated by IL-6, which unlike many other cytokines, is produced by neonates in equivalent quantities to adults following TLR2 stimulation [161, 170].

3.4 Risk factors for CoNS infection

Within the first days of life all neonates become colonised with CoNS and the number of bacteria on the skin dramatically increases within a short period of time [109, 205, 206]. Body sites with abundant CoNS colonisation include ear, axilla, nares and faeces whereas the skin on the forearm and leg, most common sites for intravascular catheters, generally have a smaller bacterial load [207].

Epidemiological analyses have identified several risk factors for CoNS infection, including low gestational age, low birth weight and procedures disrupting skin or mucosal integrity such as intravascular catheters, enteral tubes and mechanical ventilation [71, 80, 208]. Furthermore, the administration of both ante- and postnatal steroids as well as long-term administration of lipids with total parenteral nutrition (TPN) are associated with a significantly increased risk [204, 209, 210, 211, 212]. However, some of these associations may not be causal, but rather markers for the smallest and sickest infants, or may reflect the deleterious effects of the CoNS sepsis itself. Surprisingly, long-term TPN

administration is the only risk factor that has been investigated in detail. In infants, TPN impairs neutrophil phagocytosis and killing of CoNS as well as CoNS-induced tumour necrosis factor (TNF) production in an *in vitro* whole blood assay [83, 213].

3.5 Pathogenesis and virulence factors of CoNS

The formation of biofilm, an extracellular matrix consisting mainly of polysaccharides and proteins, constitutes one of the essential virulence factors of CoNS as it is critical to the successful colonization of host tissues, indwelling artificial devices and immune evasion [214, 215, 216]. Biofilm formation occurs significantly more frequently with clinically invasive strains in comparison to commensal strains [217]. The formation of biofilm consists of two phases, where CoNS first adhere unspecifically to a surface and then proliferate and produce various proteins and polysaccharides to form a complex extracellular matrix [94, 214, 218]. The numerous molecular mechanisms involved in the biofilm formation of CoNS remain incompletely characterised [219].

Biofilm provides several lines of effective protection from the host immune response and from antimicrobials. Recently, it was shown that some components of the biofilm produced by CoNS interfere with several major innate immune response mechanisms. The polysaccharide intercellular adhesin (PIA), restricted to a subpopulation of *S. epidermidis*, and poly-γ-DL-glutamic acid (PGA), ubiquitous among *S. epidermidis* strains, provide significant protection from chemotaxis, degranulation and phagocytosis of polymorphonuclear leukocytes and also confer resistance to host antimicrobial peptides, such as cathelicidin, human β–defensin 3 and dermicidin [220, 221].

Unlike Gram-negative bacteria or *S. aureus*, CoNS do not produce a large arsenal of potent toxins inducing fulminant clinical responses [214, 219]. Lipoteichoic acids and peptidoglycans of Gram-positive bacteria are significantly less potent inflammatory stimulants, typically requiring concentrations at µg/mL whereas lipopolysaccharide (LPS) at ~1,000 fold lower concentrations (~ng/mL) will elicit similar cytokine responses. In keeping with an overall reduced immune response, infection with biofilm-positive CoNS results in a 34% smaller rise in C-reactive protein, compared to biofilm-negative isolates [222]. However, a complex of bacterial peptides, derived from *S. epidermidis*, called phenol soluble modulins, have considerable pro-inflammatory potential, mediated via TLR-2 [223, 224, 225].

CoNS are frequently resistant to common antimicrobials, such as penicillins and aminoglycosides [226]. In general, biofilm producing strains of CoNS are more resistant to a broader spectrum of antibiotics than non-biofilm producing strains, probably because the biofilm poses a nearly impermeable barrier to many antibiotics [222, 227]. Furthermore, compared to other forms of growth, bacteria within a biofilm are in a state of reduced metabolic activity making them less susceptible to antimicrobials that rely on on-going cell division (e.g. β-lactams) [228, 229].

3.6 Innate immune responses

The ‘immaturity of host response mechanisms’ is often postulated to contribute to the greatly increased susceptibility to bacterial infections in preterm infants, but the mechanisms underlying such impairment are incompletely defined [230]. Compared to early-onset infections, there is a striking lack of data on the host

immune responses to pathogens causing LOS, particularly CoNS. These data are, however, of paramount importance to understand pathogenesis and ultimately develop interventions to reduce the disease burden. Here, we review the published literature on various aspects of the immune response to CoNS infections in neonates. We used the following method for reviewing the English literature: internet based PubMed search from 1985 to 2007; search terms included ‘neonate’, ‘preterm infant’, ‘sepsis’, ‘late-onset’, ‘nosocomial infection’, ‘coagulase-negative staphylococcus’, ‘*S. epidermidis*’, ‘immune response’, ‘cytokine’.

3.6.1 Transplacental antibodies

Placentally-transmitted immunoglobulin G (IgG) plays an important role as a first line defence mechanism and low IgG levels in preterm infants are associated with susceptibility to LOS [88]. The opsonic activity against *S. epidermidis* correlates with anti-staphylococcal peptidoglycan IgG and with gestational age in preterm infants [116]. However, preterm infants who subsequently acquired CoNS sepsis are indistinguishable from uninfected infants on the basis of their anti-CoNS IgG levels at birth [117], possibly due to the insufficient complement-independent opsonic activity of preterm immunoglobulin [116]. In contrast, the level of maternal anti-GBS antibodies (to both to *GBS* type Ia and III) predicts risk of neonatal *GBS* early-onset sepsis [119, 120].

3.6.2 Complement, opsonophagocytosis and bacterial killing

Many aspects of the innate immune responses to various neonatal pathogens, particularly GBS, show significant differences between neonates and adults [143, 231, 232, 233]. There are comparatively few analogous data on CoNS. In addition to significant hypogammaglobulinemia, preterm infants also display significantly reduced complement activity of both classical and alternative pathways [116, 118, 234]. Both lack of effective antibodies and decreased complement activity contribute to inefficient polymorphonuclear cell opsonophagocytosis and bacterial killing of CoNS compared to adults and term neonates [116, 235]. The deficient complement deposition on CoNS of premature sera is corrected *in vitro* by use of IVIG [236]. Furthermore, a significantly impaired capacity of preterm polymorphonuclear cells to upregulate oxidative burst in response to stimulation with CoNS has been described [237]. TLR-2 and -4 have emerged as important activators for bacterial phagocytosis, but data relating to their role in neonates are not available [238].

The effect of biofilm on efficient phagocytosis of CoNS is unclear and the effects *in vivo* are to likely differ from *in vitro* observations; biofilm formation of CoNS is significantly diminished in planktonic growth, the usual form of CoNS preparations employed for *in vitro* studies [214, 235, 237, 239, 240].

3.6.3 Cytokine production

Substantially different cytokine profiles in response to various stimuli have been observed in term and preterm neonates compared to healthy adults and this contributes significantly to the deficient innate immune responses to pathogens

in the newborn and preterm infants in particular [230]. Little is known about the neonatal cytokine responses to CoNS and only a few studies have investigated CoNS induced cytokine production *in vitro* and these data are limited to adults. Bayston et al. described strong induction of TNF□in a whole blood assay stimulated with cell-free supernatant of CoNS [241], whereas other groups used whole, heat-inactivated CoNS to induce TNF-a, IL-1b, IL-6 and IL-12 in human mononuclear leukocytes [242, 243, 244]. There are no analogous data from newborn infants, but these are clearly warranted. Studies of other neonatal pathogens suggest that cytokine profiles of *in vivo* infections show some correlation with outcome [38, 245, 246].

3.6.4 Phagocyte signalling and function

There are very limited data on the initial TLR signalling pathways which orchestrate the innate and adaptive immune response to CoNS [247]. However, since CoNS are Gram-positive, it is likely that they signal at least in part through TLR2, and possibly TLR4 [238]. Indeed, *S. epidermidis*, the most commonly isolated strain of CoNS, secretes the TLR2 agonist phenol-soluble modulin [225]. The potential involvement of either of these receptors, or indeed other TLRs, in the recognition of CoNS by the innate immune system may have serious implications for preterm infants. For example, the expression of TLR4, while similar on term neonatal and adult monocytes [159, 162], is significantly reduced in preterm infants [160]. Additionally, although the expression of TLR2 on neonatal and preterm monocytes were comparable to that in healthy adults [159, 163], reduced signalling in response to TLR2 agonists was observed in preterm cells, possibly due to lowered MyD88 expression [86]. Finally, neonatal

cytokine responses to a broad panel of TLR agonists (such as bacterial lipopeptides, heat-killed *L. monocytogenes*, zymosan particles etc.) are polarised towards a high IL-6, low TNF production [170]. These effects may be mediated via the distinct adenosine system of human newborns, in which high plasma levels of adenosine, an endogenous purine metabolite with immunomodulatory function, acts via the adenosine A3 receptor to induce cyclic AMP (cAMP), a second messenger that inhibits TLR2-mediated TNF production but preserves production of IL-6 [161]. It is not yet known whether the effects of adenosine, that are manifest towards pure TLR2 agonists (e.g., bacterial lipopeptides) as well as microbial particles with TLR2 agonist activity such as heat-killed *Listeria monocytogenes* and zymosan, also extend to CoNS.

In summary, there is very limited published information regarding innate immune responses of neonates to CoNS. Furthermore, data relating to EOS cannot be readily extrapolated to late-onset CoNS infection, as there are fundamental differences with regards to affected subjects and pathogens involved. In addition, investigations undertaken on adult cells will not adequately reflect the differential immune responses of neonates. Thus, further investigations of the immunological mechanisms underlying invasive CoNS infection in preterm infants are clearly warranted.

3.7 New therapeutics

Despite the low levels of CoNS IgG in preterm neonates and *in vitro* evidence that additional IgG might correct defective opsonophagocytosis, the administration of pooled intravenous immunoglobulin has not resulted in significant protection from invasive bacterial infections in several large trials and

is currently not recommended for routine use [177, 235, 236]. Administration of donor-selected human anti-staphylococcal immunoglobulin containing high titres of antibodies against key antigens of *S. aureus* and *S. epidermidis* resulted in a significant reduction of *S. aureus* infections, but not CoNS sepsis, in VLBW infants [176]. A small safety trial using human donor *S. aureus* immunoglobulin found no effect on the incidence of invasive *S. aureus* infections in VLBW [122], suggesting other innate effector mechanisms, possibly including TLR activation, are likely to be important [248, 249].

3.8 Conclusion and future directions

Infections with CoNS constitute the most common LOS in preterm neonates, adding significantly to morbidity, mortality and increased health care costs. Hence, it is of great importance to characterise the innate immune responses to CoNS in preterm infants, which will be essential for the development of future prophylactic and therapeutic interventions. Successful strategies to reduce CoNS infections among very preterm low birth weight infants would reduce mortality and morbidity, shorten hospital stay, reduce antibiotic usage and thereby reduce the overall human and financial costs of extreme prematurity.

Chapter 4

Histologic chorioamnionitis is associated with reduced risk of late-onset sepsis in very preterm infants

4.1 Abstract

Background: Histologic chorioamnionitis (HCA) is implicated in the onset of preterm labor and delivery. Chorioamnionitis is a known risk factor for early-onset sepsis (EOS) and may modulate postnatal immunity. Preterm infants are at greatly increased risk of late-onset sepsis (LOS), particularly with coagulase-negative staphylococci (CoNS), but the impact of HCA on the risk of LOS is unknown.

Methods: 838 preterm infants born at <30 weeks gestational age (GA) at a single tertiary center were included. Histologic examination of placenta and extra-placental membranes was performed and clinical data extracted from hospital databases. The influence of HCA on the incidence of EOS and LOS was examined using logistic regression analysis and Cox proportional-hazards regression.

Results: Mean GA was 26.9 ± 1.9 weeks and mean birth weight was 936 ± 277g. Two hundred and seventy-six (33%) of 838 infants developed LOS. The presence of fetal or maternal HCA, or maternal HCA or fetal HCA alone, was associated with a significantly decreased risk of LOS with any organism (HR 0.74 95% CI 0.57-0.95, p=0.020; HR 0.73 95% CI 0.56-0.94, p=0.016 and HR 0.67 95% CI 0.49-0.93, p=0.018, respectively). Histologic chorioamnionitis

correlated with a significantly decreased risk of CoNS LOS (HR=0.74 95% CI 0.57-0.95, p=0.020 and HR 0.73 95% CI 0.55-0.98, p=0.038, respectively).

Conclusions: Histologic chorioamnionitis is associated with a significantly reduced risk of acquiring LOS, both with CoNS and other bacteria. Perinatal inflammation may enhance the functional maturation of the preterm immune system and provide protection against LOS in high-risk preterm infants.

4.2 Introduction

The incidence of premature delivery has increased by up to 30% over the past two decades in resource-rich countries. Premature infants account for 75% of neonatal morbidity and mortality, causing considerable human and health care costs, estimated at US$26.2 billion/year in the United States alone [2, 9, 60, 250].

Histologic chorioamnionitis (HCA, inflammation of the placental chorionic disk and the extra-placental membranes) is associated with preterm delivery and is inversely correlated with gestational age (GA) and birth weight (BW) [38]. Histologic evidence of chorioamnionitis is present in approximately 30% and 60% of placentae at 29 weeks and 23-24 weeks GA, respectively [38, 251, 252, 253, 254]. Chorioamnionitis is associated with activation of fetal immune cells and increased levels of pro-inflammatory cytokines, including interleukins, tumor necrosis factor-alpha and other mediators, in cord blood and cerebrospinal fluid [255, 256, 257, 258, 259]. Perinatal inflammation can have deleterious effects, with increased neonatal morbidity, including intracranial hemorrhage, periventricular leukomalacia (PVL) and cerebral palsy [41, 42, 43]. The infectious pathogens that drive perinatal inflammation also increase the risk of vertically acquired early-onset sepsis (EOS, with onset <72 hours of age) [260]. The presence of HCA has been associated with decreased risk of acute respiratory disease in preterm infants, at least partially due to stimulation of cortisol production and accelerated lung maturation, whereas the incidence of chronic lung disease is increased by exposure to perinatal inflammation in most studies [254, 260, 261, 262, 263, 264, 265, 266, 267, 268]. Some studies suggest that HCA is more prevalent in survivors of extreme prematurity and the

improved outcome was largely attributable to decreased risk of respiratory disease [38, 269].

Infection is one of the most common clinical problems encountered in the preterm population, who have greatly heightened susceptibility [79, 270]. In this population, late-onset sepsis (LOS, age at onset >72 hours) is most frequently caused by coagulase-negative staphylococci (CoNS) and the incidence of infection is inversely correlated with GA and BW [80]. Infection with CoNS is associated with increased risk of common adverse neonatal outcomes such as chronic lung disease (CLD) and cerebral palsy and results in prolonged hospital stay and health care costs [112, 254, 271, 272].

Preterm birth is commonly complicated by perinatal inflammation of the maternal and/or fetal placental unit. Perinatal inflammation may have pervasive effects on immune function [273], and we therefore hypothesized that chorioamnionitis may influence protective immune responses to CoNS and other pathogens causing LOS in preterm infants. Here we report a large epidemiologic study of the relationship between histologic chorioamnionitis and the risk of LOS in a large cohort of very preterm infants.

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4.3 Patients and methods

This single-center, retrospective cohort study was conducted at King Edward Memorial Hospital, the only tertiary perinatal center in Western Australia (total population ~2.1 million) with approximately 6000 deliveries annually. The study was approved by the institutional review board (#0830-02/08). All infants born at less than 30 weeks GA and admitted to the Neonatal Intensive Care Unit at King Edward Memorial Hospital between January 1st, 2001, and December 31st, 2007, were identified from the neonatal database (n=1147). Infants without placental histologic examination (usually those out-born and transferred postnatally), and those with major congenital anomalies that might increase susceptibility to infection were excluded.

Sepsis was defined by a combination of i) suggestive clinical signs, ii) a positive blood culture (for the diagnosis of CoNS sepsis: only single organism blood cultures were included) [80] and iii) appropriate antibiotic treatment of ≥5 days. Blood culture isolates were categorized into 2 groups; CoNS and other pathogenic bacteria. Early-onset sepsis was defined as sepsis with onset at <72 hours of age, and LOS as sepsis with onset >72 hours of age. Chronic lung disease was defined as a requirement for supplemental oxygen at 36 weeks post-menstrual age.

4.3.1 Placental histology

Histopathologic scoring was performed on placentae that are collected and processed for histologic examination on all preterm infants born at less than 32 weeks GA, as part of routine clinical practice. Semi-quantitative histologic scoring of the extra-placental membranes, umbilical cord, chorionic plate and

placenta was performed using widely accepted guidelines [274] by an experienced perinatal histopathologist, who was blinded to clinical outcomes. Evidence of a maternal inflammatory reaction was defined as an acute inflammatory reaction (predominantly neutrophils) in the decidua, extending into the chorion in the extraplacental membranes, or from the maternal blood space into the chorionic plate. Fetal chorioamnionitis was defined as the presence of neutrophils marginating and infiltrating the umbilical vessels (usually the vein) into the cord, or from the fetal vessels of the chorionic plate towards the amnion. Funisitis is inflammation involving the umbilical cord usually from the fetal vessels.

Preterm placentae are routinely cultured for microbial pathogens by taking a sample of the chorionic plate with overlying amnion. The microbiology laboratory culture the subamniotic layer and for aerobic organisms including haemophilus, mycoplasma and ureaplasma species [275].

4.3.2 Statistical analysis

Continuous data were summarized using means and standard deviations or medians and interquartile ranges, according to normality. Categorical data were summarized using frequency distributions. Univariate group comparisons were performed using Chi-square and Fisher exact tests as appropriate. Factors associated with EOS were evaluated using logistic regression analysis. Covariate effects were summarized using odds ratios (OR) and their 95% confidence intervals (CI). Time to first infection (CoNS or other LOS) and time until death were estimated using Kaplan-Meier survival probabilities. Effects of HCA and other covariates on the time until infection were examined using Cox

proportional-hazards regression and their effects were summarized using hazard ratios (HR) and 95% confidence intervals (CI). In the analyses of time to infection, deaths were censored. All evaluations of the effects of HCA were adjusted for GA. SPSS statistical software (version 15, SPSS Inc., Chicago IL) was used for data analysis. All hypothesis tests were two-sided and p-values<0.05 were considered statistically significant.

4.4 Results

Detailed placental histology was available from 869/1147 infants (75.7%) born during the study period. Of the 278 infants excluded because placental histologic examination was not performed, many were born in peripheral hospitals or by emergency delivery. A further 31 infants were excluded due to major congenital abnormalities that may significantly alter the risk of bacterial infection (e.g. trisomy 21, gastroschisis, myelomeningocele). A total of 838 infants were therefore included in the analysis. The mean GA of the study cohort was 26.9 ± 1.9 weeks and with a mean birth weight of 936 ± 277g. Ninety-six (11.5%) infants died, and of these 45 (46.9%) died on or before day three of life.

4.4.1 Histologic chorioamnionitis

On placental histologic examination, 384/838 (45.8%) had evidence of perinatal inflammation. Combined maternal and fetal HCA was observed in 132 (34.4%) placentae, maternal HCA alone in 154/384 (40.1%), and fetal HCA alone in 16/384 (4.2%). Funisitis in the absence of maternal HCA was uncommon (16/384, 4.2%). Histologic chorioamnionitis was inversely related to GA; at 22-24 weeks GA 86/152 (56.6%) had evidence of histologic HCA, compared to 32/142 (22.5%) at 29 weeks GA ($p<0.0001$) (Figure 4.1).

Table 4.1 Characteristics of infants with and without histological chorioamnionitis.

EOS, early-onset sepsis; LOS, late-onset sepsis; OR, odds ratio; CI, confidence interval; HCA, histologic chorioamnionitis; CPAP, continuous positive airway pressure; CVL, central venous line; NEC, necrotizing enterocolitis. Data shown as n (%) or mean ± standard deviation.

	Placental inflammation		
	No HCA *n=535 (64%)*	**HCA** *n=303 (36%)*	**p value**
Gestational Age[#]	27.2±1.8	26.3±1.9	<0.001
Birth weight [#]	950±282	912±267	0.063
Birth weight z-score[#]	-0.20±1.04	0.09±0.74	<0.001
Sex (Male)	293 (55%)	154 (51%)	0.272
Apgar at 1 min <7	328 (62%)	226 (75%)	<0.001
Apgar at 5 min <7	85 (16%)	85 (28%)	<0.001
EOS	11 (2%)	14 (5%)	0.036
Any LOS	180 (34%)	96 (32%)	0.562
CoNS LOS	141 (26%)	77 (25%)	0.765
CLD	182 (34%)	110 (36%)	0.505
PVL	6 (1%)	12 (4%)	0.006
Death	58 (11%)	38 (13%)	0.458

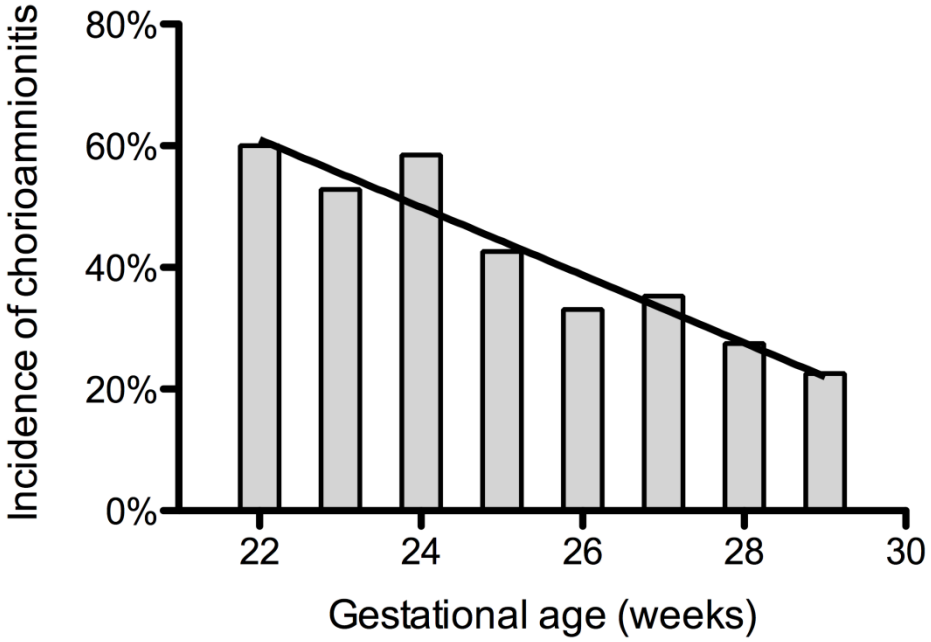

Figure 4.1 Incidence of histologic chorioamnionitis according to gestational age.

(r^2: 0.91, p=0.0002)

4.4.2 Placental culture and pathology

Placental microbiological cultures were performed in almost two-thirds of cases (536/838, 64%), and approximately one quarter of these (127, 23.7%) yielded a positive culture. *Ureaplasma urealyticum* was the most commonly isolated pathogen (53/127, 41.7%). Histologic chorioamnionitis was significantly more common in cases of positive placental culture; maternal HCA was observed in 70.1% (89/127) of cases with positive placental cultures compared to 36.9% (151/409) of cases without bacterial growth (p<0.001). Similarly fetal HCA or funisitis were more commonly associated with positive placental cultures (80/127, 63% with positive versus 135/409, 33% with negative cultures, p<0.001).

4.4.3 Neonatal sepsis

There were a total of 328 episodes of neonatal sepsis in 300 (35.8%) infants. Three percent (25/838) of infants developed EOS, with *Streptococcus agalactiae* and *Escherichia coli* the most common isolates. The basic characteristics of infants with and without EOS were similar (mean GA 26.4 (±1.8) versus 26.9 (±1.9) weeks, p=0.184, and BW 889g (±281g) versus 938g (±277g), p=0.393)(Table 4.2). Infants with EOS had lower Apgar scores than those without (Apgar <7 at 1 minute in 88% versus 66%, p=0.029, and Apgar <7 at 5 minutes in 42% versus 20%, p=0.009, respectively) (Table 4.2). Furthermore, infants with EOS had a higher incidence of any HCA (56% versus 36%, p=0.036) and maternal HCA (56% versus 34%, p=0.020) than infants without infection (Table 4.2). Importantly, none of the infants with EOS subsequently developed LOS.

Table 4.2 Risk factors associated with early-onset sepsis.

EOS, early-onset sepsis; LOS, late-onset sepsis; OR, odds ratio; CI, confidence interval; HCA, histologic chorioamnionitis; CPAP, continuous positive airway pressure; CVL, central venous line; NEC, necrotizing enterocolitis.

	EOS			
Risk factor/outcome	**No** *n=813 (97%)*	**Yes** *n=25 (3%)*	**OR (95% CI) for EOS**	**p value**
Gestational Age[#]	26.9±1.9	26.4±1.8	0.87 (0.71-1.07)	0.184
Birth weight [#]	938±277	889±281	0.52 (0.12-2.32)[*]	0.393
Birth weight z-score[#]	-0.1±0.9	-0.2 ±1.1	1.09 (0.71-1.66)	0.695
Sex (Male)	435 (54%)	12 (48%)	0.80 (0.36-1.78)	0.587
Apgar at 1 min <7	533 (66%)	21 (88%)	3.6 (1.06-12.08)	0.029
Apgar at 5 min <7	160 (20%)	10 (42%)	2.87 (1.25-6.59)	0.009
Antibiotics given to infant	509 (63%)	10 (40%)	0.40 (0.18-0.90)	0.022
HCA	289 (36%)	14 (56%)	2.31 (1.03-5.15)	0.036
Maternal chorioamnionitis	273 (34%)	14 (56%)	2.52 (1.13-5.62)	0.020
Fetal chorioamnionitis	141 (17%)	7 (28%)	1.85 (0.76-4.51)	0.135
Funisitis	189 (23%)	10 (40%)	2.18 (0.96-4.94)	0.055
HCA + Funisitis	175 (22%)	8 (32%)	2.18 (0.86-5.50)	0.106
Death[‡‡]	87 (11%)	9 (36%)	-	0.001

Data shown as n(%) or mean ±standard deviation [#]

Three hundred and four episodes of LOS occurred in 276 infants (32.9% of the total number of infants). Twenty-four of 276 (8.7%) infants developed two episodes of LOS and two infants developed three episodes. The median age of onset of all LOS episodes was 10 days. For infants with >1 episode of LOS, only the first episode was included in the regression analysis.

The majority of LOS cases were caused by CoNS (218 of 276 infants; 79%) with a median age at onset of 10 days. Sixty eight cases (68/276; 24.6%) of LOS were due to non-CoNS pathogens, of which *Staphylococcus aureus*, *E. coli*, *S. agalactiae* and *Enterococcus sp.* were the most frequent. The median age at onset

of non-CoNS LOS was 13 days of age. Infants with LOS were born significantly earlier (mean GA 26.2 (±1.8) versus 27.2 (±1.8) weeks, p<0.001) and with lower BW (848g (±240g) versus 980g (±284g), p<0.001) than those infants who did not develop LOS (Table 4.3). Infants who acquired LOS required respiratory support for longer (ventilated for median of 297 hours versus 44 hours, p<0.001 and continuous positive airway pressure for 736 hours versus 575 hours, p=0.001). These infants also required parenteral nutrition for longer (16 days versus 10 days p<0.001), took longer to reach full enteral feeds (20 days versus 13 days, p<0.001), and had a higher incidence of necrotizing enterocolitis (NEC) (8% versus 3%, p=0.004) and CLD (49% versus 28%, p<0.001) (Table 4.3).

Infants with CoNS LOS were more premature (26.2 (±1.8) versus 27.1 (±1.9) weeks, p<0.001) and had lower BW than those without CoNS LOS (854g (±244g) versus 965g (±283g), p<0.001) (Table 4.4). Furthermore, infants with CoNS LOS required respiratory support for longer (ventilated for 285 hours versus 49 hours, p<0.001 and continuous positive airway pressure for 748 hours versus 584 hours, p=0.001), needed parenteral nutrition for longer (16 days versus 10 days p<0.001), and were slower to reach full enteral feeds (20 days versus 13 days, p<0.001). In addition, infants with CoNS LOS had a higher incidence of NEC (7% versus 4%, p=0.029) and CLD (48% versus 30%, p<0.001) (Table 4.4).

Table 4.3 Risk factors associated with late-onset sepsis.

EOS, early-onset sepsis; LOS, late-onset sepsis; OR, odds ratio; CI, confidence interval; HCA, histologic chorioamnionitis; CPAP, continuous positive airway pressure; CVL, central venous line; NEC, necrotizing enterocolitis.

	Any LOS			
Risk factor/outcome	**No** *n=562 (67%)*	**Yes** *n=276 (33%)*	**HR (95% CI)**	**p value**
Gestational Age[#]	27.2 (±1.8)	26.2 (±1.8)	0.74 (0.69-0.79)	<0.001
Birth weight (g) [#]	980 (±284)	848 (±240)	0.17 (0.10-0.27)[*]	<0.001
Birth weight z-score[#]	-0.7 (±0.96)	-0.15 (±0.92)	0.93 (0.82-1.05)	0.242
Sex (Male)	299 (53%)	148 (54%)	1.07 (0.84-1.36)	0.909
Apgar at 1 min <7	368 (66%)	186 (68%)	1.22 (0.94-1.57)	0.576
Apgar at 5 min <7	118 (21%)	52 (19%)	1.04 (0.77-1.41)	0.436
HCA	207 (37%)	96 (35%)	0.97 (0.75-1.24)	0.562
Maternal chorioamnionitis	197 (35%)	90 (33%)	0.96 (0.74-1.23)	0.483
Fetal chorioamnionitis	103 (18%)	45 (16%)	0.92 (0.67-1.27)	0.464
Funisitis	136 (24%)	63 (23%)	0.92 (0.70-1.22)	0.701
HCA + Funisitis	125 (22%)	58 (21%)	0.91 (0.68-1.22)	0.845
Ventilator	297 (53%)	184 (67%)	1.36 (1.06-1.74)	<0.001
Ventilator [hours] [##ǂ]	44 [1-2415]	297 [1-1502]	1.02 (1.01-1.02) [**]	<0.001
CPAP	308 (55%)	179 (65%)	1.07 (0.83-1.36)	0.006
CPAP [hours] [##ǂ]	575 [1-1827]	736 [4-1856]	1.01 (1.00-1.01) [**]	0.001
CLD	157 (28%)	135 (49%)	1.69 (1.33-2.14)	<0.001
CVL	105 (19%)	88 (32%)	1.47 (1.14-1.89)	<0.001
Total CVL days[##ǂ]	9 [1-39]	10 [1-38]	0.98 (0.95-1.01)	0.454
Multiple CVL episodes	12 (11%)	13 (15%)	1.63 (0.93-2.85)	0.491
Parenteral nutrition	335 (60%)	189 (69%)	1.13 (0.87-1.45)	0.013
Total parenteral nutrition days[##ǂ]	10 [1-54]	16 [3-56]	1.04 (1.03-1.05)	<0.001
Enteral (tube) feeds	304 (54%)	178 (65%)	1.06 (0.83-1.36)	0.004
Time to full enteral feeds [hours][##ǂ]	13 [4-59]	20 [6-133]	1.05 (1.04-1.06)	<0.001
NEC [ǂǂ]	18 (3%)	21 (8%)	-	0.004
Death[ǂǂ]	78 (14%)	18 (8%)	-	0.002

Data shown as n(%), mean ±standard deviation [#] or median, [range] [##]

Table 4.4 Risk factors associated with CoNS late-onset sepsis.

EOS, early-onset sepsis; LOS, late-onset sepsis; OR, odds ratio; IQR, inter-quartile range; HCA, histologic chorioamnionitis; CPAP, continuous positive airway pressure; CVL, central venous line; NEC, necrotizing enterocolitis.

	CoNS LOS			
Risk factor/outcome	**No** *n=620 (74%)*	**Yes** *n=218 (26%)*	**HR (95% CI)**	**p value**
Gestational Age[#]	27.1 (±1.9)	26.2 (±1.8)	0.74 (0.69-0.80)	<0.001
Birth weight (g) [#]	965 (±283)	854 (±244)	0.19 (0.11-0.32)[*]	<0.001
Birth weight z-score[#]	-0.09 (±0.96)	-0.12 (±0.92)	0.96 (0.84-1.10)	0.710
Sex (Male)	327 (53%)	120 (55%)	1.13 (0.87-1.48)	0.558
Apgar at 1 min <7	409 (67%)	145 (67%)	1.18 (0.89-1.57)	0.824
Apgar at 5 min <7	126 (21%)	44 (20%)	1.15 (0.83-1.60)	0.946
HCA	77 (35%)	226 (37%)	0.99 (0.75-1.31)	0.765
Maternal chorioamnionitis	216 (35%)	71 (33%)	0.96 (0.72-1.28)	0.544
Fetal chorioamnionitis	108 (17%)	40 (18%)	1.08 (0.76-1.52)	0.764
Funisitis	147 (24%)	52 (24%)	0.99 (0.72-1.35)	0.906
HCA + Funisitis	48 (22%)	135 (22%)	0.96 (0.70-1.34)	0.883
Ventilator	335 (54%)	146 (67%)	1.38 (1.04-1.83)	0.001
Ventilator [hours] [##ǂ]	49 [1-2415]	285 [1-1502]	1.02 (1.01-1.02)[**]	<0.001
CPAP	344 (56%)	143 (66%)	1.11 (0.84-1.46)	0.009
CPAP [hours] [##ǂ]	584 [1-1856]	748 [4-1601]	1.01 (1.00-1.02)[**]	0.001
CLD	188 (30%)	104 (48%)	1.62 (1.24-2.12)	<0.001
CVL	129 (21%)	64 (29%)	1.31 (0.98-1.75)	0.010
Total CVL days[##ǂ]	10 [1-29]	10 [1-39]	0.97 (0.93-1.01)	0.264
Multiple CVL episodes	15 (2%)	10 (5%)	1.62 (0.86-3.06)	0.106
Parenteral nutrition	373 (60%)	151 (69%)	1.17 (0.88-1.56)	0.017
Total parenteral nutrition (days)[##ǂ]	10 [1-56]	16 [3-56]	1.04 (1.03-1.05)	<0.001
Enteral (tube) feeds	339 (55%)	143 (66%)	1.12 (0.85-1.48)	0.005
Time to full enteral feeds (days)[##ǂ]	13 [4-59]	20 [6-133]	1.05 (1.04-1.06)	<0.001
NEC [ǂǂ]	23 (4%)	16 (7%)	-	0.029
Death[ǂǂ]	81 (13%)	15 (7%)	-	0.014

Data shown as n(%), mean ±standard deviation [#] or median, [range] [##]

4.4.4 Chorioamnionitis and risk of neonatal sepsis

The presence of any HCA was associated with increased risk of EOS (OR 2.31 95% CI 1.03-5.15, p=0.036). Similarly, maternal HCA was associated with an increased risk of EOS: more than half (14/25, 56%) of infants with EOS had evidence of maternal HCA compared to 33.6% of infants without EOS (OR 2.52 95% CI 1.13-5.62, p=0.020).

Chorioamnionitis significantly reduced the risk of LOS (Table 4.5 and Figure 4.2). Late-onset sepsis caused by any organism was less common in infants with evidence of perinatal inflammation (adjusted hazard ratio (HR) for any HCA HR 0.74 95%CI 0.57-0.95, p=0.020; for maternal HCA alone HR 0.73 95% CI 0.56-0.94, p=0.016; for fetal HCA alone HR 0.67 95% CI 0.49-0.93, p=0.018) (Table 4.5).

Similarly the risk of LOS with the most common pathogen, CoNS, was reduced in infants exposed to any HCA (HR 0.74 95% CI 0.57-0.95, p=0.020) as well as maternal HCA (HR 0.73 95% CI 0.55-0.98, p=0.038). The risk of LOS due to organisms other than CoNS was significantly reduced by fetal chorioamnionitis only (HR 0.31 95% CI 0.13-0.72, p=0.006) (Table 4.5).

Table 4.5 Histological chorioamnionitis and risk of late-onset sepsis.

OS, early-onset sepsis; LOS, late-onset sepsis; GA, gestational age, HCA, histologic chorioamnionitis; HR, hazard ratio, CI, confidence interval.

Predictor variables for any LOS		**Adjusted for GA**			**Adjusted for GA and EOS**		
	n (%)	**HR**	**95%CI**	**p value**	**HR**	**95%CI**	**p value**
HCA	303 (36%)	0.74	0.57-0.95	0.020	0.75	0.58-0.97	0.028
Maternal chorioamnionitis	287 (34%)	0.73	0.56-0.94	0.016	0.74	0.57-0.96	0.024
Fetal chorioamnionitis	148 (18%)	0.67	0.49-0.93	0.018	0.67	0.48-0.93	0.016
Funisitis	199 (24%)	0.82	0.62-1.09	0.173	0.83	0.63-1.10	0.196
Predictor variables for CONS LOS							
HCA	303 (36%)	0.74	0.57-0.95	0.020	0.75	0.58-0.97	0.028
Maternal chorioamnionitis	287 (34%)	0.73	0.55-0.98	0.038	0.75	0.56-1.00	0.051
Fetal chorioamnionitis	148 (18%)	0.82	0.58-1.17	0.270	0.82	0.58-1.16	0.258
Funisitis	199 (24%)	0.90	0.66-1.23	0.490	0.91	0.66-1.24	0.532
Predictor variables for other LOS							
HCA	303 (36%)	0.74	0.44-1.23	0.243	0.75	0.45-1.26	0.274
Maternal chorioamnionitis	287 (34%)	0.81	0.48-1.35	0.417	0.83	0.49-1.38	0.468
Fetal chorioamnionitis	148 (18%)	0.31	0.13-0.72	0.006	0.31	0.13-0.72	0.006
Maternal or fetal chorioamnionitis	303 (36%)	0.74	0.44-1.23	0.243	0.75	0.45-1.26	0.274
Funisitis	199 (24%)	0.75	0.42-1.33	0.328	0.76	0.43-1.26	0.353

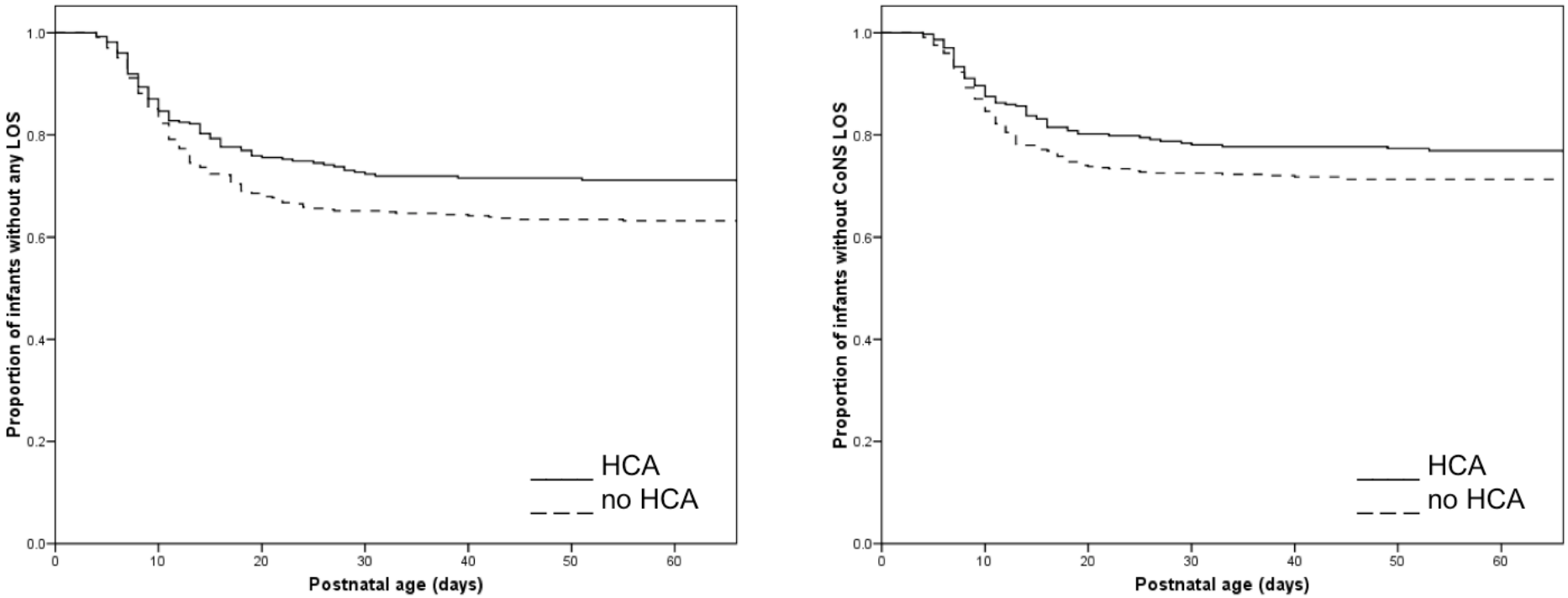

Figure 4.2 Kaplan-Meier curves showing time to late-onset sepsis by histological chorioamnionitis.

LOS with any organism (left) and LOS with coagulase-negative staphylococci (right) using Kaplan-Meier survival curves. y-axis indicates proportion of infants without LOS versus postnatal age at onset of LOS (days). HCA, histologic chorioamnionitis.

4.5 Discussion

In this large single center retrospective study we report, for the first time, that HCA is associated with a significantly decreased risk of late-onset sepsis in extremely preterm infants. Chorioamnionitis was associated with overall reduced risk of LOS and of infection with both the commonest cause of LOS in preterm infants, CoNS, and with infection due to other non-CoNS bacteria. The protective effect of HCA on LOS risk is opposite to the increased risk of EOS associated with HCA in the present and previous studies [260, 261, 262]. Our data also confirmed the well-documented risk factors for LOS and CoNS infection; extreme prematurity and its complications and management.

Early-onset sepsis follows direct colonization and invasion of the neonate with the same organism that is recovered from the placenta, following to vertical infection of the birth canal. The relatively low overall yield of routine bacterial cultures reflects the colonization/infection of the placenta with anaerobic and difficult-to-culture organisms, as suggested by recent data using 16sRNA PCR-based, culture-independent methods [31]. However the pathogen most commonly cultured in placental inflammation related to preterm birth, *U. urealyticum*, does not typically cause acute clinical disease in the neonate. This suggests that if perinatal inflammation is due to a low virulence organism, it is relatively well-controlled by the maternal immune system and does not result in bacterial invasion of the fetus and EOS, then it will enhance the maturation of the neonatal immune system. Uncontrolled infection with a virulent organism will result in direct infection of the fetus, clinically presenting as EOS.

Conversely the organisms causing perinatal inflammation rarely result in clinical LOS. Instead it appears that chorioamnionitis has significant effects on immune function, and therefore indirectly modulates the risk of LOS with nosocomial

organisms. The mechanisms by which HCA may result in maturation of the fetal and neonatal immune system are unknown, but may involve transfer of bacterial ligands and/or maternal immune mediators across the placenta. Recent animal data demonstrate that exposure to specific *Toll*-like receptor (TLR) ligands prior to induced polymicrobial sepsis significantly improves bacterial control and survival of neonatal mice [181]. Intra-amniotic injection of lipopolysaccharide in sheep produces chorioamnionitis and amplifies monocyte responsiveness to TLR agonists [276]. Therefore specific TLR agonists may be useful therapeutic tools to accelerate the maturation of neonatal immunity, once the underlying pathways and the potential risks are fully understood.

Activation of the fetal immune system by perinatal inflammation is increasingly being recognized and the effects of early life influences such as inflammation on the developing immune phenotype may be long-lasting [277]. High levels of cord blood pro-inflammatory cytokine levels are associated with a decreased risk of allergic disease in later childhood [278] and offspring of allergic mothers who developed neonatal sepsis after term delivery had a reduced incidence of allergic diseases compared to those without neonatal infection [273]. Other studies suggest that the presence of chorioamnionitis in preterm infants may be associated with increased risk of recurrent wheezing or asthma in childhood [279, 280]. In summary, these data suggest that perinatal inflammation has significant, GA- and context-dependent effects on the maturation of the immune system and clinical outcomes.

Despite the reduced risk of LOS and CLD in infants exposed to perinatal inflammation, there are also adverse associations with chorioamnionitis. For example, elevated cytokine levels in cord blood are associated with significantly increased risk of developing cerebral palsy [255, 256, 258, 259, 281]. Moreover,

we have previously described dysfunctional immune responses and aberrant regulation of inflammation in term and preterm infants [282, 283]. The complex effects of perinatal inflammation, which complicates the majority of extreme preterm deliveries, on both post-natal immune function and clinically relevant outcomes clearly warrant further investigation

We acknowledge that our study has some limitations, including the availability of placental histopathology in 75% of all babies, which could theoretically bias the findings. The sample size of our study did not allow us to analyze the effect of HCA on long-term neurological outcomes, such as intracranial hemorrhage or PVL. The strengths of our study include the relatively large sample size for a single center study, yielding clear results. This retrospective cohort from the post-surfactant era was almost universally exposed to antenatal steroids, standard management for threatened preterm delivery. The epidemiologic characteristics of our cohort and the incidence of HCA are similar to those previously reported [38, 254] and our findings are likely to be broadly relevant. Confirmation in an independent cohort is required.

In conclusion, this single-center, retrospective cohort study demonstrates that HCA is associated with significantly reduced risk of LOS in high-risk preterm infants. Understanding the mechanisms by which perinatal inflammation affects post-natal immune function is important in improving the management of infection and other adverse outcomes in extremely preterm infants.

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Chapter 5

Reduced levels of antimicrobial proteins and peptides in human cord blood plasma

5.1 Introduction

Neonates, particularly those born prematurely, are uniquely susceptible to invasive bacterial infections. The neonatal immunity relies predominantly on innate responses, including antimicrobial proteins and peptides, but cord blood plasma concentrations of antimicrobial proteins and peptides (APP) have not been investigated.

5.2 Patients and methods

The study was approved by the King Edward Memorial Hospital ethics committee. Cord blood samples, obtained by venepuncture of placental surface vessels and maternal samples by peripheral venepuncture at delivery, following written informed consent, were collected into heparinised tubes, processed within two hours and plasma was snap-frozen and stored at -80^{o}C. APP concentrations were determined by enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturers' recommendations (Hycult biotechnology, Uden, The Netherlands and Cayman Chemical, Ann Arbor, MI, USA). Comparisons between groups were by Mann-Whitney U test (2 groups)

or Kruskal-Wallis test with Dunn's post-test (>2 groups). P-levels of <0.05 were considered significant.

5.3 Results

ELISA were successfully performed for each APP on the number of samples shown (Table 5.1), and gestational age (GA)-dependent deficiencies in plasma concentrations were noted for each APP. BPI levels were significantly lower in the preterm infants of 30-33 wk GA compared to maternal levels, $p<0.05$. Calprotectin levels in cord plasma of all neonatal groups were significantly decreased compared to maternal levels, $p<0.001$. Cord plasma LL37 levels were significantly lower in the <30 wk and 30-33 wk group than in term infants and mothers, $p<0.001$ for both <30 wk and 30-33 wk vs. mothers and $p<0.01$ for both <30 wk and 30-33 wk vs. term neonates. $sPLA_2$ levels in cord plasma were significantly reduced in the very preterm group compared to maternal levels, $p<0.05$, whereas the levels in the 30-33 wk and 37-41 wk groups did not differ significantly from maternal levels. HNP 1-3 levels were at comparable levels in the neonatal groups but significantly higher in maternal plasma, $p<0.001$.

There was no correlation between neonatal APP levels and manual white cell or neutrophils counts. We found increased levels of bactericidal/permeability increasing protein (BPI) and calprotectin in peripheral blood of mothers with histological chorioamnionitis (n=15) compared to those without chorioamnionitis (n=85) (BPI: median 23.2 pg/ml (range 3.9-67.3) vs. 10.5 (0.1-90.6) and calprotectin: median 411.3 pg/ml (225.9-717.7) vs. 238.4 (97-1091)), $p=0.0017$ and $p=0.013$, respectively.) Neonatal plasma APP concentrations did not significantly differ with placental histological chorioamnionitis or with mode of

delivery (vaginal delivery (n=39) or caesarean section (n=71)). A weak correlation between maternal and neonatal APP levels was found only for human neutrophil defensins (HNP1-3) (r^2=0.19; p=0.018).

Table 5.1 Levels of antimicrobial proteins and peptides according to GA.

GA, gestational age; BW, birth weight; BPI, bactericidal/permeability inducing protein; sPLA2, secretory Phospholipase A2; HNP, human neutrophil defensins.

	<30 wk	**30-33 wk**	**37-42 wk**	**mothers**
GA	28 wk	31 wk	38 wk	N/A
	(23.2-29.0)	(30.0-33.3)	(37.0-42.1)	
BW	930 g	1630 g	3315 g	N/A
	(585-1655)	(1095-3350)	(2100-4945)	
gender	5 female,	14 female,	40 male,	N/A
	6 male	16 male	27 female	
BPI	n=11	n=30	n=67	n=100
(ng/ml)	5.3	7.3	10.1	12.7
	(0.1-64.6)	(0.1-46.2)	(0.1-95)	(0.1-90.6)
Calprotectin	n=11	n=30	n=67	n=100
(ng/ml)	116.9	90.7	134.6	336.9
	(23.8-634.3)	(0.7-514.1)	(23.8-563.4)	(97-1091)
LL37	n=11	n=30	n=67	n=100
(ng/ml)	13.8	16.7	29.7	35.6
	(3.7-41.1)	(0.1-43.3)	(8-79)	(8.5-130.3)
sPLA2	n=11	n=22	n=27	n=13
(pg/ml)	1402	2247	2760	4098
	(468.4-2651)	(502.3-7213)	(1103-6941)	(904.8-8334)
HNP1-3	n=7	n=22	n=67	n=33
(pg/ml)	4173	4011	4641	7466
	(2178-8404)	(1778-17807)	(1324-13956)	(4575-23617)

5.4 Discussion

This study demonstrates broad, GA-dependent deficiencies of APP in human cord plasma. BPI has several anti-infective properties and reduced levels of BPI in cord plasma are consistent with the cellular BPI deficiency and impaired release of BPI of neonatal neutrophils [179, 284]. Levels of calprotectin, an abundant constituent of leukocyte cytosolic protein with significant antimicrobial activity, were lower in all groups of neonates compared to maternal plasma. LL37 has broad antimicrobial activity and is found in vernix caseosa, skin and airway lining fluid of neonates [125, 127]. Levels of LL37 were reduced in preterm infants, whereas the levels in term neonates were comparable to maternal plasma content. Levels of HNP1-3 are lower in all neonates than in maternal plasma. Reduced levels of the acute-phase reactant secreted phospholipase A2 ($sPLA_2$), a potent microbicidal against Gram-positive cocci, were detected in the most preterm infants, whereas the levels in moderately preterm and term infants were similar to maternal levels.

One limitation of the study is the possibility that maternal peripheral blood plasma APP levels are generally increased by pregnancy and/or the trauma of birth. However, significant GA-related differences between cord and maternal APP levels remained when pregnancies with chorioamnionitis were excluded, suggesting that GA is a major determinant of plasma APP concentrations.

In conclusion, we report a broad deficiency of APP in human cord blood, particularly in very preterm neonates. The relationship of APP levels with the clinical risk of neonatal sepsis warrants further investigation. These data highlight novel therapeutic approaches, currently under biopharmaceutical development, to supplement these deficient host defence factors.

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Conflicts of interest: none to declare.

Chapter 6

Human monocyte responsiveness to the commensal bacterium *Staphylococcus epidermidis* develops late in gestation

6.1 Abstract

Background: The skin commensal *Staphylococcus epidermidis* (SE), which colonizes newborn infants in the first days of life, rarely causes infection in those born at term but is a leading cause of late-onset sepsis in preterm infants. We therefore investigated gestational age (GA)-dependent differences in innate immune responses to SE in preterm and term infants, and in healthy adults.

Methods: Cord and peripheral blood mononuclear cells (MNC) were stimulated with SE. Innate immune responses were assessed, including transcriptional activation of the *Toll*-like receptor (TLR) pathway by quantitative RT-PCR array. Cytokine production and expression of TLR2 and TLR4 were analysed at mRNA and protein level. Phagocytosis and intracellular cytokine induction by pHrodo-labelled SE as well as activation of kinases were determined by flow cytometry (FACS).

Results: Phagocytosis and intracellular killing of SE were similar in neonatal and adult monocytes. Cytokine gene expression and protein synthesis increased in a GA-dependent manner, which was confirmed at the single cell level. These GA-related effects were not associated to differences in expression of TLR2 or TLR4 or down-stream activation of NF-kB or MAPK pathways.

Conclusions: In conclusion, expression of *Toll*-like receptors (TLR), phagocytic capacity and intracellular killing by monocytes develops early in foetal development, whereas the ability to mount a bacterial-induced cytokine response requires full development. The functional immaturity of monocyte activation pathways in the preterm infant may underpin their exquisite susceptibility to sepsis with ubiquitous commensal bacteria like SE.

6.2 Introduction

Staphylococcus epidermidis (SE), an ubiquitous commensal of the skin and mucous membranes, has become the most commonly isolated pathogen in late-onset neonatal septicaemia (LOS, onset at >72 hours of age), accounting for approximately half of all LOS [93, 285]. Preterm infants, particularly those born at less than 30 weeks gestational age (GA), are uniquely susceptible to SE infection and the GA-dependent incidence in this population varies between 10-60%, making it several times more common than early-onset sepsis (EOS; <72 hours of age) [200]. Although the mortality from SE-infections is lower (0.3-10%) than that resulting from infection with less common neonatal pathogens, particularly Gram-negative organisms and fungi [80, 87, 286, 287], SE-septicaemia is associated with considerable morbidity, including increased risk for common adverse neonatal outcomes, prolongation of hospital stay and significantly increased health care costs [101, 102, 112, 113, 272]. The immunologic mechanisms underlying the heightened susceptibility of preterm infants to invasive SE infections remain incompletely characterised, but are central to identifying those infants at greatest risk and developing effective preventative and therapeutic interventions.

Preterm infants are believed to have deficient innate immune function, including impaired bacterial phagocytosis, intracellular killing and cytokine production associated with low levels of placentally transmitted immunoglobulins, and reduced serum complement activity. However, most data are derived from studies using pathogens that predominantly result in EOS, particularly group B streptococci, or purified agonists, such as lipopolysaccharide (LPS) [77, 288, 289, 290, 291]. The interaction of the commensal SE, the most common

neonatal pathogen, with the developing innate immune system and the resulting host responses have received comparatively less attention, despite the fact that early exposure to this organism is almost universal [109]. Extremely premature infants represent a growing population with unique, prolonged, maturation-dependent deficiencies of innate immune responsiveness that may be a useful tool to study impairments in commensal recognition. The interactions of SE with monocytes, may be particularly important as these cells represent the major cytokine-producing cells in the human bloodstream, and therefore are not only central to the early innate immune responses but also provide the essential link to adaptive immunity. One recent study suggests an impaired ability of monocytes from preterm infants to produce pro-inflammatory cytokines in response to stimulation with SE and LPS [166].

In this report, we characterised the *in vitro* innate immune responses of monocytes derived from preterm infants to SE. We show that preterm infant have normal expression of *Toll*-like receptors (TLRs), phagocytic capacity and intracellular killing, but reduced numbers of cytokine-producing monocytes after uptake of SE, resulting in broadly impaired cytokine production at both the transcriptional and translational level, a defect that may contribute to the high susceptibility of preterm newborn to SE infection.

6.3 Methods

6.3.1 Blood sampling and preparation

The King Edward Memorial Hospital ethics committee approved the study protocol and written informed consent was obtained at study entry. Clinical and demographic data were collected on all mother-infant pairs. The histology of the extra-placental membranes, umbilical cord, the chorionic plate and placenta were reviewed and histological scoring for chorioamnionitis was performed using a validated scoring system [274]. For all experiments, only MNC from infants without histological chorioamnionitis were used.

Cord blood was collected immediately following delivery into pre-heparinised syringes from both the arteries and veins of the cord and placental surface. To minimise possible contamination with maternal blood, the umbilical cord was cleaned with an alcohol swab prior to collection. Newborn infants were divided into three groups corresponding to GA that relate to differential susceptibility to bacterial infection [79]: (i) very preterm infants, <30 weeks gestational age (GA), (ii) moderately preterm infants, 31-33 weeks GA and (iii) term, 37-41 weeks GA. The total white cell and monocyte counts for the GA groups were similar on manual differential (data not shown). Control blood samples were collected from healthy non-pregnant adults by peripheral venepuncture.

Blood samples were mixed 1:1 with heparinised RPMI 1640 culture medium (Gibco, Life Technology, Paisley, Scotland). Mononuclear cells (MNC) were isolated by lymphoprep gradient centrifugation (Nycomed Pharmacia, Norway) and cryopreserved using an established method that does not significantly affect cell viability or function, as previously reported [292]. Briefly, following

centrifugation, MNC were washed once in RPMI (500 g, 10min) and then twice in RPMI/2% fetal calf serum (FCS, MultiSer Biosciences, Australia). Mononuclear cells were then resuspended in RPMI/2% FCS and manually counted. Ice-cold 15% dimethyl-sulphoxide (DMSO; BDH Chemicals, Fairy Port, Victoria, Australia) freezing solution in FCS was added drop-wise. The samples were cooled to minus 80°C at a rate of 1°C per minute in a freezing container (Nalgene, Rochester, NY) containing isopropyl alcohol (BDH Chemicals). Samples were transferred to liquid nitrogen for long-term storage. For all experiments, equal numbers from all age groups were batch analysed to reduce experimental variation.

6.3.2 Bacterial culture

S. epidermidis strain 1457 (an invasive clinical isolate, kindly provided by Dr Michael Otto, National Institute of Allergy and Infectious Diseases, Hamilton, MT) was grown to mid-log phase in brain heart infusion broth (PathWest, Perth, WA, Australia). Bacteria were heat-inactivated for 15 min. at 80°C, then washed and resuspended in sterile phosphate-buffered saline (PBS) at a bacterial concentration of $2.5x10^9$ colony-forming units (CFU)/ml, and aliquots stored at -20°C. Lack of viability was confirmed by plating serial dilutions of the inactivated bacterial suspensions onto blood agar plates. LPS contamination of SE preparations was excluded by limulus amoebocyte lysate assay (Associates of Cape Cod Inc., East Falmouth, MA) with a limit of detection of <0.01 ng/ml LPS and by lack of response by TLR4-transfected HEK293 cells (Invivogen, San Diego, CA) (data not shown).

6.3.3 Mononuclear cell cultures

Vials of cryopreserved MNC were thawed rapidly at 37^{o}C, resuspended in RPMI 1640, centrifuged, and cells resuspended to a total volume of 1ml in AIM-V (Gibco) supplemented with 2-mercaptoethanol (2-ME, Sigma, Castle Hill, Australia), as previously described [293]. Experimental conditions were optimised for bacterial dose and optimal time of cytokine output using MNC from all age groups. Mononuclear cells were counted and cultured at 10^6 live cells/ml in RPMI 1640 with 10% FCS in polypropylene round-bottom 96-well plates (Corning Inc., Corning, NY). SE were added at indicated final concentrations and the cell cultures were incubated at 37°C/5% CO_2. Culture supernatants were harvested and stored at -80°C until batch analysis.

6.3.4 TLR-pathway array

Cell pellets from cell cultures (as described above) stimulated with SE at 10^8/ml for 6 hours were resuspended and stored in RNAprotect Cell Reagent (Qiagen, Doncaster, Vic, Australia) at -80°C until RNA isolation. Cellular lysates were prepared using QIAshreddar spin column (Qiagen) and total RNA was isolated using the RNeasy kit (Qiagen). 50 ng of RNA per sample were then transcribed into cDNA using the RT-PCR array first strand Kit (SA Biosciences) and analysed on the human TLR-pathway array (SA Biosciences, Frederick, MD), using the real-time SYBR green/fluorescein PCR master mix (SA Biosciences). RT-PCR was performed on an ABI Prism 7000 (ABI, Foster City, CA) in accordance with the manufacturer's instructions. Gene expression was normalised using multiple housekeeping genes and data analysed using the

$\Delta\Delta C_t$ method [294]. Fold change in gene expression was calculated between unstimulated and SE-stimulated cells.

6.3.5 Cytokine Assays

Cytokines were measured by in-house time resolved fluorometry assay (TRF; for IL6, IL10, TNFα) or commercial enzyme-linked immunoassay (for IL1β, IL8 purchased from eBioscience, San Diego, CA) in accordance with established protocols or manufacturer's instructions. The TRF technique uses the same principle as the 'sandwich' ELISA technique, with a greater dynamic range [293]. Anti-cytokine monoclonal capture/detection antibody pairs were obtained from PharMingen (BD Biosciences, North Ryde, NSW, Australia) and TRF assays were performed on Nunc maxisorp flat-bottom plates (Roskilde, Denmark). Standard curves were generated by serially diluting recombinant cytokines purchased from PharMingen (BD Biosciences). Following incubation with biotinylated detection antibodies, Europium-labelled streptavidin (Delfia Wallac, Turku, Finland) was added, followed by enhancement solution (Delfia Wallac) and plates were kept in the dark at room temperature until analysed on a Wallac 1420 Victor^2 (PerkinElmer, Waltham, MA).

6.3.6 FACS analysis of surface expression of TLR2 and 4, phagocytosis, and intracellular cytokine staining

MNC were used directly after thawing (for basal expression of TLRs) or cultured for analysis of phagocytic capacity and cytokine induction, as above. For each experiment, we analysed samples from each GA group to minimise the effect of

day-to-day variation on inter-group differences. For the TLR staining, MNC were washed in FACS buffer (1x PBS, 2% FCS, 2% bovine serum albumin (BSA), 0.01% NaN_3) and stained with PE- and APC-labelled antibodies against TLR2 and TLR4 (eBioscience), respectively, or matched isotype controls for 30 min. at 4°C in the dark followed by a final wash and fixation in 1x BD stabilizing fixative solution (BD Biosciences).

6.3.7 pHrodo labelling of SE

For phagocytosis and intracellular cytokine experiments, SE were first labelled with the pH-sensitive fluorescent dye, pHrodo™ (Invitrogen). Bacteria labelled in this way are non-fluorescent in extracellular form but fluoresce brightly in the phagosome at pH <4.0. Heat-killed SE ($2.5x10^9$ per reaction) were incubated for 1 h with 0.5 mM of pHrodo™ succinimidyl ester and extensively washed to remove unbound dye, according to the manufacturer's instructions. pHrodo-labelled SE were thoroughly dispersed before use by rapid vortexing for 30 sec followed by 5 min in an ultrasonic water bath.

For the phagocytosis assay MNC were cultured (as described above) for 1 hour in the presence of pHrodo-labelled SE (100 bacteria/MNC) at 37^oC. After harvest, cells were washed in FACS buffer and permeabilised using 1x BD permeabilisation buffer (BD Biosciences). Cells were incubated FACS tube and non–adherent bacteria removed by washing twice with 2 ml of ice-cold PBS (500 x *g*, 5 min), before fixation in 2% (v/v) formalin solution (in PBS) for subsequent flow cytometry.

For the intracellular cytokine assay, MNC were cultured with pHrodo-labelled SE as for the phagocytosis assay for the first hour. After this, cells were incubated for a further 4 h at 37^{o}C in the presence of 6 µg/ml of Brefeldin A (eBioscience, San Diego, CA). Cells were then transferred to a round-bottom polystyrene 96-well plate and washed twice with ice-cold PBS (500 x *g*, 5 min) followed by immediate fixation using BD FACS-lyse solution (BD Biosciences) according to the manufacturers instruction. Cells were then permeabilised using 1x BD *Perm2* permeabilisation buffer for 10 min (BD Biosciences) and then washed and resuspended in FACS buffer. Cells were then incubated with FITC- or APC-labelled antibodies against TNF-α or IL-6 (eBioscience) or appropriate isotype controls for 30 min at 4°*C* in the dark, before a final two washes and fixation in 1x BD stabilizing fixative solution (BD Biosciences).

To normalise for the potential effect of opsonisation on phagocytosis, especially as preterm infants are often hypocomplementaemic, which may affect bacterial uptake [235], we performed the phagocytosis, intracellular killing and intracellular cytokine assays in the presence and absence of 10% baby rabbit complement.

For all assays, a minimum of 5000 monocytes, identified by their FSC/SSC characteristics, were acquired uncompensated using a FACSCalibur (BD Biosciences) flow cytometer. BD CompBeads were used for all antibodies at the time of acquisition to allow *post hoc* compensation and data was analysed with FlowJo software (Treestar). For the TLR expression analysis, the median fluorescence intensity (MFI) of the isotype was subtracted from the MFI after staining with the specific TLR-antibodies. For the phagocytosis assay, the proportion of monocytes positive for SE ingestion was determined by setting a

threshold marker of 1.5% based on the red fluorescence properties of unstimulated monocytes. The degree of ingestion was determined from the MFI of the positive monocyte population. For the intracellular cytokine staining, positively stained cells were identified by first setting a threshold marker of 1.5% for each fluorophore using the fluorescence properties of isotype labelled cells.

6.3.8 Cell signalling studies

Monocytes were isolated from whole MNC using negative magnetic selection with a human monocyte enrichment kit without CD16 depletion as per the manufacturer's protocol (EasySep, Melbourne, Victoria, Australia). Purified monocytes (purity routinely >85%) were incubated with the following stimuli; pHrodo labelled SE ($4x10^7$ CFU/ml) with 10% baby rabbit complement (PelFreeze, Rogers, AR, USA), 10 ng/ml LPS (Alexis, Lausanne, Switzerland) or 100 ng/ml FSL-1 (Invivogen, San Diego, CA, USA). Cells were harvested by transfer into 2 ml ice-cold PBS at the following time points: 15 min, 30 min, 60 min, 90min, 120 min and 150 min. Unstimulated cells and those stimulated with LPS or FSL-1 were incubated only for 30 min. Upon transfer, cells were centrifuged and washed once more with ice cold PBS. Cells were then fixed in 1.5% (v/v) formaldehyde in PBS for 10 min at room temperature before permeabilisation and further fixation with methanol for 10 min at 4°C. Cells were washed twice with FACS buffer before staining with NFκβ PE, P38 Alexa488 (both BD Biosciences) and JNK Alexa 647 (Cell Signalling Technologies, Danvers, MA, USA) or matched isotype controls and analysed on a FACSCalibur flow cytometer.

6.3.9 Intracellular killing assay

MNC were cultured as described above and inoculated with 10^6 CFU/ml of LSE for 30 min. in the presence or absence of rabbit complement (RbC, 10%, Sigma) or Cytochalasin D (10 µg/ml, Sigma). Cells were washed twice with ice-cold PBS (500 x *g*, 5 min) to remove non-internalised bacteria and incubated for a further 2 hours, after which cells were lysed by resuspension for 5 min in sterile water with 1% (w/v) ethylenglycolate deoxycholate (Fluka AG, Buchs, Switzerland). The lysates were serially diluted, plated onto blood agar plates (PathWest), and incubated at 37°C/5% CO_2. The CFU determined after 2 days by manual counting.

6.3.10 Statistical analysis

Unless otherwise stated in the figure legend, comparisons between groups were performed using the Kruskal-Wallis test with Dunn's post-test using Prism 5 for Mac (GraphPad, La Jolla, CA). For analysis of correlation, the Spearman r-test was used. Differences were considered significant at p-level <0.05.

6.4 Results

6.4.1 SE-induced TLR pathway gene expression is gestational age-dependent.

We characterised the expression of neonatal and adult TLR-pathway related genes in response to *in vitro* stimulation of MNC with heat-killed SE (Figure 6.1, A-D). Direct comparison of gene expression patterns between the groups demonstrated that both the number of upregulated genes and the degree of upregulation were significantly lower in the most premature infants compared to moderately preterm infants, term infants, and adults.

6.4.2 GA-dependent impairment of cytokine production in response to SE

We selected key cytokines from the RT-PCR array for further comparative analysis of the responses to SE and consistently found the lowest expression levels in the most preterm infants (Figure 6.2) (n=5 per group; $p<0.01$ for <30 weeks versus adults for IL1β, IL6, IL8, IL10 and TNFα).

Furthermore, there was a significant increase in gene expression levels with higher GA ($p=0.01$, $r^2=0.41$ for IL1β, $p=0.005$, $r^2=0.47$ for IL6, $p=0.007$, $r^2=0.44$ for IL8, $p=0.014$, $r^2=0.38$ for IL10 and $p=0.04$, $r^2=0.28$ for TNFα).

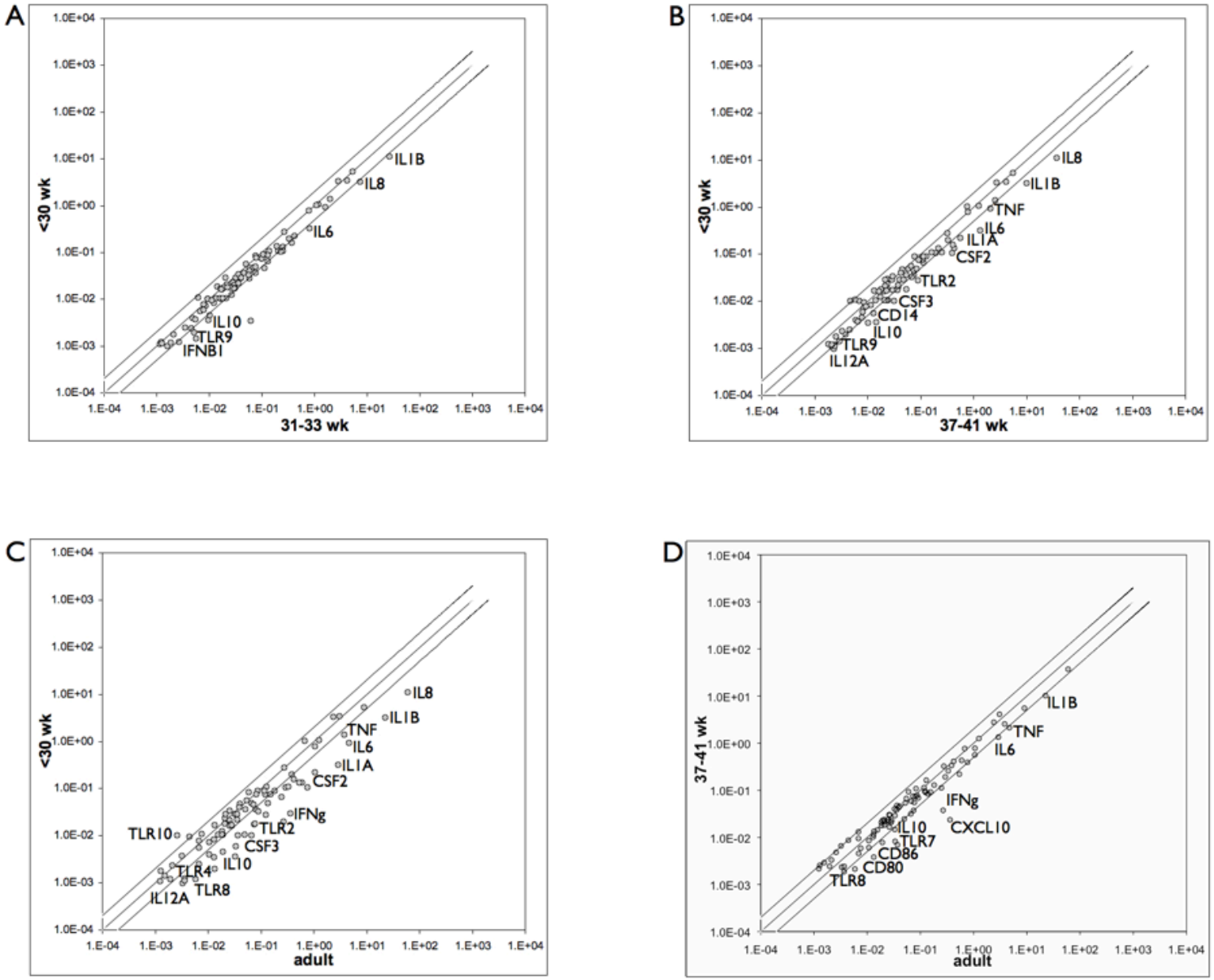

Figure 6.1 TLR-pathway associated gene expression in response to SE. CBMC and PBMC (10^6/ml) of preterm and term infants and adults, respectively, were stimulated with HKSE 10^8/ml for 6 hours. RNA was isolated, transcribed into cDNA and analysed on a human RT-PCR TLR pathway array (SuperArray). Panels (A-C) show log transformation plots of the gene expression levels of each gene between preterm, term and adult SE-stimulated CBMC/PBMC (n=5 in each group). The central lines indicate equivalence and the outer lines indicate a two-fold difference in gene expression.

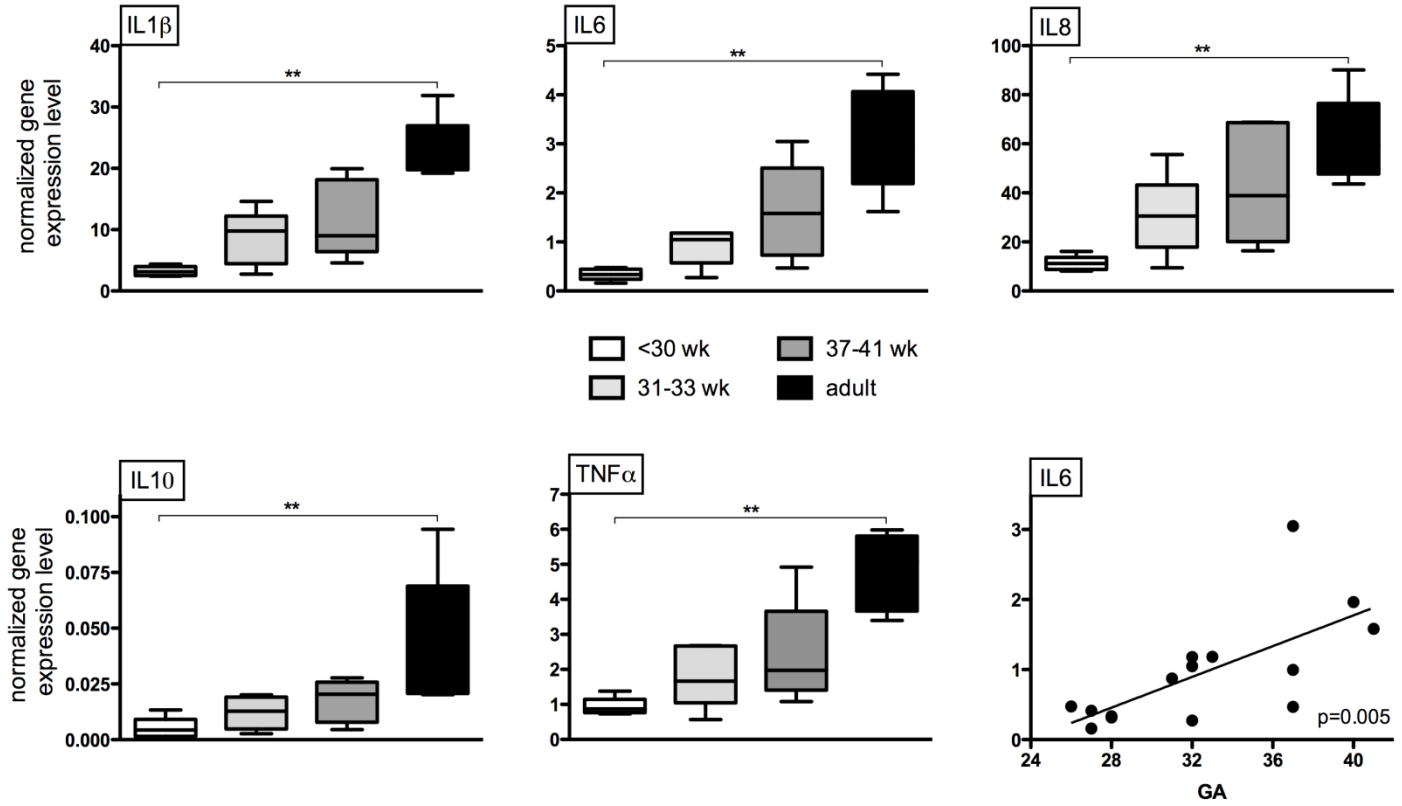

Figure 6.2 Cytokine gene expression in response to SE.

Neonatal CBMC and adult PBMC were stimulated with HKSE 10^8/ml for 6 hours. RNA was isolated, transcribed into cDNA and analysed on a human RT-PCR TLR-pathway array (SuperArray) (n=5 in each group). Graphs show mean ±SEM, **p<0.01. The last panel depicts the gene expression level of IL6 in relation to gestational age and the p-value of linear regression analysis.

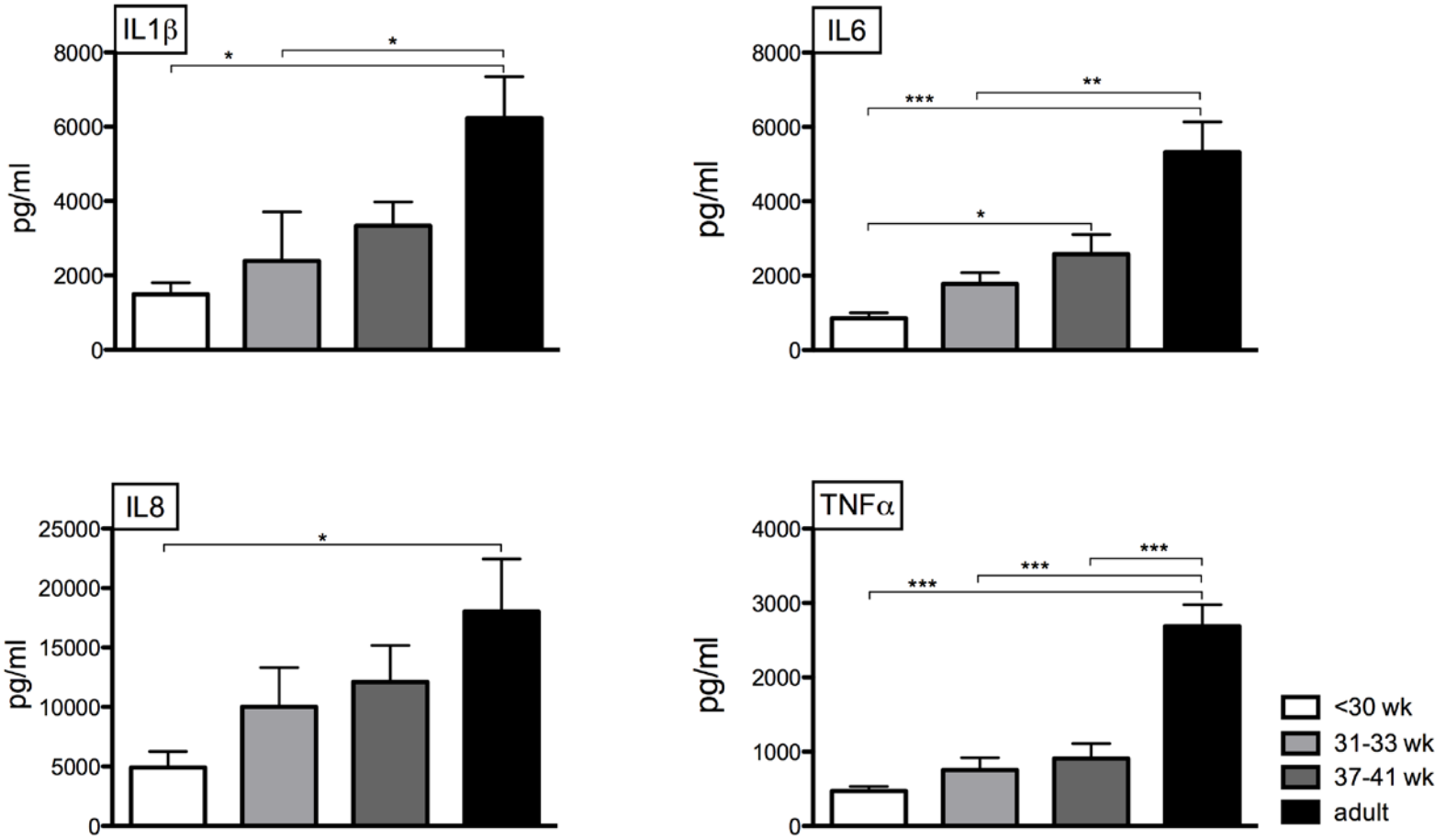

Figure 6.3 Neonatal MNCs demonstrate impaired HKSE-induced cytokine protein expression.

CBMC of preterm and term infants and adult PBMC were incubated with HKSE (10^8/ml) for 6 hours. Supernatants were harvested and cytokine levels measured by TRF (IL6, TNFα; n=14-20 per group) or ELISA (IL1β, IL8; n=4-6 per group). Graph show mean ±SEM. *p<0.05, **p<0.01, ***p<0.001.

To assess whether the differences noted in cytokine mRNA responses were also manifest at the protein level, we determined the cytokine protein concentrations in MNC culture supernatants. Concentrations of SE-induced IL1β, IL6, IL8 and TNFα secreted by the most preterm infants' MNC were significantly reduced compared to adults ($p<0.05$, $p<0.001$, $p<0.05$ and $p<0.001$, respectively) (Figure 6.3). For the moderately preterm infants, significantly lower cytokine protein levels were determined for IL1β ($p<0.05$), IL6 (<0.01) and TNFα ($p<0.001$). With the exception of reduced TNFα production ($p<0.001$), cytokine secretion of term infants was not significantly different from adults. Linear regression analysis revealed significant increases in cytokine responses with GA for IL6 ($n=45$, $r^2=0.25$, $p=0.005$), TNFα ($n=37$, $r^2=0.19$, $p=0.007$), and IL8 ($n=15$, $r^2=0.26$, $p=0.05$), and with a similar trend for IL1β ($n=15$, $r^2=0.22$, $p=0.08$).

6.4.3 Activation of monocytes by SE is GA-dependent and requires phagocytosis.

We next examined the SE-specific cytokine response at the cellular level to identify the source of cytokines and to assess whether reduced cytokine gene expression was due to a decreased number of cytokine-producing cells and/or reduced expression of cytokine per cell. We did this using a method recently developed by our laboratory that allows for reliable simultaneous detection of intraphagolysosomal bacteria and intracellular cytokine content [295]. Flow cytometry revealed that for all groups tested only monocytes positive for phagocytosed SE produced IL6 and TNFα, suggesting that phagocytosis was

absolutely required for cytokine induction. However, not all phagocytic cells produced cytokines (Figure 6.4A and 6.4B).

Both the total number of cytokine-producing monocytes and the cytokine production per cell were significantly lower in the most preterm infants compared to adults with an overall GA-dependent cytokine response (Figure 6.4C and 6.4D; IL6: n=14, r^2=0.32, p<0.05; TNFα: n=15, r^2=0.38, p<0.05).

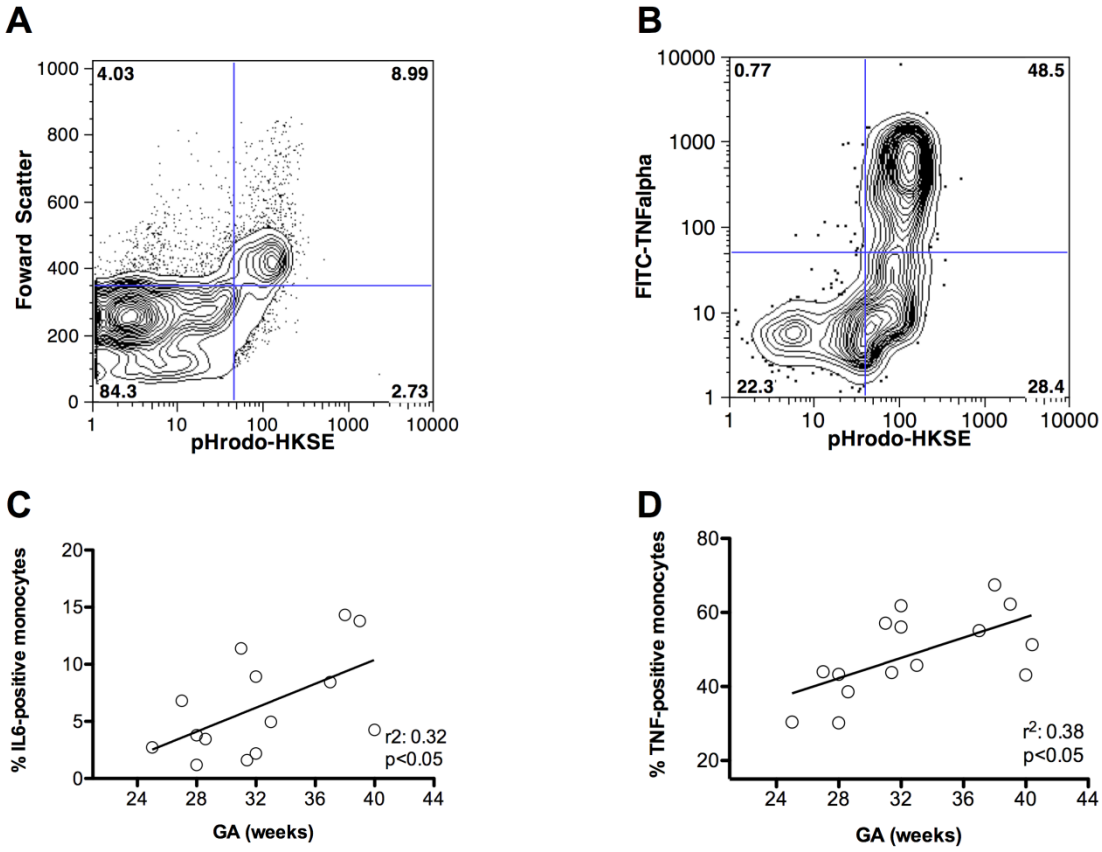

Figure 6.4 Intracellular cytokine production induced by HKSE.

Neonatal PBMC and adult PBMC were incubated with HKSE at 10^8/ml, with or without 10% rabbit complement (RbC), for 4 hours in the presence of Brefeldin A (6 µg/ml) and stained for IL6 and TNFα. Monocytes were identified by their FSC/SSC characteristics. FACS plot (A) shows the % of monocytes that phagocytosed pHrodo-labelled SE and FACS plot (B) shows the % of pHrodo-positive monocytes that produce TNFα, respectively. Panels (C) and (D) depict the % of IL6 and TNFα-positive monocytes in relation to gestational age, respectively. Graphs show mean ±SEM.

6.4.4 Monocyte phagocytic and intracellular killing capacity is GA-independent

To determine if the differences in cytokine production observed were due simply to lower levels of bacterial uptake by preterm infant monocytes, we next examined the kinetics of non-opsonic and opsonic phagocytosis. Remarkably, all groups showed similar kinetics and phagocytic indices (Figure 6.5A). Further, opsonization of SE with rabbit serum significantly and equivalently increased the uptake of SE by monocytes across all age groups (Figure 6.5B).

As neonatal monocytes take up SE equivalently to adult cells, we investigated the ability of neonatal monocytes to control *in vitro* infection with live SE. Employing a recently described method for assessment of intracellular killing capacity [296], we observed similar killing of bacteria between the GA groups with less than 0.01% of the original number of SE viable after 1 hour of incubation with MNC, indicating normal killing capacity of neonatal monocytes (Figure 6.6).

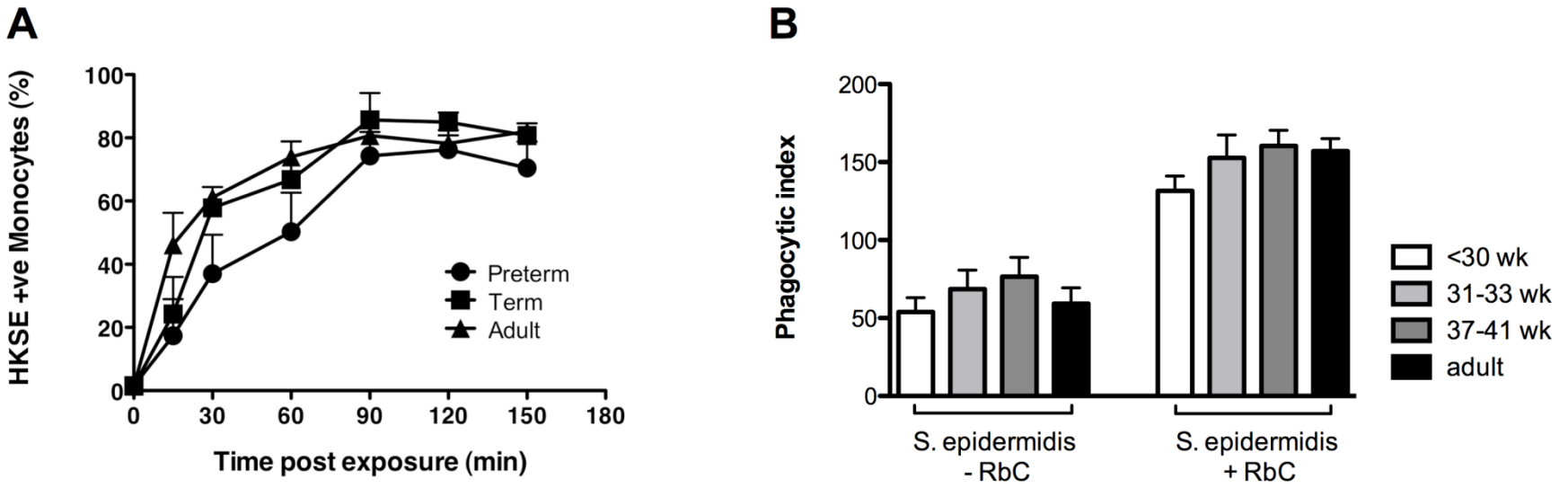

Figure 6.5 Equivalent phagocytosis of heat-killed S. epidermidis (HKSE) by neonatal and adult mononuclear cells.

Neonatal CBMC and adult PBMC were incubated with pHrodo-labelled HKSE at 10^8/ml for 60 minutes in the presence or absence of 10% rabbit complement (RbC). Monocytes were identified by their FSC/SSC characteristics and analysed for uptake of pHrodo-labelled SE. (A) depicts kinetics of uptake of pHrodo-labelled SE in extremely preterm and term infants compared to adults and (B) shows the phagocytic index of monocytes of the groups. Graphs show mean ±SEM.

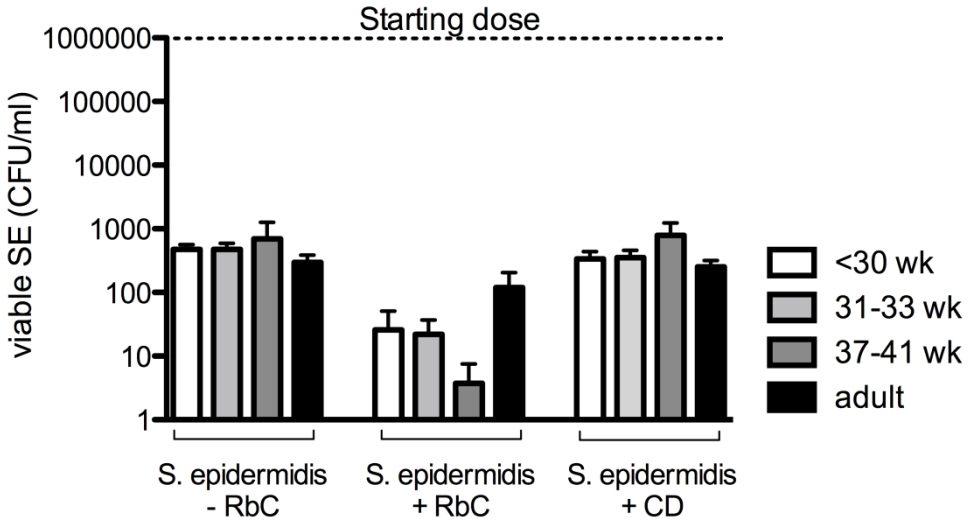

Figure 6.6 Intracellular killing capacity of neonatal whole blood.

Neonatal CBMC and adult PBMC (n=5 for each group) were inoculated with live SE at 10^6/ml for 30 min. in the presence or absence of 10% rabbit complement (RbC) and Cytochalasin D (CD, 10 µg/ml). Non-internalised bacteria were washed off and cells incubated for a further 120 min. Cells were then lysed and serial dilutions of the lysate plated onto blood agar plates. The logarithmic graph shows the mean number of viable SE/ml ±SEM in relation to the inoculated number of bacteria (starting dose).

6.4.5 Monocyte surface expression of TLRs

To investigate differential expression of innate immune receptors potentially involved for the recognition of SE, we analysed mRNA and protein expression of TLRs. RT-PCR analysis did not reveal significantly different gene expression of TLR2, 4 and 6 in preterm infants and term neonates (Figure 6.7 A-C).

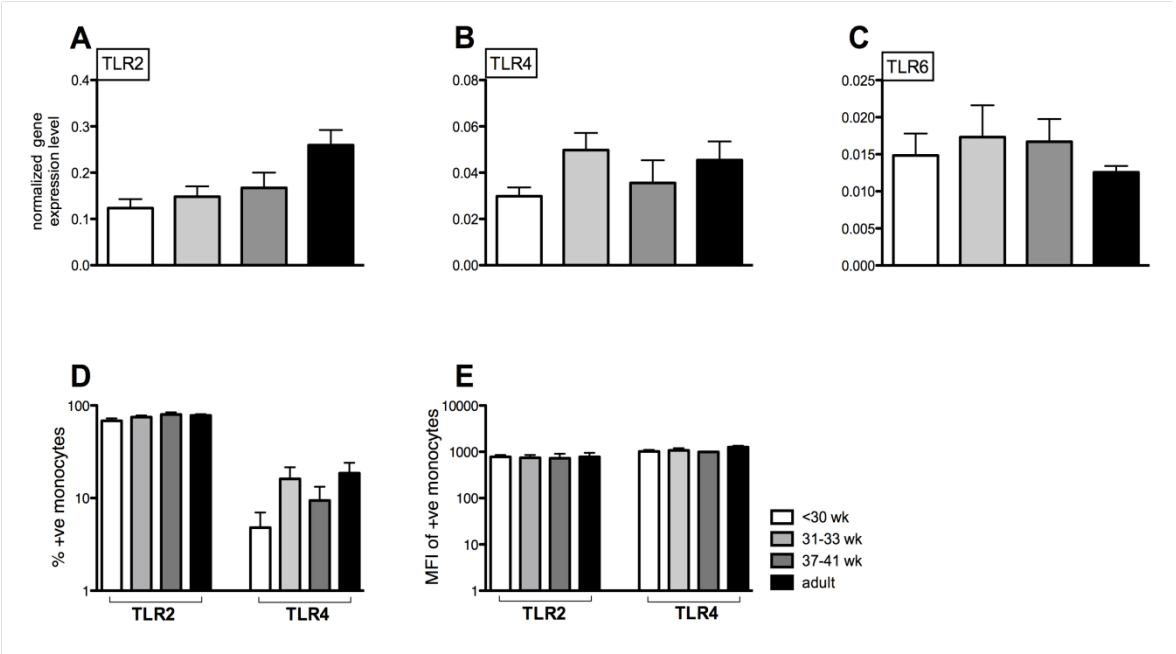

Figure 6.7 Expression of Toll-like receptors on neonatal leukocytes.

Neonatal cord blood and adult peripheral blood mononuclear cells (CBMC and PBMC) were analysed for monocyte TLR-expression. Panel D depicts the number of monocytes positive for TLR2 and TLR4. Panel 9E shows the specific median fluorescence intensity (MFI) of TLR-positive-monocytes (n=5 /group). Positive TLR expression was defined by a fluorescence signal greater than matching isotype control for each donor. Data are presented as mean ±SEM.

We next determined the expression of TLR2 and TLR4 protein on monocytes by flow cytometry (Figure 6.7D and 6.7E). No differences between the groups in either total numbers of TLR4-positive monocytes or median fluorescence intensity of TLR4-positive monocytes were observed. For TLR2, there was a non-significant trend towards increased numbers of TLR2-expressing monocytes with increasing GA; however, no differences were found for the MFI of TLR2-positive cells. We then examined the down-stream signalling events associated with TLR-dependent activation and found that the numbers of phospho-p65 positive, phospho-p38 positive and JNK positive monocytes after stimulation with SE were similar between the groups (Figure 6.8A-C).

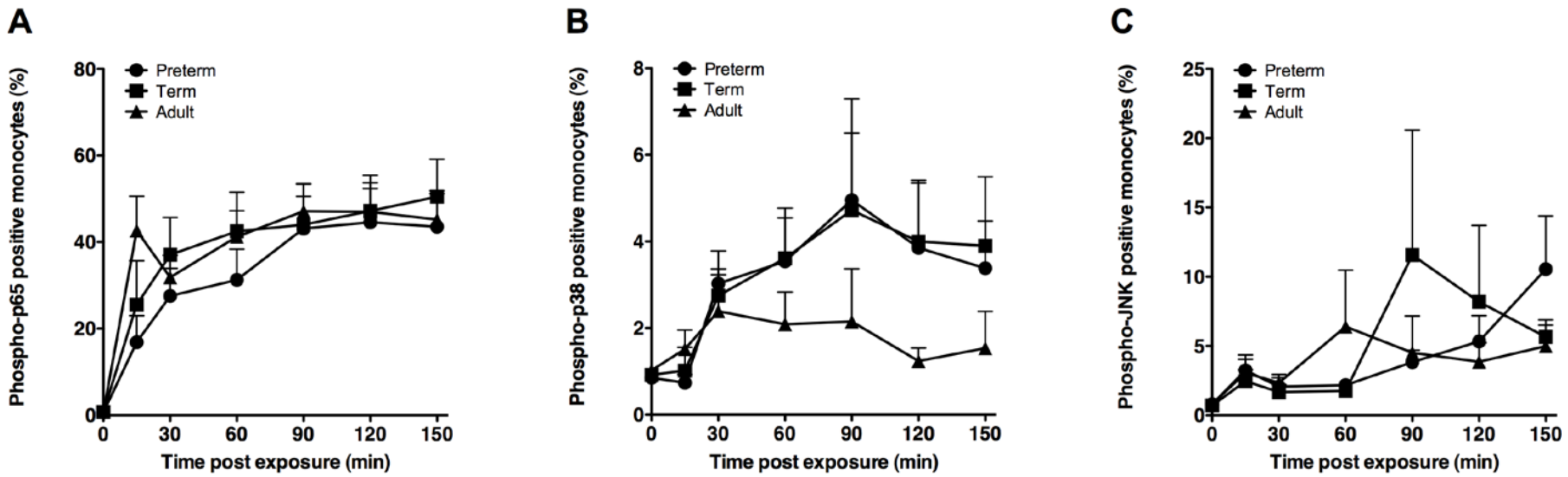

Figure 6.8 Cell signalling in response to stimulation with S. epidermidis.

Purified neonatal and adult monocytes were incubated with pHrodo-labelled SE at $4x10^7$/ml in the presence of 10% rabbit complement. Cells were harvested at 15, 30, 60, 90, 120 and 150 minutes, respectively, and stained for phospho p65, phospho p38 and JNK. Panels A-C depict the percentage of monocytes positive for p65, p38 and JNK according to time post-exposure. Data are presented as mean ±SEM.

6.5 Discussion

In characterizing *in vitro* innate immune responses of newborns to SE in relation to GA, we have demonstrated that even in extremely preterm infants, the presence of SE is reliably detected and phagocytosed, but that subsequent activation of innate immune responses are significantly impaired. These GA-related deficiencies are likely to contribute to the heightened susceptibility of extremely premature infants to SE infection.

Early response cytokines (IL1β, IL6, IL8, TNFα) are commonly associated with inflammation initiated by the innate immune system through activation of the potent transcription factor NF-kB [290]. In keeping with the limited data on SE-responses in preterm infants [166], our results indicate a significant GA-dependent defect in production of several cytokines after stimulation of neonatal MNC with SE. This was evident at both transcriptional and translation level, signifying a profound impairment in the ability of preterm infants to mount innate inflammatory responses to SE.

The clinical correlates of GA-related differences in innate responses to SE are increasingly recognised. Erythema toxicum neonatorum (ETN), a condition commonly manifesting with rash and low-grade temperature in term neonates [297, 298], is thought to result from an inflammatory innate immune response to SE that have penetrated into the dermis via hair shafts [206]. In addition to this, IL6 levels significantly increase during the first week of life in non-infected term neonates, possibly reflecting initial immune recognition of commensal bacteria, such as SE [170]. Although neonates are ubiquitously colonised with SE within days after birth [109], ETN is exceptionally rare in preterm infants [297, 298], suggesting significant GA-related differences in innate immune responses *in*

vivo. Furthermore, while term infants rarely develop invasive SE infection, the same organism represents the most common cause of late-onset septicaemia in preterm infants [80].

Engagement of phagocyte surface receptors, phagocytosis and intracellular bacterial killing are key elements of the proximal innate immune response. Neonatal monocyte phagocytosis and intracellular killing are comparable to adult MNC [167, 299, 300, 301, 302]. However, published data are derived from early-onset pathogens or latex particles and there are no analogous data on monocyte phagocytosis of SE in preterm infants. Our results are the first to demonstrate similar monocyte phagocytic capacity in preterm and mature neonates and adults, both in terms of the percentage of SE-phagocytosing monocytes and the number of ingested bacteria. Furthermore, we observed similar monocyte phagocytosis of the early-onset pathogen *Streptococcus agalactiae* [295]. Crucially, induction of TNF-α and IL6 by SE was confined solely to those monocytes that had ingested the bacteria, irrespective of GA. Furthermore, preterm infants' MNC are able to contain *in vitro* infection with live SE as effectively as term infants and adults.

As preterm infants commonly are hypocomplementaemic, we assessed the ability of their monocytes to phagocytose SE in the absence and presence of rabbit serum as a complement source. Interestingly, while opsonization resulted in increased efficiency of bacterial uptake in all age groups, it failed to normalise the deficient cytokine production of extremely preterm infants' monocytes. Furthermore, we did not detect different numbers of phospho-p65 positive monocytes in preterm infants compared to term infants and adults. Therefore, the markedly reduced cytokine levels in response to SE of preterm infants are

neither accounted for merely by insufficient uptake or intracellular killing of the bacteria, nor by reduced activation of the principle secondary phosphokinases. We therefore conclude that the reduced cytokine responses induced by phagocytosis in very preterm infants may lie in impairment downstream of NF-kB activation. Of note, we were able to detect some cytokine production in response to SE even in extremely premature infants, further indicating that preterm infants' MNC are able to engage and at least partially process SE. This is in keeping with the clinical observation of raised concentrations of C-reactive protein (CRP), an acute phase reactant transcriptionally controlled by IL6 and a clinical biomarker of inflammation, in SE sepsis in the majority of very premature infants [222, 303].

We next examined the neonatal MNC expression of TLR2 and TLR4, the predominant receptors for Gram-positive and Gram-negative bacteria, respectively [156], at both mRNA and cell surface protein level. We have previously shown that SE engages the innate immune system by TLR2-dependent and –independent signalling [304]. Monocytes of preterm infants showed abundant TLR2 expression at levels similar to those in term infants or adults. TLR4 was expressed on a smaller subset of monocytes in preterm infants, but expression was also equivalent across groups. Similar levels of mRNA and protein expression of TLR2 and TLR4 in term neonatal and adult cells where reported by Levy et al [159], whereas another study found TLR4 expression increased with GA [160]. We conclude that differences in TLR2 induced responsiveness to SE in preterm infants are unlikely to be due exclusively to relative levels of expression of this major receptor for Gram-positive bacteria.

In summary, our findings represent the most comprehensive assessment of innate immune responses to SE in infants to date. We demonstrate that monocytes from infants born early in gestation express TLR2 and 4 appropriately, and phagocytose and kill SE comparably to term and adult MNC. However, this is not reflected in mature cytokine responses, despite appropriate activation of phospho-p65. Delineation of the maturational steps necessary for completely engaging transcriptional activation of cytokine and other genes will be important in developing diagnostic, preventative and therapeutic strategies to reduce the infectious disease burden in highly susceptible populations such as preterm infants. In addition to *in vitro* experiments utilizing sophisticated techniques and isolated cells, it will be essential to investigate the complex innate immune responses to SE infection *in vivo*.

Acknowledgements

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Chapter 7

Staphylococcus epidermidis activates innate immunity via TLR2-independent and –dependent pathways

7.1 Abstract

Background: *Staphylococcus epidermidis* (SE) is a nosocomial pathogen that causes catheter-associated bacteraemia in the immunocompromised, including those at the extremes of age, motivating study of host clearance mechanisms. SE-derived soluble components engage TLR2; but additional signalling pathways have also been implicated, and TLR2 can play complex, at times detrimental, roles in host defence against other *Staphylococcal spp*. The role of TLR2 in responses of primary blood leukocytes to live SE and in clearance of SE bacteraemia, the most common clinical manifestation of SE infection, is unknown.

Methodology/Principal findings: We studied TLR2-mediated recognition of live clinical SE strain 1457 employing TLR2-transfected cells, neutralizing anti-TLR antibodies and TLR2-deficient mice. TLR2 mediated SE-induced cytokine production in human embryonic kidney cells, human whole blood and murine primary macrophages, in part via recognition of a soluble TLR2 agonist. After i.v. challenge with SE, early (1h) cytokine/chemokine production and subsequent clearance of bacteraemia (24-48h) were markedly impaired in TLR2-deficient mice.

Conclusions/Significance: TLR2 mediates recognition of live SE and clearance of SE bacteraemia *in vivo*.

7.2 Introduction

Staphylococci spp. are frequent causes of nosocomial infection [95, 305]. Coagulase-negative staphylococci, especially *S. epidermidis* (SE) are ubiquitous skin commensals and a major cause of nosocomial bacteraemia, particularly in immunocompromised patients and those with implantable medical devices [306]. The ability of *Staphylococcus spp.* to cause disease has been ascribed to immune evasion, including avoidance of opsonophagocytosis [219]. SE lacks genome islands found in *S. aureus* (SA) encoding pathogenesis factors, likely contributing to its lesser virulence [307]. Nevertheless, SE is a frequent cause of bacteraemia, especially at the extremes of age, resulting in significant morbidity and mortality [77]. SE attaches to foreign material, interacts with the host extracellular matrix and elaborates biofilm, reducing deposition of complement/IgG, susceptibility to host antimicrobial peptides and neutrophil-mediated killing [218, 306, 308].

Characterisation of the *Toll*-like receptor (TLR) system [309, 310, 311, 312] has paved the way for studies implicating TLR2 in recognition of Gram-positive bacteria [309, 310, 313]. However, additional recognition pathways of Gram-positive bacteria include ß-integrins [314, 315], lectins [316], CD36 [317], and nucleotide oligomerization domain proteins 1 and 2 (NOD1 and -2) [318], members of the NOD-like receptor (NLR) family of cytosolic sensors [319]. SA-derived factors activate NODs1 and -2 and the NLRP3 inflammasome [318, 320] and NOD2 contributes to cutaneous defence against live SA *in vivo* [321].

There is evidence both in favour and against a role for TLR2 in host defence against several Gram-positive bacteria, including SA [296, 318, 322, 323]. The complexity of the data may relate to distinct routes of infection in murine

models- e.g., i.p. versus i.v. [322, 324], differences in bacterial preparations (e.g., live vs. killed) and inoculum size, and differences in innate immunity between mice and humans [325]. Live SA subverts TLR2 to inhibit superoxide production by murine macrophages thereby prolonging survival of SA in phagosomes [296]. Thus the roles of TLR2 in innate responses to SA are context dependent, and may include detrimental roles in infection outcome.

SE also engages the innate immune system [77]. Killed SE preparations induce cytokine production from human peripheral blood mononuclear cells *in vitro* [166, 243, 326, 327], and fractions containing SE-derived phenol-soluble modulin peptides (PSMs) induce cytokine production in TLR2-transfected HEK cells and primary murine cells [328, 329, 330, 331]. SE surface polysaccharide intercellular adhesin activates human astrocytoma cells via TLR2 [332]. In contrast, SE-derived peptidoglycan (PG) activates human monocytic THP-1 cells via both TLR2 (polymeric PG) and NOD2 (monomeric PG) [333].

Although SE-derived factors can activate human cultured cells via TLR2 and NOD2, their relative contribution to responses of primary leukocytes to live SE *in vitro* or clearance of SE bacteraemia *in vivo*- experimental settings that may most closely mimic clinical infection- are undefined. As bacteraemia is the major clinical manifestation of SE infection, characterizing bloodstream clearance mechanisms is a priority. We therefore investigated the interaction of live SE with TLR2, studying both human and murine cells *in vitro* and intravenous infection of mice *in vivo*, thereby avoiding potential limitations inherent to study of killed bacteria, isolated bacteria-derived factors, or analyses relying on a single mammalian species [325, 334]. We demonstrate for the first time that live SE, which elaborates a TLR2-activating soluble factor (SE-S), can activate

primary cells via human and murine TLR2 *in vitro* and that TLR2 substantially and selectively contributes to clearance of SE bacteraemia *in vivo*.

7.3 Methods

7.3.1 Ethics statement

Blood was collected from healthy donors after written informed consent in accordance with the institutional review board-approved study protocols of Children's hospital Boston (X07-05-0223). All animal protocols were approved by the Animal Care and Use Committee of Children's Hospital Boston (08-11-1261R).

7.3.2 Bacteria

Wild-type (WT) SE strain 1457, obtained from a patient with an infected central venous catheter [335], was grown to mid-log phase in Brain Heart Infusion Broth (PathWest, Perth, Australia), collected by centrifugation and resuspended in pyrogen-free PBS. Lipopolysaccharide (LPS) contamination of SE preparations was excluded by *Limulus* amoebocyte lysate assay (Associates of Cape Cod, East Falmouth, MA) and lack of response by TLR4-transfected HEK293 cells (Invivogen, San Diego, CA). For study of the SE-derived soluble factor (SE-S), SE were grown overnight in trypticase soy broth (TSB), centrifuged at 2900xg at 4^{o}C for 5 min and the supernatant filtered (0.2 μm) and stored at -20°C until use.

7.3.3 TLR agonists

TLR agonists used in this study included fibroblast-stimulating lipopeptide-1 (FSL-1, TLR2/6, 1 μg/ml; InvivoGen), ultra-pure LPS from *Salmonella*

minnesota devoid of TLR2-stimulating activity (TLR4; 100 ng/ml; List Biological Laboratories, Campbell, CA).

7.3.4 Whole Blood

Blood was collected either in sodium heparin tubes (Vacutainer; Becton Dickinson or Greiner, Kremsmünster, Austria) and tested whole, or mixed 1:1 with RPMI 1640 (Gibco, Life Technology, Paisley, Scotland) prior to culture in polypropylene tubes or in round-bottom 96-well plates (Corning Incorporated) at 37°C/5% CO_2. Culture supernatants were harvested and stored at -20°C.

7.3.5 Antibody blocking

Heparinised whole blood was incubated with 10 µg/ml neutralizing, azide- and antibiotic-free, IgA mAbs to human TLR2 or TLR4 (Invivogen) for 30 min prior to addition of FSL-1 (1µg/ml), LPS (100 ng/ml) or SE (10^6/ml). After 4h, culture supernatants were harvested and stored at -20°C prior to IL-6 ELISA. To exclude effects on bacterial viability, blood was plated onto blood agar plates and CFUs enumerated, after overnight incubation (37°C/5% CO_2). For blocking SE-S, whole blood was incubated with neutralizing azide-free rat polyclonal Abs against human TLR2, TLR4, TLR6 or isotype control (Invivogen) for 30min prior to addition of stimulus. After 18h, culture supernatants were harvested and stored at -20^{o}C.

7.3.6 TLR-transfected human embryonic kidney cells

Stable cell lines of human embryonic kidney (HEK)-293 cells transfected with TLR2 or TLR4/MD2 were cultured in supplemented DMEM containing 10% heat-inactivated FCS and 10 µg/ml ciprofloxacin [336]. After antibiotic removal, cells were stimulated with SE (10^6–10^8 bacteria/ml) or pure TLR agonists for 24 h (5%CO_2, 37°C), and cell culture supernatants harvested for storage at -20^{o}C.

7.3.7 Cytokine Assays

Human cytokines were measured by time resolved fluorometry (TRF; IL-6), commercial ELISA (IL-8 and IL-6; eBioscience, San Diego, CA) per manufacturers' instructions, using Maxisorp flat-bottom plates (Nunc, Roskilde, Denmark) [293] or by employing multi-analyte fluorometric beads (Milliplex, Millipore) on a Luminex xMAP system (Luminex Corp., Austin, TX) using BeadView Multiplex Software v.1 (Upstate Cell Signaling Solutions, Temecula, CA).

7.3.8 Mice

TLR2-deficient (Jackson laboratories; Bar Harbor, ME) and C57BL/6 WT (Taconic; Hudson, NY) female mice were confirmed by genotype (PCR) and phenotype (impaired peritoneal macrophage response to FSL-1 with preserved response to LPS) and matched for age.

7.3.9 Bacteraemia Model

Adult mice 7–12 weeks old were injected i.v. with 10^8 SE. Blood was collected by submandibular bleed at 1, 4, 24 and 48h post-infection. For cytokine analysis, blood was diluted (5 volumes RPMI) and plasma collected (1200xg at 4°C; 5 min) and stored at -20°C.

7.3.10 Primary peritoneal macrophages

Murine macrophages were obtained from the peritoneal cavity of mice 3 days after i.p. thioglycollate injection. Adherent cells were maintained in DMEM supplemented with 10% FCS and ciprofloxacin (10μg/ml) at 5% CO_2/37°C. Following ciprofloxacin removal by washing with PBS, cells were stimulated with SE (10^4 – 10^8/ml) for 4h and cell culture supernatants stored at -20°C.

7.3.11 Trans-well assays

SE were added to the upper chamber of a 0.4 μm trans-well system in a 24 well plate (Corning Costar; Acton, MA) in DMEM with 10% FCS for 4h. There were no live SE found in the lower chamber. Cell culture supernatants were harvested and stored at -20°C prior to cytokine measurement.

7.3.12 Murine cytokines

Murine cytokines were determined by ELISA (TNF and IL-6; R&D Systems; Minneapolis, MN) or by employing multi-analyte fluorometric beads (Milliplex,

Millipore) on a Luminex xMAP system (Luminex Corp., Austin, TX) using BeadView Multiplex Software v.1 (Upstate Cell Signaling Solutions, Temecula, CA).

7.3.13 Statistical Analyses

Statistical analyses employed GraphPad Prism v5.0 for Macintosh (GraphPad, La Jolla, CA). Unless stated otherwise in the figure legend, comparisons between 2 groups were by the Mann-Whitney U test and those between >2 groups were by the Kruskal-Wallis test with Dunn's post-test. P-values <0.05 were considered statistically significant.

7.4 Results

7.4.1 Transfection of TLR2 into HEK cells confers responsiveness to SE

We first confirmed that live SE could engage TLR2 to induce cytokine production by assessing TLR2-transfected HEK cells. TLR2-transfected HEK cells demonstrated dramatically increased IL-8 production in response to SE (Figure 7.1), demonstrating that live SE engages TLR2. In contrast, transfection of HEK cells with TLR4/MD-2 did not enhance SE-induced IL-8 production (data not shown).

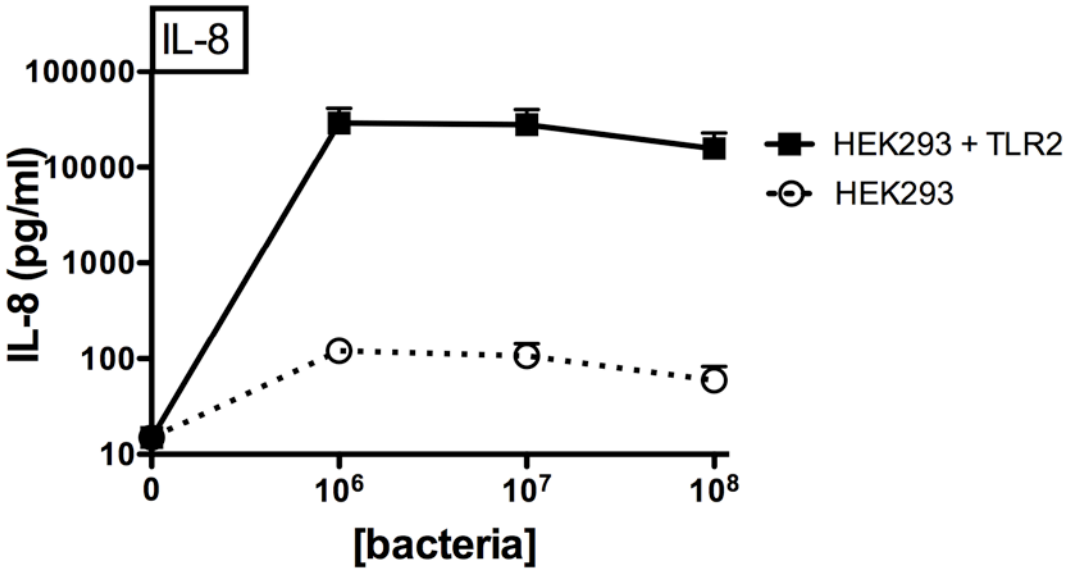

Figure 7.1 Transfection of human embryonic kidney cells with TLR2 confers responsiveness to live SE.

Transfection of HEK293 cells with TLR2 enhanced SE-induced IL-8 production after 24 h of stimulation (n = 3).

7.4.2 SE-induced cytokine production is inhibited by blocking TLR2 in whole blood

To assess the potential role of TLR2 in SE-induced innate immune responses in human blood, we investigated the effect of blocking anti-TLR Abs on SE-induced IL-6 production in human whole blood (Figure 7.2). Pre-incubation of human whole blood with neutralizing anti-TLR2 and TLR4 Abs (10 mg/ml) inhibited cytokine responses to FSL-1 and LPS, respectively (Figure 7.2 A and B). Pre-incubation of human whole blood with anti-TLR2 Ab inhibited SE-induced IL-6 production by >50%, whereas pre-incubation with anti-TLR4 had no effect (Figure 7.2 C and D). The anti-TLR2 Ab significantly inhibited the % cytokine production of TNF, IL-6, IL-10, IFNγ and G-CSF as compared to the anti-TLR4 Ab (Figure 7.2 D).

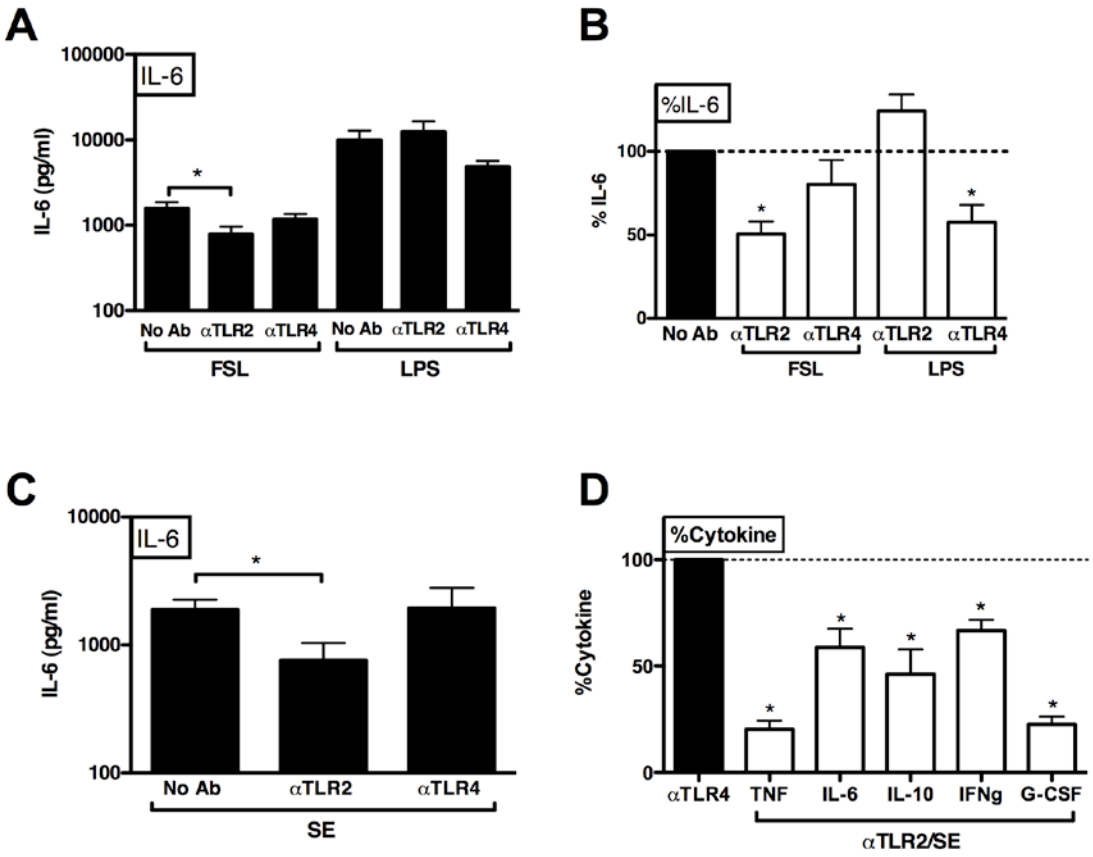

Figure 7.2 TLR2 blocking Abs inhibit SE-induced cytokine production in human blood.

Blocking anti-TLR IgA Abs were added to human whole blood (10µg/ml) 30 min prior to stimulation with SE (10^6/ml) for 4 h and extracellular IL-6 measured by ELISA. Cytokines including TNF. IL-6, IL-10, IFNγ and G-CSF were measured by milliplex. (A) Anti-TLR2 Ab selectively inhibits FSL-induced IL-6 production but not LPS. (B) Inhibition expressed by % IL-6 production (% of No Ab control) (C) Selective inhibition of SE-induced IL-6 by anti-TLR2, but not anti-TLR4 Ab. (D) Inhibition as expressed by % cytokine production (% of control Ab (anti-TLR4)). 1 sample-t-test vs. 100% (n = 4); * $p < 0.05$.

7.4.3 Impaired SE-induced TNF production in TLR2-/- murine peritoneal macrophages

We subsequently measured cytokine responses to SE in peritoneal macrophages derived from wild-type and TLR2-deficient mice. As expected, TLR2-deficient peritoneal macrophages demonstrated marked impairment in TNF production in response to FSL-1 (TLR2/6) but equivalent response to LPS (TLR4) (Figure 7.3 A). TLR2-deficient macrophages also demonstrated marked impairment in SE-induced TNF, particularly at lower concentrations of SE tested (10^4-10^6 CFU/ml; Figure 7.3 B). In contrast, TNF responses to high concentrations of SE (10^7-10^8) were largely TLR2-independent (Figure 7.3B). TLR2-deficient macrophages had markedly diminished production of IL-6, CXCL1 and CXCL2 4 h after infection with 10^5 LSE (Figure 7.3 C and D).

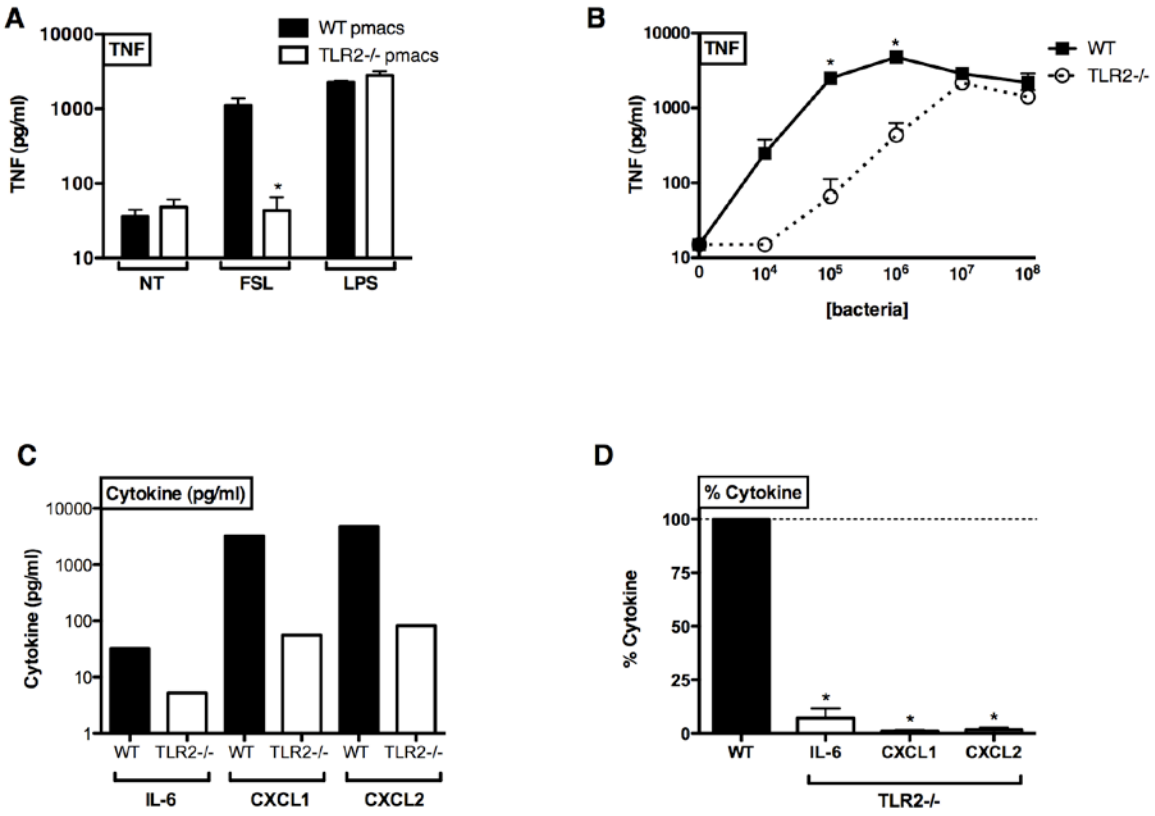

Figure 7.3 Impaired SE-induced cytokine production from primary TLR2-/- murine peritoneal macrophages.

Primary murine peritoneal macrophages (pmacs) from WT and TLR2-deficient C57BL/6 mice were stimulated with SE for 4 h. (A) Selectively impaired bacterial lipopeptide FSL-1-induced cytokine response in TLR2-deficient pmacs, with normal responses to LPS (TLR4). (B) TLR2-deficient pmacs demonstrated markedly impaired TNF production to low concentrations (10^4-10^6 bacteria/ml) of SE (n = 3-9). * $p < 0.05$. (C) SE-induced production of cytokines/chemokines is lower in TLR2-deficient pmacs (representative of 3 similar experiments). (D) Composite analysis demonstrating %cytokine/chemokine production of TLR2-deficient pmacs relative to WT (n=3, one-sample t-test versus 100%). *$p < 0.05$.

7.4.4 SE secretes a soluble TLR2 agonist

We next assessed whether TLR2 agonist activity of SE may be released as a soluble factor. In trans-well experiments in which SE were placed in the upper chamber, wild-type macrophages in the lower chamber produced TNF, CXCL1 and CXCL2 suggesting the secretion of a soluble factor that could traverse the semi-permeable membrane (Figure 7.4 A). This SE-derived secreted factor (SE-S) was highly TLR2-dependent as responses of TLR2-deficient macrophages were dramatically impaired (Figure 7.4 A, and composite analysis in Figure 7.4 B). Accordingly, TLR2-deficient pmacs demonstrated impaired SE-S-induced cytokine and chemokine production (Figure 7.4 C and D). SE-S also induced IL-6 production in human whole blood in a concentration-dependent manner (Figure 7.4 E), an activity that was inhibited by neutralizing anti-TLR2 Abs (Figure 7.4 F).

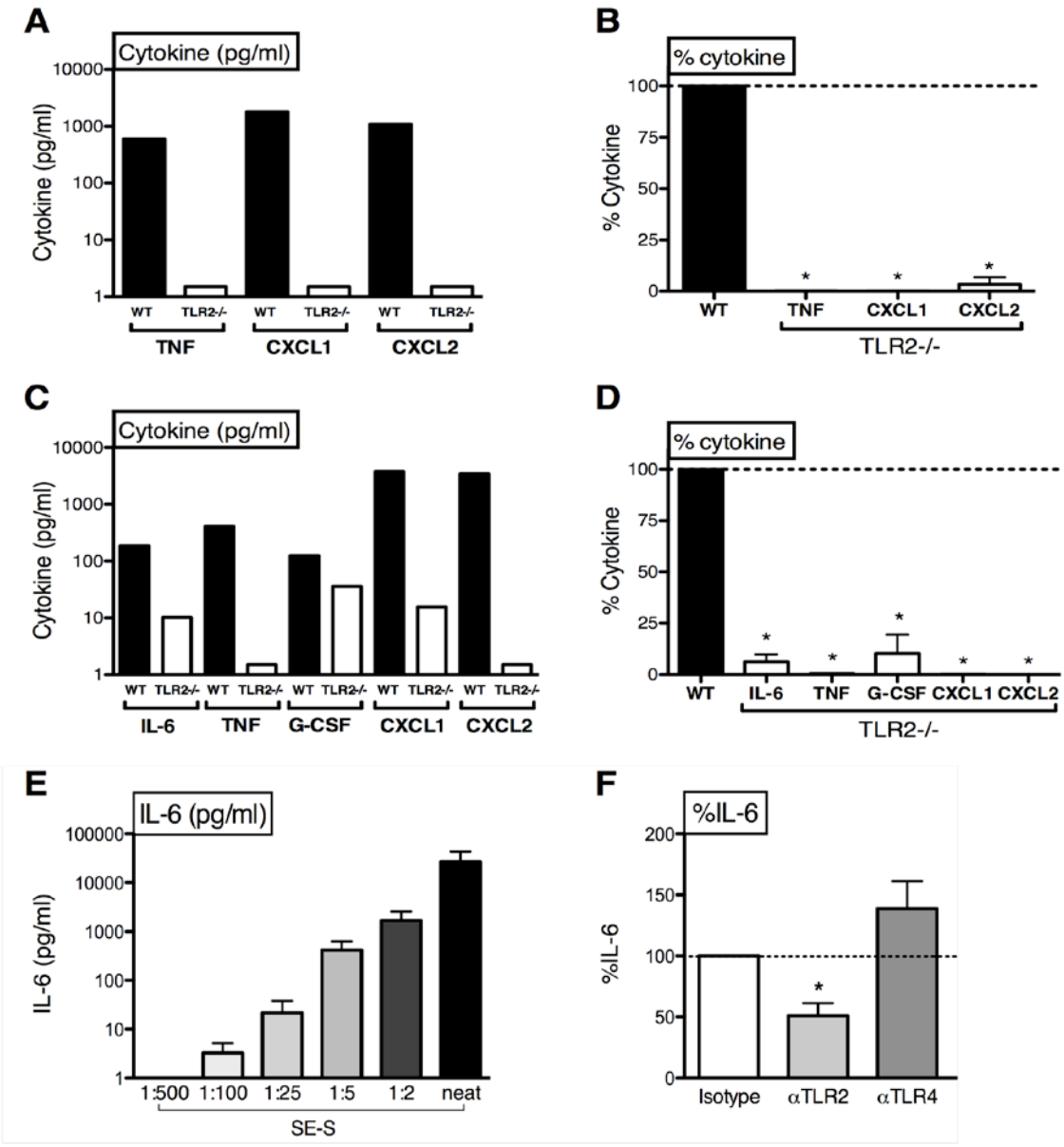

Figure 7.4 SE soluble factor activates cytokine production in a TLR2-dependent manner.

(A) SE in a trans-well upper chamber induced cytokine production in WT, but not TLR2-deficient murine peritoneal macrophages (pmacs; representative of 3 similar experiments); (B) Composite trans-well analysis expressed as %IL-6 production in TLR2-deficient vs. WT pmacs; (C) Filtered supernatants of stationary phase SE contain a soluble factor (SE-S) which induced cytokine/chemokine production from TLR2-deficient pmacs; (D) composite analysis of SE-S-induced cytokine/chemokine production in TLR2-deficient vs. WT pmacs. (E) SE-S induced concentration-dependent IL-6 production in human whole blood, (F) neutralizing anti-TLR2 polyclonal Abs blunted SE-S-induced IL-6 in human whole blood. * $p < 0.05$.

7.4.5 SE-induced cytokine production in vivo is mediated via TLR2

To test whether TLR2 contributes to SE-induced cytokine production in peripheral blood *in vivo*, we injected SE i.v. to wild-type or TLR2-deficient mice then measured cytokine production at 1h (Figure 7.5). TLR2-deficient mice demonstrated markedly diminished production of IL-6, IL-10, G-CSF, CXCL1 and CXCL2 *in vivo* (Figure 7.5 A) that was significant upon composite analysis (Figure 7.5 B).

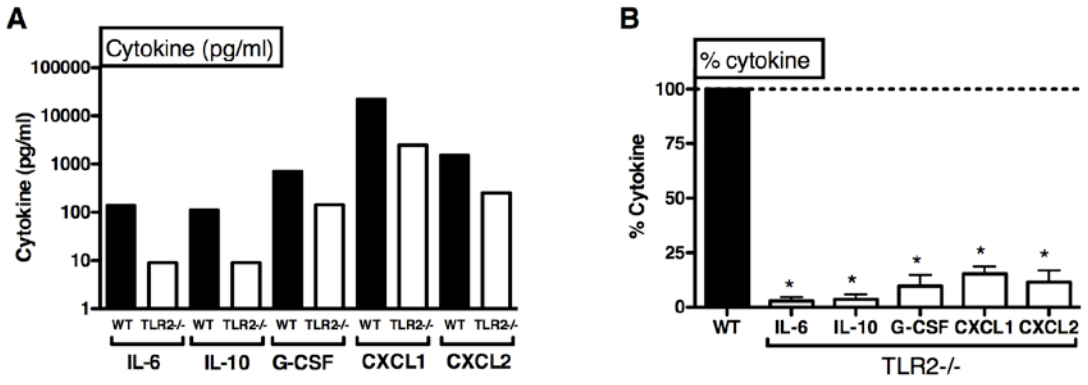

Figure 7.5 SE-induced cytokine production is TLR2-dependent *in vivo*.

Female C57BL/6 WT or TLR2-deficient mice were injected intravenously with 10^8 CFU SE prior to collection of peripheral blood at 1h for measurement of cytokines/chemokines by Milliplex assay. (A) SE-induced production of cytokines/chemokines is lower in TLR2-deficient mice (representative of 3 similar experiments). (B) Composite analysis demonstrating %cytokine/chemokine production of TLR2-deficient mice relative to WT (n=3, one-sample t-test versus 100%). *$p < 0.05$.

7.4.6 TLR2 selectively mediates clearance of SE bacteraemia in vivo

To assess the role of TLR2 in clearance of SE after blood-based dissemination, C57BL/6 wild-type or TLR2-deficient mice were injected i.v. with 10^8 of SE prior to collection of blood for analysis. TLR2-deficient mice demonstrated selectively impaired clearance of SE bacteraemia, with dramatically and significantly higher peripheral blood concentrations of SE at 24 and 48h (Figure 7.6).

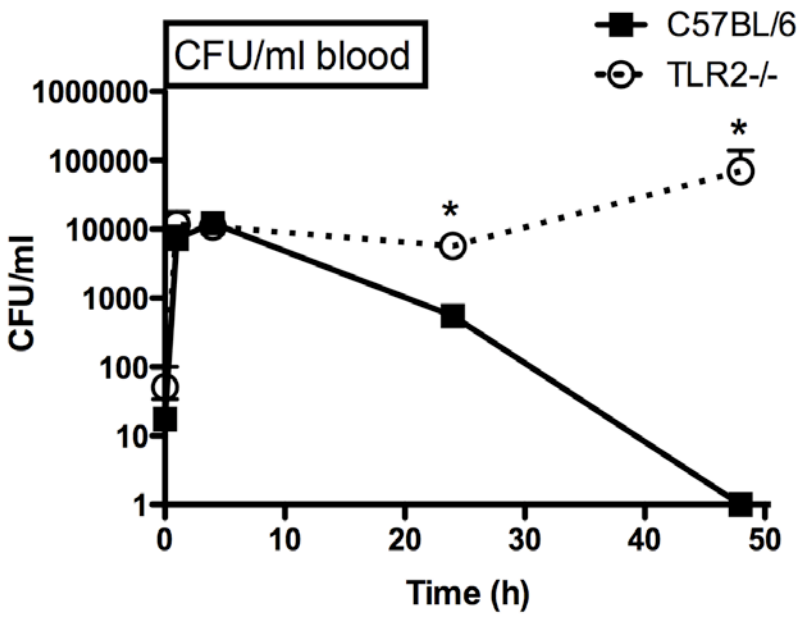

Figure 7.6 TLR2-deficient mice demonstrate a selective impairment in clearance of SE bacteraemia.

Female C57BL/6 WT or TLR2-deficient mice were injected i.v. with 10^8 CFU of SE prior to collection of peripheral blood at 1, 4, 24 or 48 h for measurement of CFUs by plating serial dilutions (n=4-9); *p<0.05.

7.5 Discussion

We have demonstrated for the first time the importance of TLR2 in recognition of live SE as measured by whole blood cytokine production and highlight a selective role of TLR2 in clearance of SE bacteraemia. TLR2 mediated SE-induced cytokine production in TLR2-transfected HEK cells, human whole blood, and murine primary peritoneal macrophages. TLR2-deficient mice injected intravenously with SE demonstrated reduced early (1h) cytokine/chemokine induction and selectively and markedly impaired subsequent clearance of bacteraemia at 24-48h.

SE releases an unknown soluble factor (SE-S) that activates both human and murine cells via TLR2 (Fig. 4C & D). We speculate that release of SE-S may contribute to responses to SE *in vivo*. Indeed, our evidence that TLR2 plays a prominent role in recognition of and responses to SE is consistent with the ability of SE-derived surface components to induce inflammation [337], including TLR2 agonists such as PSM [328, 330], surface polysaccharide intercellular adhesin [332] and peptidoglycan [333]. Whether the SE-S activity detected in our present study reflects these or other molecules [338] will be the subject of future work.

Impaired production of cytokines in response to intravenous SE was associated with impaired clearance of bacteraemia, suggesting that TLR2-mediated cytokine production may contribute to clearance of SE *in vivo*. In contrast, Raby et al recently reported that after i.p. challenge with SE, administration of inhibitory soluble TLR2 (sTLR2) reduced peritoneal PMN infiltration, but did not reduce bacterial clearance from the peritoneal cavity [329]. Our distinct results likely reflect different routes of bacterial challenge and different approaches to

TLR2 inhibition, with genetic ablation providing a more definitive approach. SE engages TLR2, but at high bacterial concentrations can activate primary murine macrophages via a TLR2-independent pathway, indicating engagement of additional pattern recognition pathways, possibly including NLRs and the inflammasome [333, 334, 339].

Multiple aspects of our studies of live SE are novel: a) SE (and SE-S) activates cytokine production via TLR2 in human whole blood, b) TLR2 is particularly important in detecting low concentrations of SE in primary murine macrophages, whereas high concentrations of SE can activate these cells in a TLR2-independent manner, c) TLR2 mediates early (1h) cytokine production after i.v. challenge with SE *in vivo* and d) TLR2 plays a crucial and selective role in mediating subsequent clearance of SE bacteraemia at 24-48 h *in vivo*. We speculate that in TLR2-deficient animals, reduced production of early (1h) plasma cytokines, including IL-6, impairs subsequent clearance of staphylococci, as has also been suggested in humans with neutralizing auto-IL-6 Abs [340].

In conclusion, our study is the first to demonstrate the importance of TLR2 in recognition of live SE in whole blood, the key site of SE infection, *in vitro* and *in vivo*. We have demonstrated a selective role of TLR2 in clearance of SE bacteraemia, the most common, harmful and costly clinical manifestation of SE infection [219, 306]. Our study may inform efforts to developing novel adjunctive approaches to prevent and/or treat SE infection [341]. Novel TLR2 antagonists in biopharmaceutical development may reduce SE-induced inflammation, but may also impair clearance of SE bacteraemia. Conversely, enhancing TLR2-mediated host defence may hasten clearance of SE bacteraemia; consistent with protection against bacteraemia afforded by hypermorphic alleles of TIRAP,

a signalling molecule downstream of TLR2 [342]. Future studies should define the roles of TLR2 in susceptibility of immunocompromised populations, particularly those at the extremes of age, to SE bacteraemia [77].

Acknowledgements

We thank Drs. Michael Wessels, Raif Geha, Richard Malley, Anthony Keil and Peter Campbell for mentorship and intellectual input. David Atlas, Cristina Farrar (Dept. Clinical Microbiology PathWest, Princess Margaret Hospital for Children), and the research assistants of the School of Paediatrics and Child Health Vaccine Trials Group provided expert technical assistance.

Chapter 8

Method of bacterial killing differentially affects the complexity of the human innate immune response to Staphylococcus epidermidis

8.1 Abstract

Background: *In vitro* investigations of human innate immune responses to extracellular bacteria commonly utilise killed preparations in preference to live organisms. The effects of the bacterial preparation method on the activation of innate signalling pathways by the common opportunistic pathogen *Staphylococcus epidermidis* (SE) are unknown.

Methods: Mononuclear cell cytokine expression patterns induced by live, heat-killed and ethanol-killed SE were characterised at the transcriptional and translational level. Toll-like receptor (TLR)-activating capacity of the preparations was analysed using TLR-transfected human embryonic kidney cells.

Results: Live SE activated NFκB, STAT1, type I interferon, and inflammasome those pathways. Killed preparations engaged the NFκB pathway, but had significantly lower capacity to activate other innate immune pathways.

Conclusions: Killing of extracellular bacteria has significant qualitative and quantitative effects on key aspects of innate responses *in vitro*. Interpretation of *in vitro* data and extrapolation of findings should take into account the potential

effects of bacterial preparation and should not assume that responses to killed bacteria are predictive of responses to live organisms.

8.2 Introduction

Killed bacterial preparations and purified bacterial ligands are commonly utilised in studies of pathogenesis employed in preference to live bacteria to minimise experimental variability and simplify experimental conditions. A variety of heat- and ethanol treatment protocols are employed used to kill bacteria for immunological investigations. Heat causes denaturation of organic molecules, including proteins, lipids and membrane structures, leading to death of most microorganisms. Ethanol kills bacteria by rapid denaturation of proteins and membranes, resulting in subsequent interference with metabolism and cell lysis. Live bacteria may utilise additional mechanisms to evade or subvert immune responses and when tested *in vitro* are likely to more closely model host-pathogen interactions *in vivo* [296]. Few studies have investigated the effects of the method of bacterial killing on the nature of the interaction with the human innate immune system. Given the major effects these killing methods have on bacterial integrity and structure, we hypothesised that both bacterial killing *per se* and the method of killing may qualitatively and quantitatively alter immune signalling and responses, compared to those generated by stimulation with live extracellular bacteria.

Coagulase-negative staphylococci and *Staphylococcus epidermidis* (SE) in particular, are the most commonly isolated organisms in the microbiology laboratory and are a leading cause of nosocomial bloodstream infections, particularly in premature infants and patients with indwelling medical devices and the immunocompromised [96, 190]. Here we report the differential effects of live, heat- and ethanol-killed SE preparations on activation of the human innate immune system using peripheral blood mononuclear cells (PBMCs) and whole

blood. We show that induction of key pathway cytokines downstream of distinct innate immune pathways by live (LSE), heat-killed (HKSE) and ethanol-killed (EKSE) SE preparations differ significantly, indicating that the method of bacterial killing profoundly influences *in vitro* responses and that live organisms co-ordinately activate multiple pathways to generate a complex anti-bacterial response.

8.3 Materials and Methods

8.3.1 Blood sampling and preparation

The Princess Margaret Hospital for Children (Perth, Australia) ethics committee approved the study protocol and written informed consent was obtained. Blood samples were collected from healthy adults by peripheral venepuncture. For isolation of peripheral blood mononuclear cells, blood was mixed 1:1 with heparinised RPMI 1640 culture medium (Gibco, Life Technology, Paisley, Scotland). Peripheral blood mononuclear cells (PBMC) were isolated by lymphoprep gradient centrifugation (Nycomed Pharmacia, Norway) and cryopreserved as previously reported [292]. Briefly, after centrifugation, cells were washed once in RPMI (500 *g*, 10 min) and then twice in RPMI/2% fetal calf serum (FCS, MultiSer Biosciences, Australia). Cells were then resuspended in RPMI/2% FCS and counted. Ice-cold 15% dimethyl-sulphoxide (DMSO; BDH Chemicals, Port Fairy, Victoria, Australia) freezing solution in FCS was added drop-wise. The samples were cooled to -80°C at a rate of 1°C/min in a freezing container (Nalgene, Rochester, NY) containing isopropyl alcohol (BDH Chemicals). Samples were then transferred to liquid nitrogen (-180^{O}C) for long-term storage and batch analysis.

8.3.2 Bacterial culture

Wild type SE *strain* 1457, originally obtained from the blood of a patient with an infected central venous catheter [335], was grown to mid-log phase in Brain Heart Infusion Broth (PathWest, Perth, Australia) and then collected by centrifugation prior to resuspension in pyrogen-free phosphate-buffered saline

(PBS) at the desired concentration. For experiments with non-viable bacteria, ethanol-killed bacteria were prepared by incubation of live bacteria in ice-cold 70% ethanol for 1 hr, rotating end-over-end. Heat-killing was achieved by incubation of the bacterial cultures in a water bath for 15 min at 80°C. Lack of viability was confirmed by plating serial dilutions of the killed bacterial suspensions onto blood agar plates. Following harvest, the bacteria were washed and then resuspended in sterile PBS at a concentration of $2.5x10^9$/ml and stored at -20°C. Concentrations of all bacterial preparations were based on colony counts following serial dilutions of LSE and verified for all preparations by manual counting using a Helber counting chamber at high magnification. Routinely conducted quality control experiments correlating OD, viable counts and CFU demonstrate a high degree of concordance (Supplemental figure 1). Lipopolysaccharide (LPS) contamination of SE preparations was excluded by *Limulus* amoebocyte lysate assay (Associates of Cape Cod Inc., East Falmouth, MA) with a limit of detection of <0.01 ng/ml, and by lack of response by TLR4-transfected human embryonic kidney (HEK) cells (Invivogen, San Diego, CA) (data not shown).

8.3.3 PBMC cultures

Vials of cryopreserved PBMC were thawed rapidly at 37^oC, resuspended in RPMI 1640, centrifuged and cells resuspended to 1 ml in AIM-V (Gibco) supplemented with ß-mercaptoethanol (Sigma; Castle Hill, Australia), as previously described [293]. 10^6cells per ml were cultured in RPMI 1640 with 10% FCS in polypropylene round-bottom 96-well plates (Corning Incorporated, Corning, NY). SE preparations were added at the indicated final concentrations

and incubated at 37°C/5% CO_2. Culture supernatants were harvested at indicated times and stored at -80°C until analysis.

8.3.4 Whole blood assay

Fresh whole blood from healthy donors was collected into heparinised tubes (Greiner, Kremsmünster, Austria), mixed 1:1 with RPMI 1640 (supplemented with 10 mM HEPES, 2 mM L-glutamine, 1 mM Na-pyruvate and 550 µM ß-mercaptoethanol). Whole blood cultures were performed in polypropylene round-bottom 96-well plates (Corning Incorporated) at 37°C/5% CO_2. Culture supernatants were harvested and stored at -80°C for batch analysis.

8.3.5 Toll-like Receptor (TLR)-pathway array

Following stimulation of PBMC cultures with LSE, HKSE or EKSE at 10^6/ml for 4 h, cell pellets were resuspended in Trizol (Invitrogen, Mount Waverley, Australia) and stored at -80°C. RNA was isolated using the PureLink RNA MiniKit (Invitrogen) in accordance with manufacturer's recommendations. Briefly, Trizol samples were thawed prior to chloroform extraction, centrifugation (12000 *g* for 15 min at 4°C), and recovery of the upper aqueous phase. An equal volume of 70% ethanol was added and the mixture was centrifuged in a silica membrane spin cartridge (12000 *g* 15 sec). RNA elution and DNA elimination were performed as per manufacturer's instructions. Purified RNA was stored at -80°C until array analysis.

We transcribed 50 ng of RNA per sample to cDNA using the RT-PCR array First Strand Kit (SA Biosciences) and analysed on the Human TLR pathway Array (SA Biosciences, Frederick, MD), using the real-time SYBR green/fluorescein PCR master mix (SA Biosciences). RT-PCR was performed on an ABI Prism 7000 (ABI, Foster City, CA) in accordance with the manufacturer's instructions. Gene expression was normalised using multiple standard housekeeping genes and data analysed using the $\Delta\Delta C_t$ method. Fold change in gene expression was calculated between unstimulated and SE-stimulated cells.

8.3.6 Human embryonic kidney cell cultures

Stable cell lines of human embryonic kidney (HEK) 293 cells transfected with TLR2, TLR3, or TLR4/MD2 (kindly provided by Dr Ashley Mansell, Institute of Medical Research, Melbourne, Australia with permission from Prof. Douglas Golenbock, University of Massachusetts Medical School, MA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 1 mM Na-pyruvate, phenol-red, 550 µM ß-mercaptoethanol, 10 mM HEPES, 0.5 mg/ml Geneticin and antibiotic/antifungal (100 units penicillin, 100 mg streptomycin, 0.25 mg amphotericin per ml) [336]. Cells were stimulated with SE preparations (10^5-10^8/ml) or with the following pure TLR agonists, which were used as positive and negative controls: TLR1/2: Pam_3Cys (10µg/mL; InvivoGen), TLR2/6: FSL-1 (10 ng/mL; InvivoGen), TLR3: Poly dI:dC (10µg/mL; InvivoGen), TLR4: ultra-pure LPS (10 ng/mL; from *E. coli,* Alexis Biochemicals, Plymouth Meeting, PA). After 18 hr incubation in a 5% saturated CO_2 incubator at 37°C, cell culture

supernatants were harvested and stored at minus 20^{o}C prior to interleukin (IL)-8 measurement.

8.3.7 Cytokine Assays

Cytokines were measured by in-house time resolved fluorometry assay (TRF; IL-6, IL-10, tumour necrosis factor-α (TNF-α)) [293]. Commercial enzyme-linked immunoassay (IL-1β, IL-8: eBioscience, San Diego, CA) or a multiplexed fluorescent bead assay (Bioplex; IP-10, MCP-1, MIP-1α) in accordance with established protocols or the manufacturer's instructions [293]. Anti-cytokine monoclonal capture/detection antibody pairs were obtained from PharMingen (BD Biosciences, North Ryde, NSW, Australia) and TRF assays were performed on Nunc Maxisorp flat-bottom plates (Roskilde, Denmark). Standard curves were generated by serially diluting recombinant cytokines purchased from PharmMingen (BD Biosciences). Following incubation with biotinylated detection antibodies, Europium-labelled streptavidin (DELFIA, Wallac, Turku, Finland) was added, followed by enhancement solution (DELFIA, Wallac) and plates were kept in the dark at room temperature until analysis using a Wallac 1420 Victor^{2} (PerkinElmer, Waltham, MA).

8.3.8 Flow cytometry analysis of intracellular cytokine induction

For the intracellular cytokine assay, PBMC were cultured with SE preparations (as above). After 30 min, Brefeldin A (6 µg/ml, eBioscience, San Diego, CA) was added to the cultures, and cells were incubated for a further 3.5 h at 37^{o}C. Cells were then transferred to a round-bottom polystyrene 96-well plate and

washed twice with ice-cold PBS (500 *g*, 5 min) followed by immediate fixation using BD FACS-Lyse solution (BD Biosciences), according to the manufacturer's instruction. Cells were permeabilised using 1x BD Perm2 permeabilisation buffer for 10 min (BD Biosciences), washed and resuspended in FACS buffer. Subsequently, cells were incubated with FITC- or APC-labelled antibodies against TNF-α or IL-6 (eBioscience) or appropriate isotype controls for 30 min at 4°*C* in the dark, prior to a final two washes and fixation in 1x BD stabilizing fixative solution (BD Biosciences). A minimum of 5000 monocytes, identified by forward/sideward scatter characteristics (FSC/SSC), were acquired using a FACSCalibur (BD Biosciences) and data analysed using Flowjo software (Treestar, Ashland, OR).

8.3.9 Cell viability assay

The relative viability of PBMC following exposure to the different preparations of SE was determined using the WST-1 assay according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany), PBMC were cultured as above in the presence of 10^8 CFU/ml of each bacterial preparation for 18 h, or left unstimulated. The culture supernatant was then removed and replaced with 200 µl of fresh RPMI 1640 with 10% FCS and a 10% WST-1 reagent for a further 4 h. Absorbance was determined at 450 nm using a Wallac 1420 Victor^2 (PerkinElmer, Waltham, MA) with a negative control containing media and WST-1 but without cells.

8.3.10 Statistical analyses

Unless otherwise stated in the figure legend, comparisons between groups were performed using the Kruskal-Wallis test with Dunn's post-test using Prism 5 for Mac (GraphPad, La Jolla, CA). Differences were considered significant at p-level <0.05.

8.4 Results

8.4.1 Heat-killed and live SE preparations induce distinct patterns of cytokine production by human PBMCs and whole blood

We characterised the cytokine-inducing capacities of three SE preparations in both human PBMC and fresh whole blood. Each of the SE preparations induced dose-dependent production of cytokines. The cytokine pattern induced by the SE preparations was similar in PBMC and fresh whole blood (Figure 8.1). Intracellular cytokine staining showed that monocytes were exclusively responsible for the production of TNF-α and IL-6 and were the major cellular source of IL-10 and IL-1b (data not shown). We also investigated the survival of LSE in whole blood and found no evidence of bacterial overgrowth; <5% of inoculated SE were viable after 1hr and no additional bacterial growth was observed after up to 18 h (data not shown).

The responses to increasing bacterial concentrations differed significantly between SE preparations (Figure 8.1); HKSE and EKSE (10^{5-8}/ml) induced proportional increases in IL-6, IL-10 and TNF secretion, whereas maximum levels of cytokines were achieved by lower inocula of LSE (10^{5-7}/ml) and cytokine concentrations decreased following stimulation with higher doses of LSE (10^{8}/ml).

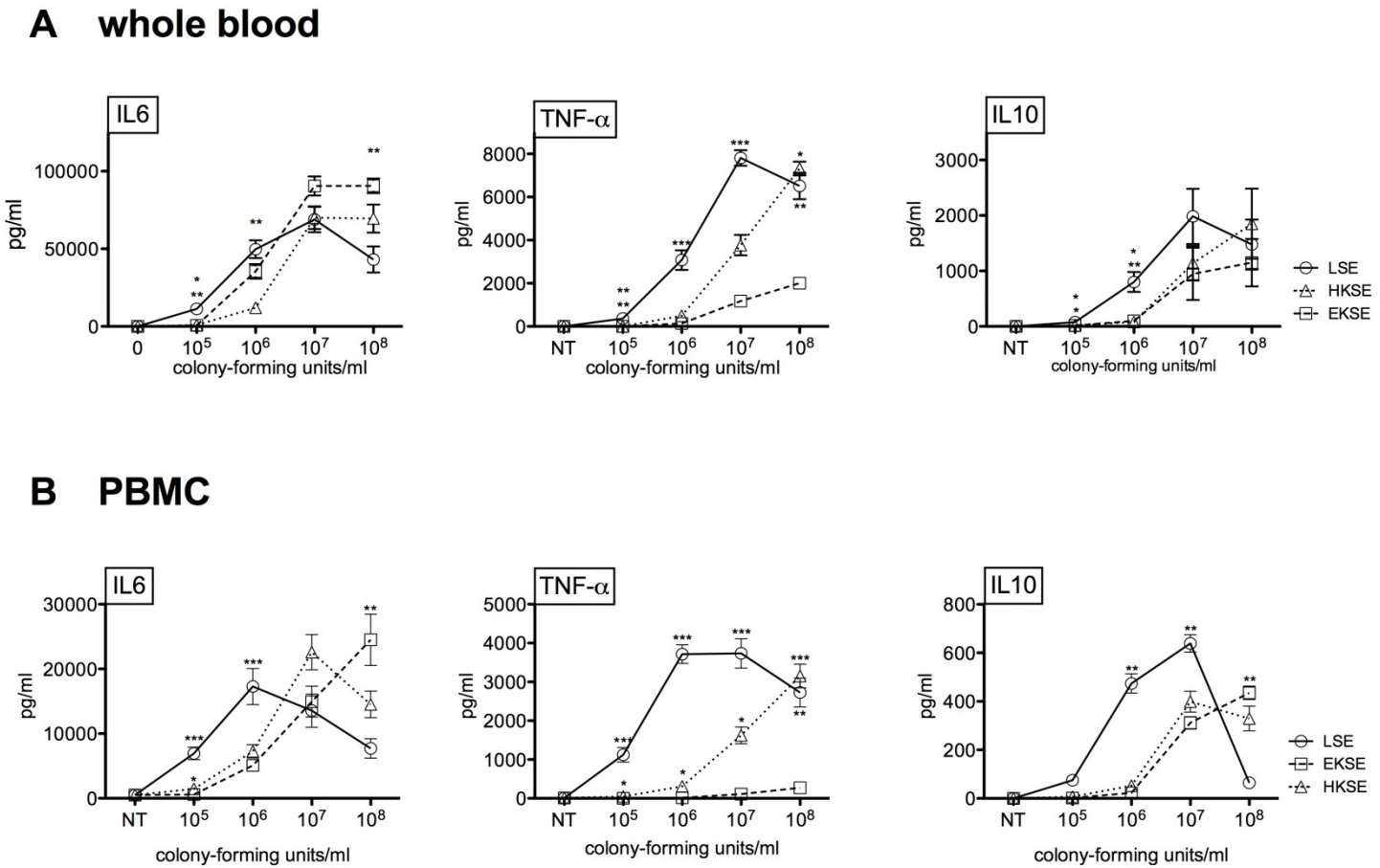

Figure 8.1 Cytokine protein responses to SE preparations in PBMC and whole blood.

Whole blood (n=15, Fig. 1A) and PBMC (n=8, Fig. 1B) were stimulated with indicated concentrations of SE preparations for 18 h. Cytokine levels were analysed by means of time-resolved fluorometry (TRF) (IL-6, IL-10, TNF-α). Graphs show mean ±SEM. *p<0.05, **p<0.01, ***p<0.001.

The most distinct significant differences were observed for TNF-α-inducing capacity in both PBMC and whole blood: EKSE incubation induced relatively little TNF-α compared to both LSE and HKSE, whereas LSE induced peak TNF-α concentrations at doses of 10^5-10^7/ml, similar to HKSE at 10^8/ml. HKSE was significantly more potent in TNF-α induction than EKSE. These SE preparation-dependent differences in cytokine induction were consistent after 4, 18 and 48 h of incubation in both PBMC and whole blood (data not shown).

8.4.2 SE preparations activate TLR2-transfected HEK cells

To investigate if the differences in the cytokine-inducing potency of the SE preparations were due to changes in their TLR-activating capacity, we used stable HEK cell lines, over-expressing TLR2, TLR3 (Figure 8.2) or TLR4/MD2 (data not shown). All SE preparations induced IL-8 production in TLR2-transfected HEK cells in a similar dose-dependent manner (Figure 8.2). None of the SE preparations activated HEK cells over expressing TLR3 or TLR4/MD2, whereas Gram-negative bacteria (*E. coli* and *H. influenzae*) and purified TLR ligands (Poly I:C for TLR3 and LPS for TLR4) induced dose-dependent IL-8 production in the HEK cells over expressing the respective TLRs (data not shown).

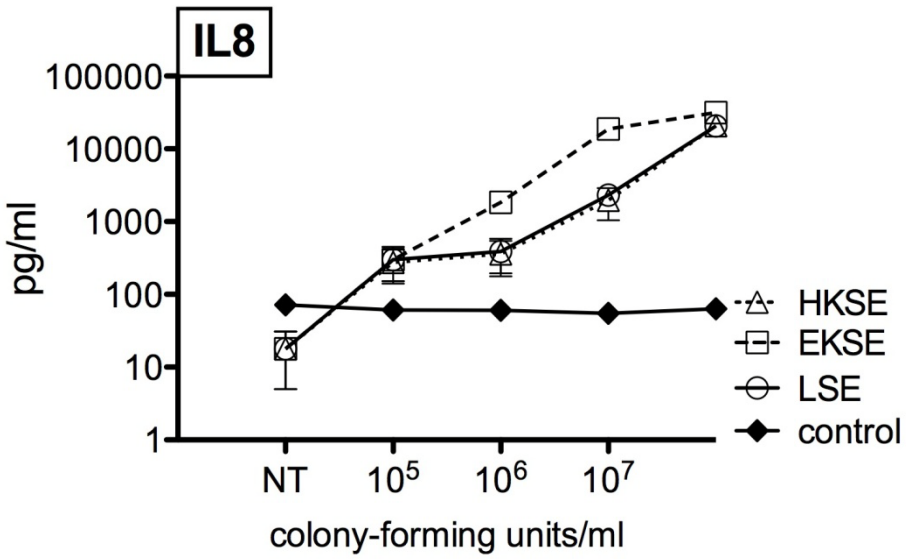

Figure 8.2 TLR2-activating capacity of SE preparations.

LSE, HKSE and EKSE were used at indicated concentrations to stimulate HEK293 cells transfected with human TLR2 or TLR3 for 18 h. Supernatants were harvested and IL-8 levels analysed by means of ELISA. Graphs depict results of duplicate wells of one representative experiment of three and indicate mean ±SEM.

8.4.3 EKSE and bacterial lipopeptides induce a high ratio of IL6/TNF

The cytokine pattern induced by EKSE was distinct from that triggered by LSE and HKSE and very similar to that induced by purified bacterial lipopeptides (BLPs) that are TLR1/2 or -2/6 agonists, Pam_3Cys and FSL-1, respectively (Fig. 3A). Similar to the BLPs, EKSE induced a significantly higher ratio of IL-6/TNF-α, cytokines that are divergently regulated and have distinct function than did LSE or HKSE (Figure 8.3) [111, 170].

The observed differences between EKSE and LSE/HKSE were not due to toxic effects of ethanol, as similar results were obtained by incubation with both ethanol concentrations significantly in excess of those expected as residual levels, and an extensively washed EKSE preparation (data not shown). In addition, the responses to EKSE were inhibited by anti-TLR2 antibodies (data not shown).

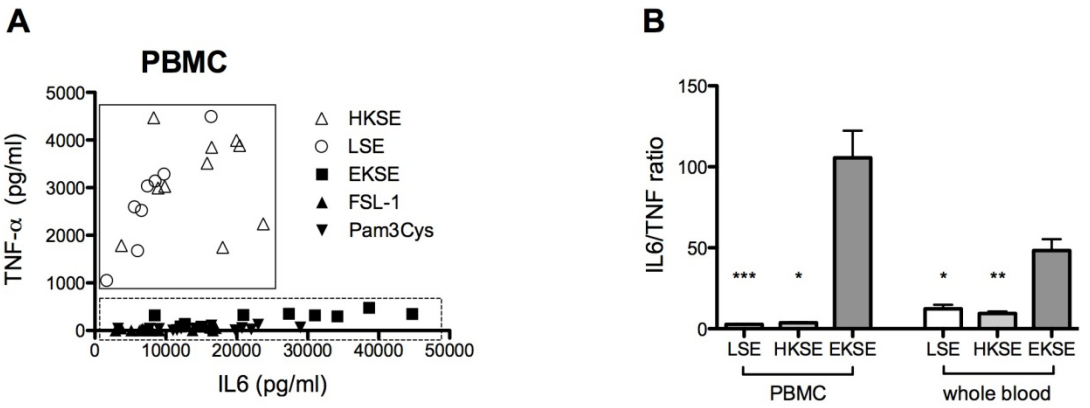

Figure 8.3 Cytokine pattern induced by EKSE is similar to that of TLR2-agonists.

(A) depicts production of the distinct TNF-α (y-axis) and IL-6 (x-axis) patterns induced by SE preparations (10^6 CFU/ml) and TLR1/2 (Pam3Cys; 10 µg/ml) and TLR2/6 (FSL-1; 10 ng/ml) agonists in PBMC (10^6/ml, n=8) after 18 h. Supernatants were harvested, stored at -80C and concentrations of IL-6, TNF-α determined by TRF. (B) shows the IL-6/TNF-α-ratio for SE preparations (10^6 CFU/ml) in PBMC and whole blood. Graphs show mean ±SEM. *p<0.05, **p<0.01, ***p<0.001.

8.4.4 Live SE selectively engage IL-1ß and type I IFN pathways

As EKSE appeared to generate a more limited cytokine profile than the other preparations, we investigated the ability of the SE preparations to induce secretion of a range of cytokines that are transcriptionally coordinated by the activity of several signalling pathways and/or by post-transcriptional regulation. The production of IL-8 was measured as an indicator of canonical NFκB activity. LSE induced greater production of IL-1β, CXCL10 (IP-10) and CCL3 (MIP-1α) than either EKSE or HKSE (Figure 8.4). Despite the induction of IP-10, there was no detectable induction of IFN-a by any of the preparations. In contrast, all three preparations induced equivalent levels of IL-8. LSE induced lower concentrations of CCL2 (MCP-1) than EKSE or HKSE.

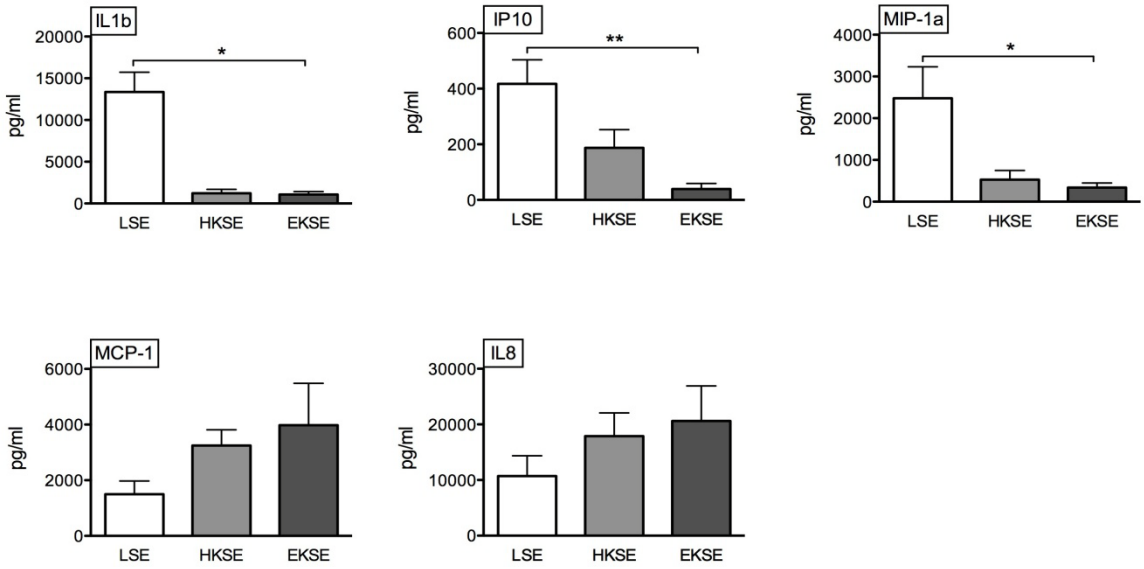

Figure 8.4 Differential engagement of cytokine pathways by SE-preparations.

PBMC (10^6/ml, n=6) were stimulated with SE preparations (10^6 CFU/ml) for 18 h. Supernatants were harvested, stored at -80^{o}C and cytokine concentrations determined by ELISA (IL-1β, IL-8) or Multiplex assay (IP-10, MCP-1, MIP-1α).

8.4.5 SE preparations differentially alter the expression IL-1ß, IFN and other TLR pathway-related genes

We used a RT-PCR array to investigate the differential effect of SE preparations on the expression of human TLR pathway-associated genes (Figure 8.5). LSE induced significantly higher expression of several pro-inflammatory cytokine genes compared to HKSE and EKSE. Notably the induction of the IL-1 genes (*IL-1a* and *IL-1*β) and IFNβ and IFNγ were among the most significantly different between LSE and other preparations. The expression of *CCL2* (MCP-1) was induced by all three preparations but was significantly lower in the LSE response in keeping with the observed protein levels for this chemokine (Figure 8.4). Relatively few genes were down regulated by bacterial stimulation. However *CD14* was significantly more down regulated in response to LSE than to the other SE preparations. A complete description of the gene expression levels in response to stimulation with LSE, HKSE and EKSE is given in Table 8.1.

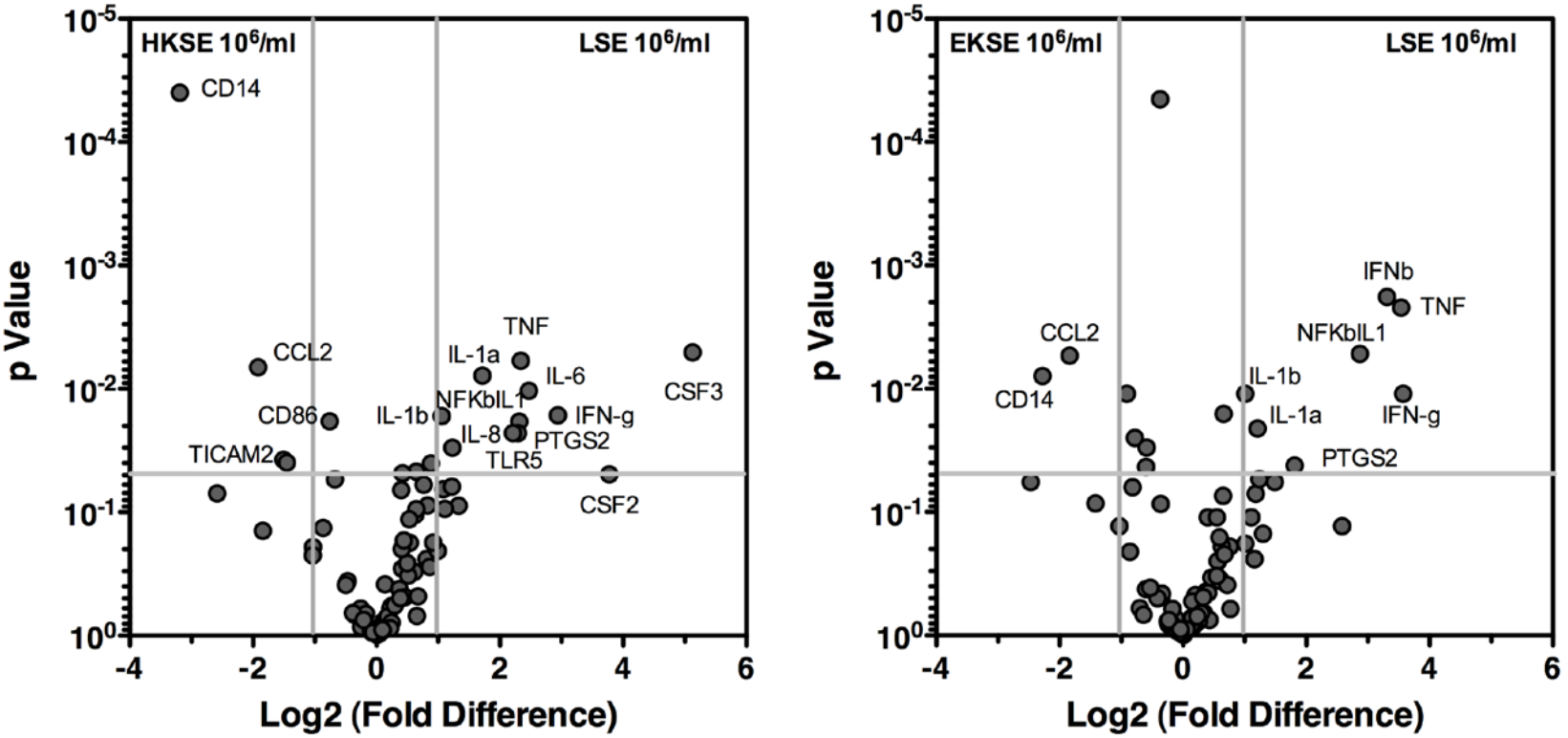

Figure 8.5 TLR pathway-associated gene expression in response to SE.

PBMC (10^6/ml) were stimulated with SE preparations at 10^6/ml for 4 h (n=3). RNA was isolated, transcribed into cDNA and analysed on a human RT-PCR TLR pathway array (SuperArray™). Volcano plots show log transformation of the gene expression levels of each gene comparing LSE with HKSE (Fig. 1A) and EKSE (Fig. 1B). The central vertical line indicates equivalence and the outer vertical lines indicate a two-fold difference in gene expression. The horizontal line indicates a t-test p-value of <0.05.

Table 8.1 The gene expression levels in human PBMC in response to stimulation with LSE, HKSE and EKSE.

Upregulated							*No change*							*Downregulated*					
	LSE		***HKSE***		***EKSE***			***LSE***		***HKSE***		***EKSE***			***LSE***		***HKSE***		***EKSE***	
Gene	*Fold∆*	*p-value*	*Fold∆*	*p-value*	*Fold∆*	*p-value*	***Gene***	*Fold∆*	*p-value*	*Fold∆*	*p-value*	*Fold∆*	*p-value*	***Gene***	*Fold∆*	*p-value*	*Fold∆*	*p-value*	*Fold∆*	*p-value*
IL6	874.3	0.000	146.7	0.000	344.9	0.000	**JUN**	1.9	0.123	-1.1	0.670	-1.2	0.699	**TLR4**	-2.4	0.100	-1.3	0.500	1.0	0.927
TNF	382.1	0.000	70.2	0.000	30.6	0.000	**CCL2**	1.9	0.182	6.8	0.012	6.4	0.012	**TNFRSF1A**	-2.5	0.002	-1.5	0.288	-1.6	0.135
CSF2	274.3	0.001	18.6	0.010	42.8	0.004	**TBK1**	1.9	0.151	1.3	0.251	1.1	0.562	**TICAM2**	-2.7	0.027	-1.0	0.831	-3.4	0.012
IL1A	144.8	0.000	41.0	0.000	58.2	0.000	**MAP4K4**	1.8	0.104	1.3	0.291	1.6	0.155	**CD86**	-4.4	0.038	-1.7	0.253	-3.1	0.084
IFNG	79.6	0.002	9.6	0.020	6.2	0.046	**MYD88**	1.7	0.126	1.9	0.081	1.4	0.204	**CD180**	-4.7	0.030	-2.5	0.196	-3.4	0.036
CSF3	73.7	0.001	2.0	0.241	27.8	0.000	**IRF1**	1.7	0.165	1.6	0.203	1.4	0.347	**TLR9**	-5.1	0.038	-4.2	0.128	-4.9	0.047
PTGS2	60.4	0.000	11.5	0.001	16.1	0.000	**MAP3K7IP1**	1.6	0.125	-1.0	0.970	1.3	0.574	**TLR8**	-5.8	0.057	-1.7	0.313	-1.1	0.781
IL1B	31.4	0.000	14.0	0.000	14.5	0.000	**PPARA**	1.6	0.312	1.4	0.327	1.2	0.656	**CD14**	-9.9	0.001	-1.2	0.443	-2.2	0.090
IFNB1	28.7	0.000	17.1	0.001	2.7	0.069	**IKBKB**	1.6	0.249	1.1	0.693	-1.4	0.381							
IL8	17.2	0.000	6.8	0.000	10.4	0.000	**IL10**	1.5	0.714	-1.1	0.864	2.2	0.153							
EIF2AK2	12.6	0.000	10.9	0.001	10.6	0.001	**UBE2N**	1.5	0.063	1.0	0.834	1.4	0.090							
NFKBIL1	10.3	0.003	1.9	0.095	1.3	0.273	**HSPD1**	1.5	0.223	-1.3	0.010	-1.1	0.752							
CD80	8.0	0.021	3.7	0.086	5.9	0.045	**FOS**	1.5	0.135	1.0	0.820	1.1	0.882							
RIPK2	7.9	0.000	5.1	0.002	4.7	0.000	**SIGIRR**	1.4	0.576	1.2	0.599	1.4	0.584							
TLR7	6.3	0.006	3.8	0.022	4.0	0.038	**RELA**	1.4	0.321	1.8	0.041	1.7	0.090							
CLEC4E	6.2	0.000	4.2	0.001	5.0	0.000	**CASP8**	1.3	0.430	-1.4	0.456	-1.2	0.656							
CXCL10	5.4	0.128	30.3	0.015	8.2	0.091	**MAPK8**	1.3	0.384	-1.1	0.668	1.1	0.642							
NFKBIA	5.0	0.000	2.7	0.001	2.9	0.000	**LTA**	1.3	0.793	1.5	0.224	-1.1	0.766							
IRAK2	3.8	0.002	2.3	0.010	2.4	0.005	**IFNA1**	1.2	0.504	-1.2	0.630	1.5	0.333							
NFKB1	3.1	0.006	2.1	0.000	2.6	0.002	**PRKRA**	1.2	0.487	-1.2	0.445	-1.2	0.555							

HSPA1A	3.0	0.025	1.5	0.292	1.2	0.456
TLR3	2.7	0.002	1.2	0.688	1.5	0.472
NFKB2	2.3	0.002	2.5	0.018	2.1	0.042
PELI1	2.2	0.055	1.4	0.107	1.6	0.026
TICAM1	2.2	0.016	1.5	0.026	1.4	0.193
REL	2.1	0.063	-1.2	0.353	1.3	0.404
MAP2K3	2.1	0.009	1.1	0.297	1.3	0.163
TLR5	2.0	0.073	-2.5	0.097	-1.2	0.765
TLR2	1.2	0.636	1.8	0.147	1.7	0.161
MAP3K1	1.2	0.833	-1.1	0.909	-1.1	0.862
IL2	1.1	0.775	-2.4	0.076	-1.6	0.164
ECSIT	1.1	0.894	-1.2	0.741	1.5	0.570
FADD	1.1	0.848	-1.8	0.152	-2.8	0.007
LY96	1.1	0.791	-1.1	0.303	1.3	0.096
TOLLIP	1.0	0.969	-1.1	0.781	1.1	0.785
MAPK8IP3	1.0	0.964	-1.2	0.566	1.0	0.965
HRAS	1.0	0.997	-1.0	0.961	-1.2	0.404
IL12A	-1.0	0.964	-1.3	0.465	1.6	0.082
IRF3	-1.0	0.947	-1.2	0.390	-1.4	0.215
NR2C2	-1.0	0.861	-1.2	0.368	1.2	0.378
CHUK	-1.1	0.797	-1.3	0.394	-1.5	0.101
TRAF6	-1.1	0.875	1.0	0.926	-1.1	0.440
MAP3K7	-1.1	0.770	-1.3	0.309	1.1	0.705
MAP2K4	-1.1	0.824	-1.3	0.351	-1.1	0.655
ELK1	-1.2	0.737	-1.3	0.076	-1.2	0.039
BTK	-1.3	0.680	-1.1	0.881	-1.4	0.278
NFRKB	-1.4	0.631	-1.4	0.330	-1.2	0.533
TLR10	-1.4	0.692	-1.8	0.363	-2.7	0.239
SARM1	-1.5	0.351	-1.5	0.435	1.1	0.718
TIRAP	-1.5	0.382	-1.5	0.159	-1.4	0.148
TLR6	-1.5	0.526	-1.7	0.155	-1.4	0.164
LY86	-1.6	0.032	-1.2	0.441	-1.6	0.028

	HMGB1	-1.6	0.220	-1.9	0.099	-1.7	0.287
	TLR1	-1.8	0.371	-3.1	0.012	-4.4	0.003
	IRAK1	-1.9	0.177	-1.2	0.583	-1.1	0.778
	UBE2V1	-2.0	0.113	-3.1	0.024	-1.1	0.919

8.4.6 SE preparations differentially affect monocyte viability

Differences in cell morphology induced by the different preparations were observed during the flow cytometric analyses. We therefore investigated the effects on cell viability of SE challenge with the different preparations. No cytotoxicity was observed for the LSE and EKSE preparations (Figure 8.6), but a significant reduction in cell viability was observed for cells exposed to HKSE. Analysis of cell frequency and bacterial phagocytosis by flow cytometry revealed that loss of the monocyte population accounted for the majority of this reduction with no differences in the relative uptake of HKSE or EKSE evident (data not shown).

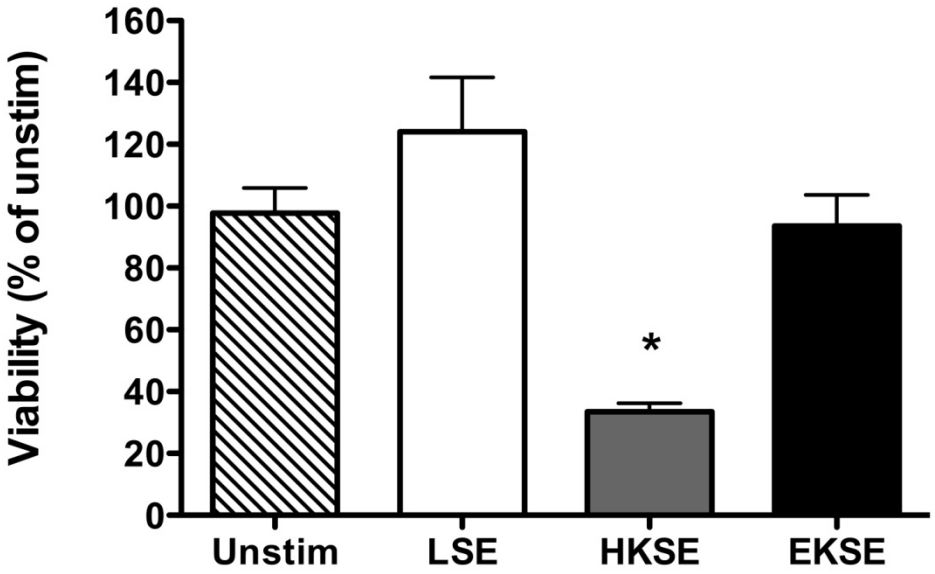

Figure 8.6 Cytotoxicity of SE preparations.

PBMC were incubated for 18 h with 10^8/ml of either: LSE, HKSE or EKSE and viability determined using the wst-1 assay (n=5). Data show mean ±SEM. *p<0.05.

8.5 Discussion

Bacteria are often killed in order to study their induction of *in vitro* immune responses as this offers the advantage of standardizing conditions and avoiding confounding effects inherent to differences in bacterial proliferation or overt contamination of the culture environment. We have demonstrated that heat- and ethanol-killing significantly alter the interaction of *S. epidermidis* with the human innate immune system. These data have important implications for the interpretation and biological relevance of studies that use killed bacterial preparations to induce *in vitro* immune responses as a model for *in vivo* responses.

Most studies of human innate immune responses to bacteria use killed bacteria or purified bacterial products, such as lipopolysaccharides or lipoproteins. The few data directly comparing the immunological properties of killed bacterial preparations to their live counterpart suggest that the killing of bacteria significantly influences the characteristics of innate immune responses.

Data derived from the stimulation of transfected HEK cell lines and both fresh human whole blood and PBMC suggest that EKSE is more TLR2-dependent in HEK cells than both LSE and HKSE. Furthermore, we observed that LSE, but not killed SE preparations, activated both the IL1-β and IFN-β pathways, both of which are associated with intracellular or cytosolic activation [343]. Analogous findings are reported for other Gram-positive organisms. For example, hemolysins secreted by live *S. aureus* (SA) induce the production of mature IL1-β in by human monocytes. Furthermore, TLR-independent activation of caspase-1 via the NLRP-3 inflammasome also requires the presence of lipoproteins, which are only secreted by live SA [320].

Heat-killed GBS does not activate TLR2 but does engage the complement/ß-integrin pathway, whereas TLR2-engagement is observed for ethanol-killed GBS and the supernatant from GBS culture [344, 345]. Unlike heat-killed GBS, live GBA activate type I interferon production in a TLR/nuclear oligomerisation domain (NOD)/RIG-like receptor-independent pathway, possibly by activation of a putative intracellular DNA sensor [346]. Killed preparations of *S. pneumoniae* also affect TLR recognition; TLR4-activity is demonstrated only for ethanol-killed [346] but not for heat-killed preparations [347]. Moreover, killed preparations of *S. pneumoniae* are inferior stimulators of the NOD2 pathway compared to live *S. pneumoniae* [348]. Heat-killed *S. pyogenes* preparations induce lower cytokine levels in human dendritic cells than live bacteria [349]. However equivalent cytokine induction is observed for heat- and antibiotic killed preparations of a number of Gram-positive and -negative pathogens [350]. Divergent results are reported for heat-killed compared to UV-killed bacteria used on human cell lines [351, 352]. Overall it appears that for extracellular bacteria and particularly Gram-positive bacteria, there are significant differences in the innate immune signalling pathways induced by live as opposed to killed organisms.

Analogous differences in signalling following killing are also reported for intracellular pathogens. *L. monocytogenes*, expresses TLR2-dependent lipoproteins [353] and is TLR2-dependent in its heat-killed form [354], whereas TLR2 may not be obligatory for recognition of the live organism [355]. Similarly live *Borrelia burgdorferi* (Bb) results in a more intense, broader and largely TLR2-independent response in human PBMC and monocytes, as well as in murine macrophages. In contrast signalling by Bb lysates is largely TLR2-dependent [356]. Only live preparations of *Chlamydia* induce secretion IL1β,

whereas IL6 and TNF induction are similar between live and heat-killed preparations [357]. Taken together, there is increasing evidence from a variety of bacteria that different killing methods, including (but not limited to) heat- and ethanol-treatment may lead to distinct patterns of release and/or loss of PAMPs, such as hemolysins, lipoproteins, and bacterial DNA, resulting in markedly differential activation of the innate immune system [358]. A general and largely consistent pattern is that experiments relying on killed bacterial preparations may over-estimate the contribution of TLR-mediated signalling and overlook other innate signalling pathways.

Our study has some limitations, most obviously that we have not extended these experiments to an *in vivo* model. However murine innate immune responses may differ significantly from those in humans and *in vivo* experimental human infections are not generally feasible [325]. Further studies might address the effect of bacterial killing on responses in various animal models, although the relevance to human disease is uncertain.

In summary, we provide evidence for significant differences in innate immune responses to live, heat- and ethanol-killed preparations of *S. epidermidis*. The different cytokine patterns resulting from differential engagement and activation of several innate immune pathways were consistently observed in both human whole blood and PBMC, with monocytes being the main responder cells. Our observations suggest that the altered recognition of killed bacteria by monocytes *per se* might dramatically significantly change overall innate immune responses. It would seem prudent to be circumspect in extrapolating these *in vitro* data to responses *in vivo*. Experimental conditions should include conditions that attempt to replicate as far as possible those occurring *in vivo*. Live bacterial preparations and whole blood may therefore be the most

biologically and clinically relevant model to employ in studies of the effects of clinically relevant human pathogens on the human innate immune system. These experimental conditions will likely assist in the interpretation of data derived from using purified ligands and purified cell lineages.

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Chapter 9

General Discussion

For the growing population of uniquely vulnerable infants, often requiring several months of hospitalisation in the neonatal intensive care unit, invasive bacterial infections remain one of the key challenges for further improvements in long-term outcomes. Gestational age at birth is the most important risk factor for nosocomial, late-onset sepsis and at the limits of viability (~23-24 weeks GA) up to 60% of infants will experience at least one episode of bloodstream infection (Figure 1.1). Late-onset sepsis significantly contributes to morbidity and mortality in this population, both directly and indirectly via the distant effects of infection-related inflammation. Coagulase-negative staphylococci, now recognised as true pathogens, are isolated in up to 80% of LOS cases, and although the associated mortality is comparatively low, these infections are not trivial, but contribute significantly to the incidence and severity of common adverse outcomes of prematurity. As approximately 80% of CoNS infections are caused by *Staphylococcus epidermidis*, we have focussed on this organism in the experiments presented in this thesis.

Despite the high incidence of LOS with SE, the relevant immunological mechanisms have received comparatively limited attention. Numerous virulence factors of SE have been elucidated, but the interaction of SE with the human immune system is incompletely defined and data on neonatal innate immune responses to this organism are particularly scarce (**Chapter 3**) [77]. In contrast to EOS, where trans-placental antibody levels correlate with the neonatal risk of infection with GBS, neonatal serum levels of anti-staphylococcal antibodies show no correlation with LOS risk [117, 119, 120]. Furthermore, prophylactic

administration to preterm infants of either pooled immunoglobulins or donor-selected anti-staphylococcal antibodies, despite significantly increased antibody serum levels, does not offer protection against LOS [121, 122, 359]. As therapeutic normalisation of immunoglobulins levels, an essential function of the adaptive immune system, does not reduce the incidence of LOS, we hypothesised that characterisation of the interaction of SE with the neonatal innate immune system will be instrumental to indentifying the immunological mechanisms that underpin the preterm infants' vulnerability to LOS.

The unique susceptibility of preterm infants to infection is often attributed to the 'immaturity' of their immune responses, but as we have outlined (**Chapter 2**), prematurity is more complex than immaturity *per se* [360]. In addition to the increased incidence of sepsis, there are additional clinical indicators of reduced immune function in preterm infants. For example, very preterm infants only very rarely show signs of *erythema toxicum neonatorum* (ETN), a condition common in term infants, that probably is reflective of skin colonisation with CoNS which invade the epidermis via hair shafts [206]. Coincident with the rapid bacterial colonisation of the skin during the first week of life, predominantly with staphylococcal species [109], healthy, non-infected term infants display increased blood levels of pro-inflammatory cytokines during that period of time [170]. The lack of clinical manifestations of ETN in preterm infants, such as erythema or raised body temperature, may reflect their inability to mount an inflammatory response. In this context, the innate immune responses to *S. epidermidis* are of particular interest for several reasons: firstly, SE constitutes the main commensal of skin and mucous membranes; secondly, despite up 1000-fold lower cutaneous bacterial load in preterm infants compared to adults, the former are at greatly increased risk of invasive infection with this organism

and thirdly, data on the interaction between the innate immune system and SE are very limited. However, the lack of clinical signs in response to bacterial colonisation is not necessarily suggestive of the complete absence of an immune response. Indeed, our group and others have shown that while preterm infants produce significantly lower levels of proinflammatory cytokines in response to various stimuli, cytokine production is not absent [160, 166]. On the contrary, there are several indicators of functional immune responses in preterm infants, including the clinical observation that in bloodstream infection even the most preterm infants typically generate significantly raised levels of C-reactive protein, which is transcriptionally controlled by IL6 [303, 361]. In addition, both innate and adaptive immune functions have the capacity to mature even in the most premature infants, as evidenced by increasing immune function with increasing GA and postnatal age [160, 166, 167]. This is further supported by the observation that preterm infants generally respond to most (though not all) routine immunisations and successfully produce adequate protective levels of neutralising antibodies [362].

While the incidence of LOS is inversely proportional to GA and several epidemiological risk factors have been identified, many preterm infants remain free of invasive infection and frequently these infants cannot be distinguished from those who acquire LOS on the basis of clinical risk factors for infection alone. This indicates that preterm infants are not uniformly ‘immunodeficient’, but that the risk of infection is determined by an intricate interaction of clinical risk factors and individual immune responsiveness.

It is recognised that exposure to perinatal inflammation, which is significantly associated with decreasing GA, alters the risk of several neonatal conditions. Preterm infants born to women with evidence of chorioamnionitis have

significantly reduced incidence and severity of respiratory disease and exposure to chorioamnionitis has been linked to increased overall survival [38, 254, 264]. In contrast, the risk of other common adverse outcomes of prematurity, such as cerebral palsy and impaired neurodevelopmental outcome, is significantly increased and has been related to the direct toxic effects of inflammation-related mediators on the development of the central nervous system [105].

Activation of the fetal immune system by perinatal inflammation is increasingly being recognised and the effects of early life influences such as inflammation on the developing immune system may be pervasive. High levels of cord blood pro-inflammatory cytokine levels are associated with a decreased risk of allergic disease in later childhood and offspring of allergic mothers who developed neonatal sepsis after term delivery had a reduced incidence of allergic diseases compared to those without neonatal infection [273, 278]. Other studies suggest that the presence of chorioamnionitis in preterm infants may be associated with increased risk of recurrent wheezing or asthma in childhood [279, 280]. In order to test the hypothesis that the risk for development of LOS in a high-risk population of very preterm infants is influenced by the exposure to histological chorioamnionitis (HCA), we conducted a large retrospective cohort study (**Chapter 4**). The results of this study clearly indicate that perinatal inflammation has significant, GA- and context-dependent effects on the maturation of the neonatal immune system and relevant clinical outcomes. Chorioamnionitis was associated with a reduced risk of LOS in preterm infants both with the commonest cause of LOS, CoNS, and with infection due to other non-CoNS bacteria. The protective effect of HCA on LOS risk is opposite to the increased risk of EOS associated with HCA in the present and previous studies [260, 261, 262]. Early-onset sepsis follows direct colonization and invasion of the neonate

with the same virulent organism that is recovered from the placenta, subsequent to vertical infection of the birth canal. Conversely the organisms causing perinatal inflammation typically are of low virulence and rarely result in clinical disease of the newborn. Instead it appears that chorioamnionitis has significant effects on postnatal immune function and maturation, and therefore indirectly affects the risk of LOS with nosocomial organisms. The mechanisms by which HCA modulates the maturation of the fetal and neonatal immune system remain unknown, but may involve transfer of bacterial ligands and/or maternal immune mediators across the placenta. Recent animal data provide support for this hypothesis; Wynn et al. demonstrated that exposure to specific *Toll*-like receptor (TLR) ligands prior to induced polymicrobial sepsis significantly improves bacterial control and survival of neonatal mice and in sheep, the intra-amniotic injection of lipopolysaccharide produces chorioamnionitis and amplifies monocyte responsiveness to TLR agonists [181, 276].

Future work should aim to understand the mechanisms by which perinatal inflammation affects postnatal immune function, as this will provide information crucial to further improvements of the management of infection and other inflammation-related adverse outcomes in extremely preterm infants. Our group is currently undertaking experiments investigating several aspects of innate immunity by comparing cellular immune responses of infants with and without exposure to chorioamnionitis. The translational potential of resulting data includes further stratification of this high-risk population with a view to prophylactic and preventative interventions, such as enhanced surveillance and targeted antibiotic therapy. Furthermore, specific TLR and other innate immune agonists maybe identified as primary mediators of immune maturation, these

may prove useful as therapeutic tools to accelerate the maturation of neonatal immunity, once the underlying pathways and the potential risks are fully understood. For example, several compounds known to have TLR-activating capacity, are being investigated for potential use as new adjuvants for neonatal vaccines [182].

Our group is currently establishing a prospective cohort study designed to further investigate the immediate and long-term immunological effects of exposure to perinatal inflammation. Importantly, this study will also provide detailed data on related clinical infectious outcomes not limited to the neonatal period but throughout infancy.

As previously outlined, appropriate innate immune responses are critical to the risk of LOS, but the underlying mechanisms and deficiencies are incompletely characterised. Studies of soluble factors of the innate immune system have described a number of deficiencies, such as low levels of placentally transferred IgG, hypocomplementaemia and reduced levels of MBL (**Chapter 2**) [360]. Antimicrobial proteins and peptides (APPs) also play an integral part of the early innate immune response to microbial invasion [123]. In addition to their direct bactericidal properties, APPs have recently been found also to interact with other key aspects of the innate immune response, including the TLR- and chemotactic pathways and these immunomodulatory functions may be as important as their antimicrobial activity [123]. Given the significance of APPs in the early innate immune response to bacterial infection and the paucity of data on APPs in neonates, we aimed to characterise the cord blood levels of the principal APPs BPI, Calprotectin, LL-37, $sPLA_2$ and HNP1-3 in term and preterm neonates (**Chapter 5**).

Our study is the first to demonstrate a broad deficiency of APPs in human cord blood with the lowest levels consistently detected in the most premature infants [124]. In the context of the common occurrence of LOS with the CoNS, the reduced levels of LL37, HNP1-3 and sPLA2 are of particular interest, as all of these have distinct activity against Gram-positive bacteria [123], and therefore might contribute to the unique susceptibility of very preterm infants to LOS.

Interestingly, the APP levels in cord blood of infants exposed to chorioamnionitis were similar to those without chorioamnionitis. In contrast, the levels of BPI and Calprotectin were significantly higher in peripheral blood of mothers with chorioamnionitis compared to those without. This discrepancy may be due to the fact that most cases of chorioamnionitis involved the maternal membranes only, and therefore, selectively induced a maternal but no detectable fetal inflammatory response. Alternatively, the lack of elevated levels of APPs may indicate the inability of the neonatal innate immune system to adequately raise the production of APPs in response to chorioamnionitis.

The findings of our study add to our understanding of the neonatal innate immune system and suggest that APP deficiency may contribute to the increased risk of invasive bacterial infection in preterm infants. Our study has some of limitations, namely the limited number of samples available for some of the APP and the fact that we were able to assess the APP levels in cord plasma only and not the APP contained in leukocytes. The postnatal ontogeny of APP production and the relationship of APP levels with the clinical risk of neonatal sepsis warrant further investigation. In fact, our group is currently recruiting very premature infants into an ongoing study that will allow an assessment of the postnatal maturation of APP levels over the first month of life. If subsequent studies demonstrate that APP levels are correlated with sepsis risk in this

population, then early determination of APP level(s) may add to risk stratification. Furthermore, the administration of recombinant APP(s), already undergoing clinical evaluation in other contexts, may offer an adjunctive prophylactic and/or therapeutic modality for preterm neonates at high risk of invasive bacterial infection [180]. Replacement of APP(s) appears promising, especially taking their additional immunomodulatory properties into consideration, but given that prophylactic treatment with immunoglobulins have been unsuccessful, the results of appropriately conducted clinical trials will need to be awaited.

One crucial factor underlying the failure of prophylactic interventions for LOS in preterm infants to date could lie in the fact that the trialled biological interventions, such as passive immunisation with immunoglobulin, does not restore monocyte responsiveness to microbial stimuli. Monocytes are able to phagocytose and intracellularly kill bacteria, present antigens to other immune cells and are the major cytokine-producing cells in the human bloodstream. In conclusion, monoyctes are not only central to the early innate immune responses but also provide the essential link to adaptive immunity.

Therefore, we next focussed on characterising essential aspects of monocyte responsiveness to the most common LOS organism, *S. epidermidis* (**Chapter 6**). The interaction of the commensal SE with the developing innate immune system and the resulting host responses have received limited attention, despite the almost universal early exposure to this organism [109]. In addition to addressing one of the key challenges for further improvements in outcome of extremely premature infants, this population displays prolonged, maturation-dependent deficiencies of innate immune responses and may therefore in itself

represent a unique biological model to study impairments in human commensal recognition.

In characterising the *in vitro* innate immune responses of newborns to SE in relation to GA, we have shown that even in extremely preterm infants, the presence of SE is reliably detected and phagocytosed, but that subsequent activation of innate immune responses are significantly impaired. In detail, our results are the first to demonstrate similar monocyte phagocytic capacity in preterm and mature neonates and adults, both in terms of the percentage of SE-phagocytosing monocytes and the number of ingested bacteria. In addition, we observed similar monocyte phagocytosis of the early-onset pathogen GBS [295]. Crucially, induction of TNF-α and IL6 by SE was confined solely to those monocytes that had ingested the bacteria, irrespective of GA. Furthermore, preterm infants' MNC are able to contain *in vitro* infection with live SE as effectively as term infants and adults.

In contrast to those preserved innate immune functions, the consequent expression of cytokine genes and secretion of cytokine protein increased in a GA-dependent manner, which was confirmed at the single cell level using flow cytometry. Importantly, this effect was observed also in fresh whole blood and persisted in isolated MNC even after supplementation with rabbit complement, indicating that inefficient opsonisation is unlikely to be an underlying mechanism.

Early response cytokines (IL1β, IL6, IL8, TNFα) are commonly associated with inflammation initiated by the innate immune system through activation of the potent transcription factor NF-kB [290]. In keeping with the limited data on SE-responses in preterm infants [166], our results indicate a significant GA-

dependent defect in production of several cytokines after stimulation of neonatal MNC with SE. These GA-related effects were neither associated with differences in expression of the principal *Toll*-like receptors for recognition of Gram-positive and Gram-negative organisms, TLR2 or TLR4, respectively, nor to a lack of activation of down-stream NF-kB or MAPK pathways. In particular, we did not detect different numbers of phospho-p65, phospho-p38 or phospho-JNK positive monocytes in preterm infants compared to term infants and adults. Therefore, the markedly reduced cytokine levels in response to SE of preterm infants are neither accounted for merely by insufficient uptake or intracellular killing of the bacteria, nor by reduced activation of the principle secondary phosphokinases.

We therefore conclude that the reduced cytokine responses induced by phagocytosis in very preterm infants may lie in impairment downstream of NF-kB activation. This profound deficiency of an essential monocyte function, is relevant not only for the activation of down-stream innate immune responses, but also for the activation of adaptive immune responses and hence, long-lasting immunity.

We have not yet been able to identify the crucial mechanism responsible for the observed GA-dependent deficiencies in innate immune function. However, based the previous findings, our group is performing ongoing mechanistic experiments aimed at delineating the principal defect. These include further analysis of downstream signalling events, including translocation of elicited signals into the nucleus and RNA stability. Another mechanism that has been implicated by studies of interferon-gamma production of cord blood T-cells involves epigenetic control of gene transcription by methylation of CpG motifs [363]. Understanding the principal mechanisms would not only provide a major

advance of basic science, but may pave the way for the design of compounds able to restore or accelerate the maturation of the deficient immune function.

The innate immune pathways relevant to the interaction of SE with the human innate immune system, particularly the neonatal immune system, are incompletely understood. Characterisation of the *Toll*-like receptor (TLR) system has paved the way for studies implicating TLR2 in recognition of Gram-positive bacteria [309, 310, 311, 312, 313]. However, additional recognition pathways of Gram-positive bacteria include ß-integrins, lectins, CD36, and nucleotide oligomerisation domain proteins 1 and 2 (NOD1 and -2), members of the NOD-like receptor (NLR) family of cytosolic sensors [317, 319, 344, 364, 365, 366]. There is evidence both in favour and against a role for TLR2 in host defence against several Gram-positive bacteria, including staphylococci [296, 318, 322, 323]. Early cytokine production following stimulation of peripheral blood MNC, TLR2-transfected human embryonic kidney cells and primary murine cells following stimulation with killed preparations of *Staphylococcus epidermidis* or SE-derived soluble factors clearly indicate engagement of the innate immune system by this organism [166, 243, 326, 327, 328, 329, 330, 331].

In order to characterise the interaction of killed preparations and live SE with TLR2, we examined both human and murine cells *in vitro* and intravenous infection of mice *in vivo*, thereby avoiding potential limitations inherent to study of killed bacteria, isolated bacteria-derived factors, or analyses relying on a single mammalian species (**Chapter 7**) [304, 325, 334]. Our results demonstrate that while in the murine *in vivo* infection model TLR2 was indispensable for recognition and clearance of SE-bacteremia, the interaction of SE with the human innate immune system is more complex and involves TLR2-dependent and additional, TLR2-independent pathways [304]. This is in keeping

with our previously described findings of significantly impaired cytokine responses of preterm infants' monocytes despite normal mRNA and surface expression of TLR2. In this set of experiments we focussed on TLR2, but based on our results, we have since started evaluating the contribution of other innate immune pathways, including nuclear oligomerization domain (NOD) receptors, NOD-like (NLR) receptors and detection of intracellular bacterial DNA. The identification of additional pathways involved in SE recognition is relevant to the understanding of the interaction of SE with the human innate immune system and has obvious translational potential. For example, detailed understanding of the critical immune pathways may be very informative for the development of new vaccine strategies and the design of novel vaccine adjuvants, as well as opening the possibility of immunomodulatory therapies that augment the host response to infection in preterm infants.

Based on our findings on the interaction of SE with various pathways of the human innate immune system, we next investigated the relevance of the method of bacterial killing on the complexity of the resulting innate immune responses (**Chapter 8**) [367]. Killed bacterial preparations and purified bacterial ligands are commonly utilised in studies of bacterial pathogenesis in preference to live bacteria to minimise experimental variability and simplify experimental conditions. However, live bacteria may engage additional mechanisms to evade or subvert immune responses and when tested *in vitro* are likely to more closely model host-pathogen interactions *in vivo* [296]. The limited available data directly comparing the immunological properties of killed bacterial preparations to their live counterparts suggest that the killing of bacteria significantly influences the characteristics of innate immune responses. Indeed, our experiments demonstrate that the induction of key pathway cytokines

downstream of distinct innate immune pathways by live (LSE), heat-killed (HKSE) and ethanol-killed (EKSE) SE preparations differ significantly. In detail, data derived from the stimulation of transfected HEK cell lines and both fresh human whole blood and PBMC suggest that EKSE is more TLR2-dependent in HEK cells than both LSE and HKSE. Furthermore, we observed that LSE, but not killed SE preparations, activated both the IL1-β and IFN-β pathways, both of which are associated with intracellular or cytosolic activation [343]. Live SE activated NFκB, STAT1, type I interferon, and inflammasome pathways. Killed preparations engaged the NFκB pathway, but had significantly lower capacity to activate other innate immune pathways.

Analogous findings are reported for other Gram-positive organisms. For example, hemolysins secreted by live *S. aureus* (SA) induce the production of mature IL1-β in by human monocytes. Furthermore, TLR-independent activation of caspase-1 via the NLRP-3 inflammasome also requires the presence of lipoproteins, which are only secreted by live SA [320]. Furthermore, heat-killed GBS does not activate TLR2 but does engage the complement/ß-integrin pathway, whereas TLR2-engagement is observed for ethanol-killed GBS and the supernatant from GBS culture [344, 345]. Unlike heat-killed GBS, live GBA activate type I interferon production in a TLR/nuclear oligomerisation domain (NOD)/RIG-like receptor-independent pathway, possibly by activation of a putative intracellular DNA sensor. Methods of killing in preparations of *S. pneumoniae* also affect TLR recognition; TLR4-activity is demonstrated only for ethanol-killed but not for heat-killed preparations [346, 347]. Moreover, killed preparations of *S. pneumoniae* are inferior stimulators of the NOD2 pathway compared to live *S. pneumoniae* [348].

One limitation of this study is the incomplete number of bacterial preparations investigated. Ideally, further methods of bacterial inactivation, such as UV-irradiation or antibiotic treatment, could have been included. However, we have characterised the immune responses to the most commonly utilised bacterial preparations, those inactivated by heat or ethanol and have provided the first data directly comparing these preparation to live SE.

In conclusion, our experiments indicate that the method of bacterial killing profoundly influences *in vitro* innate immune responses and that live organisms co-ordinately activate multiple pathways and generate a complex anti-bacterial response. For extracellular bacteria and particularly Gram-positive bacteria, there are significant differences in the innate immune signalling pathways induced by live as opposed to killed organisms. Our observations suggest that the altered recognition of killed bacteria by monocytes *per se* might dramatically change overall innate immune responses. It would seem prudent to be circumspect in extrapolating these *in vitro* data to responses *in vivo*. Therefore, our findings have some evident implications: experimental set-up should include conditions that attempt to replicate those occurring *in vivo* as far as possible. Future studies investigating the interaction of bacteria with the innate immune system should not be limited to purified bacterial ligands, killed bacteria and cell lines, but should integrate the use of fresh whole blood and whole, live bacteria. These experimental conditions will likely assist in the interpretation of data derived from using purified ligands and purified cell lineages. Taking these findings into consideration, our group has established the PREDICT study, supported by an NHMRC grant largely based on the findings presented in this thesis, which investigates various aspects of the maturation of innate immune

responses of extremely preterm infants utilising fresh cord and peripheral blood as well as live SE in real-time assays.

In summary, the data presented in this thesis contribute significantly to our understanding of the developing neonatal innate immune system, particularly relating to the interaction with the most common LOS pathogen, *S. epidermidis*. Future work aimed at further delineating the biological mechanisms underlying the preterm infants' impaired innate immune responses will be instrumental in developing new diagnostic, preventative and therapeutic avenues. In order to achieve these long-term goals, our group is currently addressing the following specific questions:

1. *Identification of the specific innate immune deficiencies relating to LOS*

In order to identify the specific mechanisms central to the preterm infants' inability to adequately respond to CoNS, our group is currently carrying out mechanistic experiments designed to address the activation of further downstream signalling pathways, nuclear translocation of the elicited signal and stability of the resulting RNA. Further understanding of the mechanisms defining the relevant immune responses in extremely preterm infants may allow for the development of new formulations of vaccines and/or vaccine adjuvants specifically targeting effective pathways.

2. *Maturation of the innate immune system and risk of late-onset sepsis*

The susceptibility of preterm infants to invasive infection most likely is caused not exclusively by a static defect in a single innate immune mechanism, but a combination of early functional deficiencies and delayed or insufficient maturation of innate immune responses. Therefore, we are currently in the final stages of recruiting extremely preterm infants into the largest and most comprehensive longitudinal study of immune maturation and its determinants to date. This study specifically studies (i) the maturation of multiple monocyte cellular responses to LOS pathogens, (ii) the postnatal ontogeny of soluble components of the innate immune system, including APPs and (iii) relates these to the risk of LOS. Importantly, this ongoing study also acknowledges our findings on the impact of bacterial preparations on the quality of *in vitro* immune responses and utilises live bacteria to assess function of fresh cord and peripheral blood in real-time assays. The findings of this study will significantly advance our understanding of immune maturation in this high-risk population and may identify innate immune parameters crucial to LOS risk that could add to risk stratification and targeted monitoring or early therapy.

3. Perinatal inflammation, immune maturation and risk of infection

Our group is currently undertaking *in vitro* experiments investigating several aspects of innate immunity by comparing cellular immune responses of infants with and without exposure to chorioamnionitis. Furthermore, we are in the process of establishing a prospective cohort study to facilitate longitudinal characterisation of the immediate and long-term effects of exposure to perinatal inflammation on immune maturation. Importantly, this study will also provide

detailed data on related clinical infectious outcomes not limited to the neonatal period but into early childhood. The results of this study will allow further understanding of the interaction of perinatal inflammation, immune maturation and risk of infection and the identification of the underlying pathways may reveal novel avenues for vaccine adjuvants or interventions aimed at immune maturation.

References

1. Laws, P.G., N. Sullivan, E. A., *Australia's mothers and babies 2004*, A.N.P.S. Unit, Editor. 2006: Sydney. p. 45-85.

2. Goldenberg, R.L., et al., *Epidemiology and causes of preterm birth.* Lancet, 2008. **371**(9606): p. 75-84.

3. Tucker, J.M., et al., *Etiologies of preterm birth in an indigent population: is prevention a logical expectation?* Obstet Gynecol, 1991. **77**(3): p. 343-7.

4. Jackson, R.A., et al., *Perinatal outcomes in singletons following in vitro fertilization: a meta-analysis.* Obstet Gynecol, 2004. **103**(3): p. 551-63.

5. Ananth, C.V., et al., *Trends in preterm birth and perinatal mortality among singletons: United States, 1989 through 2000.* Obstet Gynecol, 2005. **105**(5 Pt 1): p. 1084-91.

6. Smith, R., *Parturition.* N Engl J Med, 2007. **356**(3): p. 271-83.

7. Muglia, L.J. and M. Katz, *The enigma of spontaneous preterm birth.* N Engl J Med, 2010. **362**(6): p. 529-35.

8. McCormick, M.C., *The contribution of low birth weight to infant mortality and childhood morbidity.* N Engl J Med, 1985. **312**(2): p. 82-90.

9. Arias, E., et al., *Annual summary of vital statistics--2002.* Pediatrics, 2003. **112**(6 Pt 1): p. 1215-30.

10. Stoll, B.J., et al., *Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network.* Pediatrics, 2010. **126**(3): p. 443-56.

11. Engle, W.A., K.M. Tomashek, and C. Wallman, *"Late-preterm" infants: a population at risk.* Pediatrics, 2007. **120**(6): p. 1390-401.

12. Engle, W.A. and M.A. Kominiarek, *Late preterm infants, early term infants, and timing of elective deliveries.* Clin Perinatol, 2008. **35**(2): p. 325-41, vi.

13. Bird, T.M., et al., *Late Preterm Infants: Birth Outcomes and Health Care Utilization in the First Year.* Pediatrics, 2010. **126**(2): p. e311-319.

14. Labor, T.C.o.S., *Respiratory Morbidity in Late Preterm Births.* JAMA: The Journal of the American Medical Association, 2010. **304**(4): p. 419-425.

15. Kramer, M.S., et al., *The contribution of mild and moderate preterm birth to infant mortality. Fetal and Infant Health Study Group of the Canadian Perinatal Surveillance System.* Jama, 2000. **284**(7): p. 843-9.

16. Doyle, L.W. and P.J. Anderson, *Adult Outcome of Extremely Preterm Infants.* Pediatrics, 2010. **126**(2): p. 342-351.

17. Saroj, S. and W.D. Lex, *An overview of mortality and sequelae of preterm birth from infancy to adulthood.* The Lancet, 2008. **371**(9608): p. 261-269.

18. Bode, M.M., et al., *Outcome of Extreme Prematurity: A Prospective Comparison of 2 Regional Cohorts Born 20 Years Apart.* Pediatrics, 2009. **124**(3): p. 866-874.

19. Park, H.S., et al., *Histologic Chorioamnionitis is More Common after Spontaneous Labor than after Induced Labor at Term.* Placenta, 2010. **31**(9): p. 792-795.

20. Haddad, R., et al., *Human spontaneous labor without histologic chorioamnionitis is characterized by an acute inflammation gene expression signature.* American Journal of Obstetrics and Gynecology, 2006. **195**(2): p. 394-394.

21. Houben, M.L., et al., *The Association between Intrauterine Inflammation and Spontaneous Vaginal Delivery at Term: A Cross-Sectional Study.* PLoS One, 2009. **4**(8): p. e6572.

22. Mesiano, S., *Myometrial progesterone responsiveness and the control of human parturition.* J Soc Gynecol Investig, 2004. **11**(4): p. 193-202.

23. Varner, M.W. and M.S. Esplin, *Current understanding of genetic factors in preterm birth.* BJOG, 2005. **112 Suppl 1**: p. 28-31.

24. Romero, R., et al., *The preterm parturition syndrome.* BJOG, 2006. **113 Suppl 3**: p. 17-42.

25. Mercer, B.M., et al., *The preterm prediction study: effect of gestational age and cause of preterm birth on subsequent obstetric outcome. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network.* Am J Obstet Gynecol, 1999. **181**(5 Pt 1): p. 1216-21.

26. Copper, R.L., et al., *The preterm prediction study: maternal stress is associated with spontaneous preterm birth at less than thirty-five weeks' gestation. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network.* Am J Obstet Gynecol, 1996. **175**(5): p. 1286-92.

27. Langridge, A.T., et al., *Social and racial inequalities in preterm births in Western Australia, 1984 to 2006.* Paediatr Perinat Epidemiol. **24**(4): p. 352-62.

28. Shah, P.S., et al., *Pregnancy and neonatal outcomes of aboriginal women: a systematic review and meta-analysis.* Womens Health Issues, 2011. **21**(1): p. 28-39.

29. Goldenberg, R.L., et al., *The Alabama Preterm Birth Project: placental histology in recurrent spontaneous and indicated preterm birth.* Am J Obstet Gynecol, 2006. **195**(3): p. 792-6.

30. Romero, R., et al., *Intraamniotic infection and the onset of labor in preterm premature rupture of the membranes.* Am J Obstet Gynecol, 1988. **159**(3): p. 661-6.

31. Han, Y.W., et al., *Uncultivated bacteria as etiologic agents of intra-amniotic inflammation leading to preterm birth.* J Clin Microbiol, 2009. **47**(1): p. 38-47.

32. Gardella, C., et al., *Identification and sequencing of bacterial rDNAs in culture-negative amniotic fluid from women in premature labor.* Am J Perinatol, 2004. **21**(6): p. 319-23.

33. Romero, R., et al., *Inflammation in pregnancy: its roles in reproductive physiology, obstetrical complications, and fetal injury.* Nutr Rev, 2007. **65**(12 Pt 2): p. S194-202.

34. Romero, R., et al., *The relationship between acute inflammatory lesions of the preterm placenta and amniotic fluid microbiology.* Am J Obstet Gynecol, 1992. **166**(5): p. 1382-8.

35. Goldenberg, R.L., J.C. Hauth, and W.W. Andrews, *Intrauterine infection and preterm delivery.* N Engl J Med, 2000. **342**(20): p. 1500-7.

36. Wenstrom, K.D., et al., *Elevated second-trimester amniotic fluid interleukin-6 levels predict preterm delivery.* Am J Obstet Gynecol, 1998. **178**(3): p. 546-50.

37. Kasper, D.C., et al., *The bacterial load of Ureaplasma parvum in amniotic fluid is correlated with an increased intrauterine inflammatory response.* Diagnostic Microbiology and Infectious Disease, 2010. **67**(2): p. 117-121.

38. Lahra, M.M. and H.E. Jeffery, *A fetal response to chorioamnionitis is associated with early survival after preterm birth.* Am J Obstet Gynecol, 2004. **190**(1): p. 147-51.

39. Watts, D.H., et al., *The association of occult amniotic fluid infection with gestational age and neonatal outcome among women in preterm labor.* Obstet Gynecol, 1992. **79**(3): p. 351-7.

40. Hillier, S.L., et al., *A case-control study of chorioamnionic infection and histologic chorioamnionitis in prematurity.* N Engl J Med, 1988. **319**(15): p. 972-8.

41. Shatrov, J.G., et al., *Chorioamnionitis and cerebral palsy: a meta-analysis.* Obstet Gynecol, 2010. **116**(2 Pt 1): p. 387-92.

42. Adams-Chapman, I. and B.J. Stoll, *Neonatal infection and long-term neurodevelopmental outcome in the preterm infant.* Curr Opin Infect Dis, 2006. **19**(3): p. 290-7.

43. Aziz, N., Y.W. Cheng, and A.B. Caughey, *Neonatal outcomes in the setting of preterm premature rupture of membranes complicated by chorioamnionitis.* J Matern Fetal Neonatal Med, 2009. **22**(9): p. 780-4.

44. Berger, A., et al., *Intrauterine infection with Ureaplasma species is associated with adverse neuromotor outcome at 1 and 2 years adjusted age in preterm infants.* J Perinat Med, 2009. **37**(1): p. 72-8.

45. Steel, J.H., et al., *Bacteria and inflammatory cells in fetal membranes do not always cause preterm labor.* Pediatr Res, 2005. **57**(3): p. 404-11.

46. Winkvist, A., I. Mogren, and U. Hogberg, *Familial patterns in birth characteristics: impact on individual and population risks.* Int J Epidemiol, 1998. **27**(2): p. 248-54.

47. Porter, T.F., et al., *The risk of preterm birth across generations.* Obstet Gynecol, 1997. **90**: p. 63-7.

48. Macones, G.A., et al., *A polymorphism in the promoter region of TNF and bacterial vaginosis: preliminary evidence of gene-environment interaction in the etiology of spontaneous preterm birth.* Am J Obstet Gynecol, 2004. **190**(6): p. 1504-8; discussion 3A.

49. Engel, S.A., et al., *Risk of small-for-gestational age is associated with common anti-inflammatory cytokine polymorphisms.* Epidemiology, 2005. **16**(4): p. 478-86.

50. Jones, N.M., et al., *Interplay of cytokine polymorphisms and bacterial vaginosis in the etiology of preterm delivery.* Journal of Reproductive Immunology, 2010. **87**(1-2): p. 82-89.

51. Genc, M.R., et al., *A disproportionate increase in IL-1beta over IL-1ra in the cervicovaginal secretions of pregnant women with altered vaginal microflora correlates with preterm birth.* Am J Obstet Gynecol, 2004. **190**(5): p. 1191-7.

52. Krediet, T.G., et al., *Toll-like receptor 2 polymorphism is associated with preterm birth.* Pediatr Res, 2007. **62**(4): p. 474-6.

53. Bodamer, O.A., et al., *Evidence for an association between mannose-binding lectin 2 (MBL2) gene polymorphisms and pre-term birth.* Genet Med, 2006. **8**(8): p. 518-24.

54. Hartel, C., et al., *Polymorphisms of genes involved in innate immunity: association with preterm delivery.* Mol. Hum. Reprod., 2004. **10**(12): p. 911-915.

55. Lorenz, E., et al., *Association between the Asp299Gly polymorphisms in the Toll-like receptor 4 and premature births in the Finnish population.* Pediatr Res, 2002. **52**(3): p. 373-6.

56. Rey, G., et al., *Toll receptor 4 Asp299Gly polymorphism and its association with preterm birth and premature rupture of membranes in a South American population.* Mol Hum Reprod, 2008. **14**(9): p. 555-9.

57. Jay, D.I., et al., *Primary, secondary, and tertiary interventions to reduce the morbidity and mortality of preterm birth.* The Lancet, 2008. **371**(9607): p. 164-175.

58. Tucker, J. and W. McGuire, *Epidemiology of preterm birth.* Bmj, 2004. **329**(7467): p. 675-8.

59. Slattery, M.M. and J.J. Morrison, *Preterm delivery.* Lancet, 2002. **360**(9344): p. 1489-97.

60. Galson, S.K., *Preterm birth as a public health initiative.* Public Health Rep, 2008. **123**(5): p. 548-50.

61. Zupan, J. and E. Aahman, *Perinatal mortality for the year 2000: estimates developed by the WHO.* 2005.

62. Lawn, J.E., et al., *Why are 4 million newborn babies dying each year?* Lancet, 2004. **364**(9432): p. 399-401.

63. Thaver, D. and A.K. Zaidi, *Burden of neonatal infections in developing countries: a review of evidence from community-based studies.* Pediatr Infect Dis J, 2009. **28**(1 Suppl): p. S3-9.

64. *Clinical signs that predict severe illness in children under age 2 months: a multicentre study.* The Lancet, 2008. **371**(9607): p. 135-142.

65. Stoll, B.J. and N. Hansen, *Infections in VLBW infants: studies from the NICHD Neonatal Research Network.* Semin Perinatol, 2003. **27**(4): p. 293-301.

66. Klein, J., *Bacterial sepsis and meningitis.*, in *Infectious diseases of the fetus and newborn infant.*, J. Remington and J. Klein, Editors. 2006, WB Saunders: Philadelphia. p. 247-95.

67. Ganatra, H.A., B.J. Stoll, and A.K. Zaidi, *International perspective on early-onset neonatal sepsis.* Clin Perinatol, 2010. **37**(2): p. 501-23.

68. van den Hoogen, A., et al., *Long-term trends in the epidemiology of neonatal sepsis and antibiotic susceptibility of causative agents.* Neonatology, 2010. **97**(1): p. 22-8.

69. Verani, J.R., L. McGee, and S.J. Schrag, *Prevention of perinatal group B streptococcal disease--revised guidelines from CDC, 2010.* MMWR Recomm Rep, 2010. **59**(RR-10): p. 1-36.

70. Bizzarro, M.J., et al., *Seventy-five years of neonatal sepsis at Yale: 1928-2003.* Pediatrics, 2005. **116**(3): p. 595-602.

71. Stoll, B.J., et al., *Late-Onset Sepsis in Very Low Birth Weight Neonates: The Experience of the NICHD Neonatal Research Network.* Pediatrics, 2002. **110**(2): p. 285-291.

72. Schrag, S.J. and B.J. Stoll, *Early-onset neonatal sepsis in the era of widespread intrapartum chemoprophylaxis.* Pediatr Infect Dis J, 2006. **25**(10): p. 939-40.

73. Stoll, B.J., et al., *Very low birth weight preterm infants with early onset neonatal sepsis: the predominance of gram-negative infections continues in the National Institute of Child Health and Human Development Neonatal Research Network, 2002-2003.* Pediatr Infect Dis J, 2005. **24**(7): p. 635-9.

74. Klinger, G., et al., *Outcome of early-onset sepsis in a national cohort of very low birth weight infants.* Pediatrics. **125**(4): p. e736-40.

75. Stoll, B.J., et al., *Changes in pathogens causing early-onset sepsis in very-low-birth- weight infants.* N Engl J Med, 2002. **347**(4): p. 240-7.

76. Daley, A.J. and D. Isaacs, *Ten-year study on the effect of intrapartum antibiotic prophylaxis on early onset group B streptococcal and Escherichia coli neonatal sepsis in Australasia.* Pediatr Infect Dis J, 2004. **23**(7): p. 630-4.

77. Strunk, T., et al., *Neonatal immune responses to coagulase-negative staphylococci.* Curr Opin Infect Dis, 2007. **20**(4): p. 370-5.

78. McIntire, D.D. and K.J. Leveno, *Neonatal mortality and morbidity rates in late preterm births compared with births at term.* Obstet Gynecol, 2008. **111**(1): p. 35-41.

79. Stoll, B.J., et al., *Late-onset sepsis in very low birth weight neonates: the experience of the NICHD Neonatal Research Network.* Pediatrics, 2002. **110**(2 Pt 1): p. 285-91.

80. Isaacs, D., *A ten year, multicentre study of coagulase negative staphylococcal infections in Australasian neonatal units.* Arch Dis Child Fetal Neonatal Ed, 2003. **88**(2): p. F89-93.

81. Craft, A. and N. Finer, *Nosocomial coagulase negative staphylococcal (CoNS) catheter-related sepsis in preterm infants: definition, diagnosis, prophylaxis, and prevention.* J Perinatol, 2001. **21**(3): p. 186-92.

82. Perlman, S.E., L. Saiman, and E.L. Larson, *Risk factors for late-onset health care-associated bloodstream infections in patients in neonatal intensive care units.* Am J Infect Control, 2007. **35**(3): p. 177-82.

83. Okada, Y., et al., *Bactericidal activity against coagulase-negative staphylococci is impaired in infants receiving long-term parenteral nutrition.* Ann Surg, 2000. **231**(2): p. 276-81.

84. Okada, Y., et al., *Total parenteral nutrition directly impairs cytokine production after bacterial challenge.* J Pediatr Surg, 1999. **34**(2): p. 277-80.

85. Beck-Sague, C.M., et al., *Bloodstream infections in neonatal intensive care unit patients: results of a multicenter study.* Pediatr Infect Dis J, 1994. **13**(12): p. 1110-6.

86. Saiman, L., et al., *Risk factors for candidemia in Neonatal Intensive Care Unit patients. The National Epidemiology of Mycosis Survey study group.* Pediatr Infect Dis J, 2000. **19**(4): p. 319-24.

87. Healy, C.M., et al., *Features of invasive staphylococcal disease in neonates.* Pediatrics, 2004. **114**(4): p. 953-61.

88. Fanaroff, A.A., et al., *Incidence, presenting features, risk factors and significance of late onset septicemia in very low birth weight infants. The National Institute of Child Health and Human Development Neonatal Research Network.* Pediatr Infect Dis J, 1998. **17**(7): p. 593-8.

89. Modi, N., et al., *A case definition for national and international neonatal bloodstream infection surveillance.* Arch Dis Child Fetal Neonatal Ed, 2009. **94**(1): p. F8-12.

90. Viemann, D., et al., *Expression of toll-like receptors in neonatal sepsis.* Pediatr Res, 2005. **58**(4): p. 654-9.

91. Ng, P.C., et al., *IP-10 is an early diagnostic marker for identification of late-onset bacterial infection in preterm infants.* Pediatr Res, 2007. **61**(1): p. 93-8.

92. Ng, P.C. and H.S. Lam, *Diagnostic markers for neonatal sepsis.* Curr Opin Pediatr, 2006. **18**(2): p. 125-31.

93. Vergnano, S., et al., *Neonatal infections in England: the NeonIN surveillance network.* Arch Dis Child Fetal Neonatal Ed, 2011. **96**(1): p. F9-F14.

94. Costa, S.F., M.H. Miceli, and E.J. Anaissie, *Mucosa or skin as source of coagulase-negative staphylococcal bacteraemia?* Lancet Infect Dis, 2004. **4**(5): p. 278-86.

95. Venkatesh, M.P., F. Placencia, and L.E. Weisman, *Coagulase-negative staphylococcal infections in the neonate and child: an update.* Semin Pediatr Infect Dis, 2006. **17**(3): p. 120-7.

96. Bearman, G.M. and R.P. Wenzel, *Bacteremias: a leading cause of death.* Arch Med Res, 2005. **36**(6): p. 646-59.

97. Makhoul, I.R., et al., *Pathogen-specific early mortality in very low birth weight infants with late-onset sepsis: a national survey.* Clin Infect Dis, 2005. **40**(2): p. 218-24.

98. Downey, L.C., P.B. Smith, and D.K. Benjamin, Jr., *Risk factors and prevention of late-onset sepsis in premature infants.* Early Hum Dev, 2010. **86 Suppl 1**: p. 7-12.

99. Stoll, B.J., et al., *Neurodevelopmental and growth impairment among extremely low-birth-weight infants with neonatal infection.* Jama, 2004. **292**(19): p. 2357-65.

100. Benjamin, D.K., Jr., et al., *Neonatal candidiasis among extremely low birth weight infants: risk factors, mortality rates, and neurodevelopmental outcomes at 18 to 22 months.* Pediatrics, 2006. **117**(1): p. 84-92.

101. Gray, J.E., et al., *Coagulase-negative staphylococcal bacteremia among very low birth weight infants: relation to admission illness severity, resource use, and outcome.* Pediatrics, 1995. **95**(2): p. 225-30.

102. Payne, N.R., et al., *Marginal increase in cost and excess length of stay associated with nosocomial bloodstream infections in surviving very low birth weight infants.* Pediatrics, 2004. **114**(2): p. 348-55.

103. Leviton, A. and F.H. Gilles, *An epidemiologic study of perinatal telencephalic leucoencephalopathy in an autopsy population.* J Neurol Sci, 1973. **18**(1): p. 53-66.

104. Volpe, J.J., *Neurobiology of periventricular leukomalacia in the premature infant.* Pediatr Res, 2001. **50**(5): p. 553-62.

105. Khwaja, O. and J.J. Volpe, *Pathogenesis of cerebral white matter injury of prematurity.* Archives of Disease in Childhood - Fetal and Neonatal Edition, 2008. **93**(2): p. F153-F161.

106. Shah, D.K., et al., *Adverse neurodevelopment in preterm infants with postnatal sepsis or necrotizing enterocolitis is mediated by white matter abnormalities on magnetic resonance imaging at term.* J Pediatr, 2008. **153**(2): p. 170-5, 175 e1.

107. Kiechl-Kohlendorfer, U., et al., *Adverse neurodevelopmental outcome in preterm infants: risk factor profiles for different gestational ages.* Acta Paediatr, 2009. **98**(5): p. 792-6.

108. Barton, L., J.E. Hodgman, and Z. Pavlova, *Causes of death in the extremely low birth weight infant.* Pediatrics, 1999. **103**(2): p. 446-51.

109. Keyworth, N., M.R. Millar, and K.T. Holland, *Development of cutaneous microflora in premature neonates.* Arch Dis Child, 1992. **67**(7 Spec No): p. 797-801.

110. Adkins, B., C. Leclerc, and S. Marshall-Clarke, *Neonatal adaptive immunity comes of age.* Nat Rev Immunol, 2004. **4**(7): p. 553-64.

111. Levy, O., *Innate immunity of the newborn: basic mechanisms and clinical correlates.* Nat Rev Immunol, 2007. **7**(5): p. 379-90.

112. Mittendorf, R., et al., *Association between cerebral palsy and coagulase-negative staphylococci.* Lancet, 1999. **354**(9193): p. 1875-6.

113. Gonzalez, A., et al., *Influence of infection on patent ductus arteriosus and chronic lung disease in premature infants weighing 1000 grams or less.* J Pediatr, 1996. **128**(4): p. 470-8.

114. Bassler, D., et al., *Using a count of neonatal morbidities to predict poor outcome in extremely low birth weight infants: added role of neonatal infection.* Pediatrics, 2009. **123**(1): p. 313-8.

115. Stoll, B.J., *Infections of the neonatal infant*, in *Nelson Textbook of Pediatrics*, R.M. Kliegmann, Behrman, R.E., Jenson, H.B., Stanton, B.F., Editor. 2007, Elsevier Saunders: Philadelphia. p. 794-811.

116. Fleer, A., et al., *Opsonic defense to Staphylococcus epidermidis in the premature neonate.* J Infect Dis, 1985. **152**(5): p. 930-7.

117. Krediet, T.G., et al., *Antibody responses and opsonic activity in sera of preterm neonates with coagulase-negative staphylococcal septicemia and the effect of the administration of fresh frozen plasma.* Pediatr Res, 1998. **43**(5): p. 645-51.

118. Notarangelo, L.D., et al., *Activity of classical and alternative pathways of complement in preterm and small for gestational age infants.* Pediatr Res, 1984. **18**(3): p. 281-5.

119. Lin, F.Y., et al., *Level of maternal antibody required to protect neonates against early- onset disease caused by group B Streptococcus type Ia: a multicenter, seroepidemiology study.* J Infect Dis, 2001. **184**(8): p. 1022-8.

120. Lin, F.Y., et al., *Level of maternal IgG anti-group B streptococcus type III antibody correlated with protection of neonates against early-onset disease caused by this pathogen.* J Infect Dis, 2004. **190**(5): p. 928-34.

121. DeJonge, M., et al., *Clinical trial of safety and efficacy of INH-A21 for the prevention of nosocomial staphylococcal bloodstream infection in premature infants.* J Pediatr, 2007. **151**(3): p. 260-5, 265 e1.

122. Benjamin, D.K., Jr, et al., *A blinded, randomized, multicenter study of an intravenous Staphylococcus aureus immune globulin.* 2006. **26**(5): p. 290-295.

123. Levy, O., *Antimicrobial proteins and peptides: anti-infective molecules of mammalian leukocytes.* J Leukoc Biol, 2004. **76**(5): p. 909-925.

124. Strunk, T., et al., *Reduced levels of antimicrobial proteins and peptides in human cord blood plasma.* Arch Dis Child Fetal Neonatal Ed, 2009. **94**(3): p. F230-1.

125. Schaller-Bals, S., A. Schulze, and R. Bals, *Increased levels of antimicrobial peptides in tracheal aspirates of newborn infants during infection.* Am J Respir Crit Care Med, 2002. **165**(7): p. 992-5.

126. Starner, T.D., et al., *Expression and Activity of {beta}-Defensins and LL-37 in the Developing Human Lung.* J Immunol, 2005. **174**(3): p. 1608-1615.

127. Marchini, G., et al., *The newborn infant is protected by an innate antimicrobial barrier: peptide antibiotics are present in the skin and vernix caseosa.* Br J Dermatol, 2002. **147**(6): p. 1127-34.

128. Kai-Larsen, Y., et al., *Antimicrobial components of the neonatal gut affected upon colonization.* Pediatr Res, 2007. **61**(5 Pt 1): p. 530-6.

129. Turner, M.W., *Mannose-binding lectin: the pluripotent molecule of the innate immune system.* Immunol Today, 1996. **17**(11): p. 532-40.

130. Ip, W.K., et al., *Mannose-binding lectin enhances Toll-like receptors 2 and 6 signaling from the phagosome.* J Exp Med, 2008. **205**(1): p. 169-81.

131. Dahl, M., et al., *A Population-based Study of Morbidity and Mortality in Mannose-binding Lectin Deficiency.* J. Exp. Med., 2004. **199**(10): p. 1391-1399.

132. Sutherland, A.M., K.R. Walley, and J.A. Russell, *Polymorphisms in CD14, mannose-binding lectin, and Toll-like receptor-2 are associated with increased prevalence of infection in critically ill adults.* Crit Care Med, 2005. **33**(3): p. 638-44.

133. Garred, P., et al., *Association of mannose-binding lectin polymorphisms with sepsis and fatal outcome, in patients with systemic inflammatory response syndrome.* J Infect Dis, 2003. **188**(9): p. 1394-403.

134. Eisen, D.P. and R.M. Minchinton, *Impact of mannose-binding lectin on susceptibility to infectious diseases.* Clin Infect Dis, 2003. **37**(11): p. 1496-505.

135. Dzwonek, A.B., et al., *The role of mannose-binding lectin in susceptibility to infection in preterm neonates.* Pediatr Res, 2008. **63**(6): p. 680-5.

136. Frakking, F.N., et al., *High prevalence of mannose-binding lectin (MBL) deficiency in premature neonates.* Clin Exp Immunol, 2006. **145**(1): p. 5-12.

137. Frakking, F.N., et al., *Low mannose-binding lectin (MBL) levels in neonates with pneumonia and sepsis.* Clin Exp Immunol, 2007. **150**(2): p. 255-62.

138. Dean, M.M., et al., *Mannose binding lectin acute phase activity in patients with severe infection.* J Clin Immunol, 2005. **25**(4): p. 346-52.

139. Gessler, P., et al., *Neonatal neutropenia in low birthweight premature infants.* Am J Perinatol, 1995. **12**(1): p. 34-8.

140. Koenig, J.M. and R.D. Christensen, *Incidence, neutrophil kinetics, and natural history of neonatal neutropenia associated with maternal hypertension.* N Engl J Med, 1989. **321**(9): p. 557-562.

141. Carr, R., *Neutrophil Production and Function in Newborn Infants.* British Journal of Haematology, 2000. **110**(1): p. 18-28.

142. Falconer, A.E., R. Carr, and S.W. Edwards, *Impaired neutrophil phagocytosis in preterm neonates: lack of correlation with expression of immunoglobulin or complement receptors.* Biol Neonate, 1995. **68**(4): p. 264-9.

143. Kallman, J., et al., *Impaired phagocytosis and opsonisation towards group B streptococci in preterm neonates.* Arch Dis Child Fetal Neonatal Ed, 1998. **78**(1): p. F46-50.

144. Peden, D.B., et al., *Diminished chemiluminescent responses of polymorphonuclear leukocytes in severely and moderately preterm neonates.* J Pediatr, 1987. **111**(6 Pt 1): p. 904-6.

145. Driscoll, M.S., et al., *Longitudinal evaluation of polymorphonuclear leukocyte chemiluminescence in premature infants.* J Pediatr, 1990. **116**(3): p. 429-34.

146. Ambruso, D.R., et al., *Increased activity of the respiratory burst in cord blood neutrophils: kinetics of the NADPH oxidase enzyme system in subcellular fractions.* Pediatr Res, 1987. **21**(2): p. 205-10.

147. Yamazaki, M., et al., *Increased production of superoxide anion by neonatal polymorphonuclear leukocytes stimulated with a chemotactic peptide.* Am J Hematol, 1988. **27**(3): p. 169-73.

148. Yost, C.C., et al., *Impaired neutrophil extracellular trap (NET) formation: a novel innate immune deficiency of human neonates.* Blood, 2009. **113**(25): p. 6419-27.

149. Carr, R., et al., *Granulocyte-macrophage colony stimulating factor administered as prophylaxis for reduction of sepsis in extremely preterm, small for gestational age neonates (the PROGRAMS trial): a single-blind, multicentre, randomised controlled trial.* Lancet, 2009. **373**(9659): p. 226-33.

150. Carr, R., N. Modi, and C. Dore, *G-CSF and GM-CSF for treating or preventing neonatal infections.* Cochrane Database Syst Rev, 2003(3): p. CD003066.

151. Ahmad, M., et al., *In vivo effect of recombinant human granulocyte colony-stimulating factor on phagocytic function and oxidative burst activity in septic neutropenic neonates.* Biol Neonate, 2004. **86**(1): p. 48-54.

152. Christian Gille, B.S.L.T.C.F.P.T.O., *A new method to quantify phagocytosis and intracellular degradation using green fluorescent protein-labeled <I>Escherichia coli</I>: Comparison of cord blood*

macrophages and peripheral blood macrophages of healthy adults. Cytometry Part A, 2006. **69A**(3): p. 152-154.

153. Hallwirth, U., et al., *Monocyte switch in neonates: high phagocytic capacity and low HLA-DR expression in VLBWI are inverted during gestational aging.* Pediatric Allergy and Immunology, 2004. **15**(6): p. 513-516.

154. Jones, C.A., J.A. Holloway, and J.O. Warner, *Phenotype of fetal monocytes and B lymphocytes during the third trimester of pregnancy.* J Reprod Immunol, 2002. **56**(1-2): p. 45-60.

155. Holloway, J.A., et al., *Phenotypic analysis of circulating dendritic cells during the second half of human gestation.* Pediatr Allergy Immunol, 2009. **20**(2): p. 119-25.

156. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity.* Cell, 2006. **124**(4): p. 783-801.

157. Kawai, T. and S. Akira, *Toll-like receptor and RIG-I-like receptor signaling.* Ann N Y Acad Sci, 2008. **1143**: p. 1-20.

158. Fusunyan, R.D., et al., *Evidence for an innate immune response in the immature human intestine: toll-like receptors on fetal enterocytes.* Pediatr Res, 2001. **49**(4): p. 589-93.

159. Levy, O., et al., *Selective impairment of TLR-mediated innate immunity in human newborns: neonatal blood plasma reduces monocyte TNF-alpha induction by bacterial lipopeptides, lipopolysaccharide, and imiquimod, but preserves the response to R-848.* J Immunol, 2004. **173**(7): p. 4627-34.

160. Forster-Waldl, E., et al., *Monocyte toll-like receptor 4 expression and LPS-induced cytokine production increase during gestational aging.* Pediatr Res, 2005. **58**(1): p. 121-4.

161. Levy, O., et al., *The Adenosine System Selectively Inhibits TLR-Mediated TNF-{alpha} Production in the Human Newborn.* J Immunol, 2006. **177**(3): p. 1956-1966.

162. Yan, S.R., et al., *Role of MyD88 in diminished tumor necrosis factor alpha production by newborn mononuclear cells in response to lipopolysaccharide.* Infect Immun, 2004. **72**(3): p. 1223-9.

163. Sadeghi, K., et al., *Immaturity of infection control in preterm and term newborns is associated with impaired toll-like receptor signaling.* J Infect Dis, 2007. **195**(2): p. 296-302.

164. Lorenz, E., et al., *Association between the Asp299Gly Polymorphisms in the Toll-like Receptor 4 and Premature Births in the Finnish Population.* Pediatr Res, 2002. **52**(3): p. 373-376.

165. Vitoratos, N., et al., *Elevated circulating IL-1beta and TNF-alpha, and unaltered IL-6 in first-trimester pregnancies complicated by threatened abortion with an adverse outcome.* Mediators Inflamm, 2006. **2006**(4): p. 30485.

166. Hartel, C., et al., *Characterisation of the host inflammatory response to Staphylococcus epidermidis in neonatal whole blood.* Arch Dis Child Fetal Neonatal Ed, 2008. **93**(2): p. F140-5.

167. Strunk, T., et al., *Differential maturation of the innate immune response in human fetuses.* Pediatr Res, 2004. **56**(2): p. 219-26.

168. Schibler, K.R., et al., *Diminished transcription of interleukin-8 by monocytes from preterm neonates.* J Leukoc Biol, 1993. **53**(4): p. 399-403.

169. Yachie, A., et al., *Defective production of interleukin-6 in very small premature infants in response to bacterial pathogens.* Infect. Immun., 1992. **60**(3): p. 749-753.

170. Angelone, D.F., et al., *Innate immunity of the human newborn is polarized toward a high ratio of IL-6/TNF-alpha production in vitro and in vivo.* Pediatr Res, 2006. **60**(2): p. 205-9.

171. Kenzel, S., et al., *c-Jun kinase is a critical signaling molecule in a neonatal model of group B streptococcal sepsis.* J Immunol, 2006. **176**(5): p. 3181-8.

172. Karlsson, H., C. Hessle, and A. Rudin, *Innate immune responses of human neonatal cells to bacteria from the normal gastrointestinal flora.* Infect Immun, 2002. **70**(12): p. 6688-96.

173. Marodi, L., *Innate cellular immune responses in newborns.* Clin Immunol, 2006. **118**(2-3): p. 137-44.

174. Yerkovich, S.T., et al., *Postnatal development of monocyte cytokine responses to bacterial lipopolysaccharide.* Pediatr Res, 2007. **62**(5): p. 547-52.

175. LeBouder, E., et al., *Modulation of neonatal microbial recognition: TLR-mediated innate immune responses are specifically and differentially modulated by human milk.* J Immunol, 2006. **176**(6): p. 3742-52.

176. Bloom, B., et al., *Multicenter study to assess safety and efficacy of INH-A21, a donor-selected human staphylococcal immunoglobulin, for prevention of nosocomial infections in very low birth weight infants.* Pediatr Infect Dis J, 2005. **24**(10): p. 858-66.

177. Ohlsson, A. and J.B. Lacy, *Intravenous immunoglobulin for preventing infection in preterm and/or low-birth-weight infants.* Cochrane Database Syst Rev, 2004(1): p. CD000361.

178. Ohlsson, A. and J.B. Lacy, *Intravenous immunoglobulin for suspected or subsequently proven infection in neonates.* Cochrane Database Syst Rev, 2004(1): p. CD001239.

179. Levy, O., et al., *Impaired innate immunity in the newborn: newborn neutrophils are deficient in bactericidal/permeability-increasing protein.* Pediatrics, 1999. **104**(6): p. 1327-33.

180. Levin, M., et al., *Recombinant bactericidal/permeability-increasing protein (rBPI21) as adjunctive treatment for children with severe meningococcal sepsis: a randomised trial. rBPI21 Meningococcal Sepsis Study Group.* Lancet, 2000. **356**(9234): p. 961-7.

181. Wynn, J.L., et al., *Defective innate immunity predisposes murine neonates to poor sepsis outcome but is reversed by TLR agonists.* Blood, 2008. **112**(5): p. 1750-8.

182. Philbin, V.J. and O. Levy, *Developmental Biology of the Innate Immune Response: Implications for Neonatal and Infant Vaccine Development.* Pediatric Research, 2009. **65**(5 Part 2): p. 98R-105R 10.1203/PDR.0b013e31819f195d.

183. Wille-Reece, U., et al., *Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime-boost immunization in nonhuman primates.* J Exp Med, 2006. **203**(5): p. 1249-58.

184. Wynn, J.L., et al., *Potential of immunomodulatory agents for prevention and treatment of neonatal sepsis.* J Perinatol, 2009. **29**(2): p. 79-88.

185. Deshpande, G., S. Rao, and S. Patole, *Probiotics for prevention of necrotising enterocolitis in preterm neonates with very low birthweight: a systematic review of randomised controlled trials.* Lancet, 2007. **369**(9573): p. 1614-20.

186. Hooper, L.V. and J.I. Gordon, *Commensal host-bacterial relationships in the gut.* Science, 2001. **292**(5519): p. 1115-8.

187. Marschan, E., et al., *Probiotics in infancy induce protective immune profiles that are characteristic for chronic low-grade inflammation.* Clin Exp Allergy, 2008. **38**(4): p. 611-8.

188. Bell, S.G., *Immunomodulation, Part V: probiotics.* Neonatal Netw, 2007. **26**(1): p. 57-60.

189. Dani, C., et al., *Probiotics feeding in prevention of urinary tract infection, bacterial sepsis and necrotizing enterocolitis in preterm infants. A prospective double-blind study.* Biol Neonate, 2002. **82**(2): p. 103-8.

190. Wisplinghoff, H., et al., *Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study.* Clin Infect Dis, 2004. **39**(3): p. 309-17.

191. Yalaz, M., et al., *Neonatal nosocomial sepsis in a level-III NICU: evaluation of the causative agents and antimicrobial susceptibilities.* Turk J Pediatr, 2006. **48**(1): p. 13-8.

192. Vuong, C. and M. Otto, *Staphylococcus epidermidis infections.* Microbes Infect, 2002. **4**(4): p. 481-9.

193. Lorenz, J.M., *The outcome of extreme prematurity.* Semin Perinatol, 2001. **25**(5): p. 348-59.

194. Wilson-Costello, D., et al., *Improved Survival Rates With Increased Neurodevelopmental Disability for Extremely Low Birth Weight Infants in the 1990s.* Pediatrics, 2005. **115**(4): p. 997-1003.

195. Dizon-Townson, D.S., *Preterm labour and delivery: a genetic predisposition.* Paediatr Perinat Epidemiol, 2001. **15 Suppl 2**: p. 57-62.

196. Donoghue, D.A. and A.C. Cust. *Report of the Australian & New Zealand Neonatal Network, 1999.* 2001; Available from: www.usyd.edu.au/cphsr/anznn.

197. Ferguson, J.K. and A. Gill, *Risk-stratified nosocomial infection surveillance in a neonatal intensive care unit: report on 24 months of surveillance.* J Paediatr Child Health, 1996. **32**(6): p. 525-31.

198. Stoll, B.J., et al., *Early-onset sepsis in very low birth weight neonates: a report from the National Institute of Child Health and Human Development Neonatal Research Network.* J Pediatr, 1996. **129**(1): p. 72-80.

199. Carrieri, M.P., I. Stolfi, and M.L. Moro, *Intercenter variability and time of onset: two crucial issues in the analysis of risk factors for nosocomial sepsis.* Pediatr Infect Dis J, 2003. **22**(7): p. 599-609.

200. Weisman, L.E., *Coagulase-negative staphylococcal disease: emerging therapies for the neonatal and pediatric patient.* Curr Opin Infect Dis, 2004. **17**(3): p. 237-41.

201. Gray, J.E., et al., *Coagulase-negative staphylococcal bacteremia among very low birth weight infants: relation to admission illness severity, resource use, and outcome.* Pediatrics, 1995. **95**(2): p. 225-30.

202. Mittendorf, R., et al., *Association between cerebral palsy and coagulase-negative staphylococci.* Lancet, 1999. **354**(9193): p. 1875-6.

203. Scheifele, D.W., et al., *Delta-like toxin produced by coagulase-negative staphylococci is associated with neonatal necrotizing enterocolitis.* Infect Immun, 1987. **55**(9): p. 2268-73.

204. Maayan-Metzger, A., et al., *Clinical and laboratory impact of coagulase-negative staphylococci bacteremia in preterm infants.* Acta Paediatr, 2000. **89**(6): p. 690-3.

205. D'Angio, C.T., et al., *Surface colonization with coagulase-negative staphylococci in premature neonates.* J Pediatr, 1989. **114**(6): p. 1029-34.

206. Marchini, G., et al., *Erythema toxicum neonatorum is an innate immune response to commensal microbes penetrated into the skin of the newborn infant.* Pediatr Res, 2005. **58**(3): p. 613-6.

207. Eastick, K., et al., *Reservoirs of coagulase negative staphylococci in preterm infants.* Arch Dis Child Fetal Neonatal Ed, 1996. **74**(2): p. F99-104.

208. Haque, K.N., et al., *Pattern of culture-proven neonatal sepsis in a district general hospital in the United Kingdom.* Infect Control Hosp Epidemiol, 2004. **25**(9): p. 759-64.

209. Avila-Figueroa, C., et al., *Intravenous lipid emulsions are the major determinant of coagulase-negative staphylococcal bacteremia in very low birth weight newborns.* Pediatr Infect Dis J, 1998. **17**(1): p. 10-7.

210. Freeman, J., et al., *Association of intravenous lipid emulsion and coagulase-negative staphylococcal bacteremia in neonatal intensive care units.* N Engl J Med, 1990. **323**(5): p. 301-8.

211. Stoll, B.J., et al., *Dexamethasone therapy increases infection in very low birth weight infants.* Pediatrics, 1999. **104**(5): p. e63.

212. Wright, L.L., et al., *Antenatal corticosteroid administration and neonatal outcome in very low birth weight infants: the NICHD Neonatal Research Network.* Am J Obstet Gynecol, 1995. **173**(1): p. 269-74.

213. Okada, Y., et al., *Total parenteral nutrition directly impairs cytokine production after bacterial challenge.* J Pediatr Surg, 1999. **34**(2): p. 277-80.

214. Otto, M., *Virulence factors of the coagulase-negative staphylococci.* Front Biosci, 2004. **9**: p. 841-63.

215. Costerton, J.W., P.S. Stewart, and E.P. Greenberg, *Bacterial biofilms: a common cause of persistent infections.* Science, 1999. **284**(5418): p. 1318-22.

216. Johnson, G.M., et al., *Interference with granulocyte function by Staphylococcus epidermidis slime.* Infect Immun, 1986. **54**(1): p. 13-20.

217. Ziebuhr, W., et al., *Nosocomial infections by Staphylococcus epidermidis: how a commensal bacterium turns into a pathogen.* Int J Antimicrob Agents, 2006. **28 Suppl 1**: p. S14-20.

218. von Eiff, C., G. Peters, and C. Heilmann, *Pathogenesis of infections due to coagulase-negative staphylococci.* Lancet Infect Dis, 2002. **2**(11): p. 677-85.

219. Foster, T.J., *Immune evasion by staphylococci.* Nat Rev Microbiol, 2005. **3**(12): p. 948-58.

220. Vuong, C., et al., *Polysaccharide intercellular adhesin (PIA) protects Staphylococcus epidermidis against major components of the human innate immune system.* Cell Microbiol, 2004. **6**(3): p. 269-75.

221. Kocianova, S., et al., *Key role of poly-{gamma}-dl-glutamic acid in immune evasion and virulence of Staphylococcus epidermidis.* J. Clin. Invest., 2005. **115**(3): p. 688-694.

222. Klingenberg, C., et al., *Coagulase-negative staphylococcal sepsis in neonates. Association between antibiotic resistance, biofilm formation and the host inflammatory response.* Pediatr Infect Dis J, 2005. **24**(9): p. 817-22.

223. Mehlin, C., C.M. Headley, and S.J. Klebanoff, *An inflammatory polypeptide complex from Staphylococcus epidermidis: isolation and characterization.* J Exp Med, 1999. **189**(6): p. 907-18.

224. Liles, W.C., et al., *Stimulation of human neutrophils and monocytes by staphylococcal phenol-soluble modulin.* J Leukoc Biol, 2001. **70**(1): p. 96-102.

225. Hajjar, A.M., et al., *Cutting Edge: Functional Interactions Between Toll-Like Receptor (TLR) 2 and TLR1 or TLR6 in Response to Phenol-Soluble Modulin.* J Immunol, 2001. **166**(1): p. 15-19.

226. Klingenberg, C., et al., *Phenotypic and genotypic aminoglycoside resistance in blood culture isolates of coagulase-negative staphylococci from a single neonatal intensive care unit, 1989-2000.* J Antimicrob Chemother, 2004. **54**(5): p. 889-96.

227. Gristina, A.G., et al., *Comparative in vitro antibiotic resistance of surface-colonizing coagulase-negative staphylococci.* Antimicrob Agents Chemother, 1989. **33**(6): p. 813-6.

228. Mah, T.F. and G.A. O'Toole, *Mechanisms of biofilm resistance to antimicrobial agents.* Trends Microbiol, 2001. **9**(1): p. 34-9.

229. Yao, Y., D.E. Sturdevant, and M. Otto, *Genomewide analysis of gene expression in Staphylococcus epidermidis biofilms: insights into the pathophysiology of S. epidermidis biofilms and the role of phenol-soluble modulins in formation of biofilms.* J Infect Dis, 2005. **191**(2): p. 289-98.

230. Lewis, D.B. and C.B. Wilson, *Developmental Immunology and role of host defenses in fetal and neonatal susceptibility to infection*, in *Infectious Diseases of the fetus and newborn infant*, J.S. Remington and J. Klein, Editors. 2006, Elsevier Saunders: Philadelphia. p. 87-211.

231. Henneke, P. and R. Berner, *SIRS and group-B streptococcal sepsis in newborns: pathogenesis and perspectives in adjunctive therapy.* Semin Fetal Neonatal Med, 2006. **11**(5): p. 333-42.

232. Stroobant, J., et al., *Diminished bactericidal capacity for group B Streptococcus in neutrophils from "stressed" and healthy neonates.* Pediatr Res, 1984. **18**(7): p. 634-7.

233. Bialek, R. and P. Bartmann, *Is there an effect of immunoglobulins and G-CSF on neutrophil phagocytic activity in preterm infants?* Infection, 1998. **26**(6): p. 375-8.

234. Baker, C.J., et al., *Role of intravenous immunoglobulin in prevention of late-onset infection in low-birth-weight neonates. The Neonatal IVIG Study Group.* Rev Infect Dis, 1990. **12 Suppl 4**: p. S463-8; discussion S468-9.

235. Schutze, G.E., et al., *Role of neutrophil receptors in opsonophagocytosis of coagulase-negative staphylococci.* Infect Immun, 1991. **59**(8): p. 2573-8.

236. Correa, A.G., et al., *Immunoglobulin G enhances C3 degradation on coagulase-negative staphylococci.* Infect Immun, 1994. **62**(6): p. 2362-6.

237. Bjorkqvist, M., et al., *Defective neutrophil oxidative burst in preterm newborns on exposure to coagulase-negative staphylococci.* Pediatr Res, 2004. **55**(6): p. 966-71.

238. Takeda, K., T. Kaisho, and S. Akira, *Toll-like receptors.* Annu Rev Immunol, 2003. **21**: p. 335-76.

239. Kristinsson, K.G., J.G. Hastings, and R.C. Spencer, *The role of extracellular slime in opsonophagocytosis of Staphylococcus epidermidis.* J Med Microbiol, 1988. **27**(3): p. 207-13.

240. Augustinsson, A., et al., *Interaction of Staphylococcus epidermidis from infected hip prostheses with neutrophil granulocytes.* Scand J Infect Dis, 2001. **33**(6): p. 408-12.

241. Bayston, K., M. Tomlinson, and J. Cohen, *In-vitro stimulation of TNF-alpha from human whole blood by cell-free supernatants of gram-positive bacteria.* Cytokine, 1992. **4**(5): p. 397-402.

242. Lina, G., et al., *Coagulase-negative staphylococci isolated from two cases of toxic shock syndrome lack superantigenic activity, but induce cytokine production.* FEMS Immunol Med Microbiol, 1996. **13**(1): p. 81-6.

243. Megyeri, K., et al., *Induction of cytokine production by different Staphylococcal strains.* Cytokine, 2002. **19**(4): p. 206-12.

244. Timmerman, C.P., et al., *Induction of release of tumor necrosis factor from human monocytes by staphylococci and staphylococcal peptidoglycans.* Infect Immun, 1993. **61**(10): p. 4167-72.

245. Romero, R., et al., *Bacterial vaginosis, the inflammatory response and the risk of preterm birth: a role for genetic epidemiology in the prevention of preterm birth.* Am J Obstet Gynecol, 2004. **190**(6): p. 1509-19.

246. Schultz, C., et al., *Enhanced interleukin-6 and interleukin-8 synthesis in term and preterm infants.* Pediatr Res, 2002. **51**(3): p. 317-22.

247. Levy, O., *Innate immunity of the human newborn: distinct cytokine responses to LPS and other Toll-like receptor agonists.* J Endotoxin Res, 2005. **11**(2): p. 113-6.

248. Blander, J.M., *Signalling and phagocytosis in the orchestration of host defence.* Cell Microbiol, 2007. **9**(2): p. 290-9.

249. Parker, L.C., et al., *The expression and roles of Toll-like receptors in the biology of the human neutrophil.* J Leukoc Biol, 2005. **77**(6): p. 886-92.

250. Gilbert, W.M., T.S. Nesbitt, and B. Danielsen, *The cost of prematurity: quantification by gestational age and birth weight.* Obstet Gynecol, 2003. **102**(3): p. 488-92.

251. Mueller-Heubach, E., D.N. Rubinstein, and S.S. Schwarz, *Histologic chorioamnionitis and preterm delivery in different patient populations.* Obstet Gynecol, 1990. **75**(4): p. 622-6.

252. Elimian, A., et al., *Histologic chorioamnionitis, antenatal steroids, and perinatal outcomes.* Obstet Gynecol, 2000. **96**(3): p. 333-6.

253. Gupta, M., et al., *Impact of clinical and histologic correlates of maternal and fetal inflammatory response on gestational age in preterm births.* J Matern Fetal Neonatal Med, 2007. **20**(1): p. 39-46.

254. Lahra, M.M., P.J. Beeby, and H.E. Jeffery, *Intrauterine inflammation, neonatal sepsis, and chronic lung disease: a 13-year hospital cohort study.* Pediatrics, 2009. **123**(5): p. 1314-9.

255. Rogers, B.B., et al., *Umbilical vein interleukin-6 levels correlate with the severity of placental inflammation and gestational age.* Hum Pathol, 2002. **33**(3): p. 335-40.

256. Yoon, B.H., et al., *Interleukin-6 concentrations in umbilical cord plasma are elevated in neonates with white matter lesions associated with periventricular leukomalacia.* Am J Obstet Gynecol, 1996. **174**(5): p. 1433-40.

257. Hecht, J.L., et al., *Relationship Between Neonatal Blood Protein Concentrations and Placenta Histologic Characteristics in Extremely Low GA Newborns.* Pediatr Res, 2011. **69**(1): p. 68-73.

258. D'Alquen, D., et al., *Activation of umbilical cord endothelial cells and fetal inflammatory response in preterm infants with chorioamnionitis and funisitis.* Pediatr Res, 2005. **57**(2): p. 263-9.

259. Laborada, G. and M. Nesin, *Interleukin-6 and interleukin-8 are elevated in the cerebrospinal fluid of infants exposed to chorioamnionitis.* Biol Neonate, 2005. **88**(2): p. 136-44.

260. Korbage de Araujo, M.C., et al., *A risk factor for early-onset infection in premature newborns: invasion of chorioamniotic tissues by leukocytes.* Early Hum Dev, 1999. **56**(1): p. 1-15.

261. Gonzalez-Luis, G., et al., *[Neonatal morbidity and mortality in very low birth weight infants according to exposure to chorioamnionitis].* An Esp Pediatr, 2002. **56**(6): p. 551-5.

262. Martius, J.A., et al., *Risk factors associated with early-onset sepsis in premature infants.* Eur J Obstet Gynecol Reprod Biol, 1999. **85**(2): p. 151-8.

263. Lau, J., et al., *Chorioamnionitis with a fetal inflammatory response is associated with higher neonatal mortality, morbidity, and resource use than chorioamnionitis displaying a maternal inflammatory response only.* Am J Obstet Gynecol, 2005. **193**(3 Pt 1): p. 708-13.

264. Lahra, M.M., P.J. Beeby, and H.E. Jeffery, *Maternal versus fetal inflammation and respiratory distress syndrome: a 10-year hospital cohort study.* Arch Dis Child Fetal Neonatal Ed, 2009. **94**(1): p. F13-6.

265. Dempsey, E., et al., *Outcome of neonates less than 30 weeks gestation with histologic chorioamnionitis.* Am J Perinatol, 2005. **22**(3): p. 155-9.

266. Leviton, A., et al., *Microbiologic and histologic characteristics of the extremely preterm infant's placenta predict white matter damage and later cerebral palsy. the ELGAN study.* Pediatr Res, 2009. **67**(1): p. 95-101.

267. Lee, H.J., et al., *Chorioamnionitis, respiratory distress syndrome and bronchopulmonary dysplasia in extremely low birth weight infants.* J Perinatol, 2011. **31**(3): p. 166-70.

268. Paul, D.A., et al., *Reduced mortality and increased BPD with histological chorioamnionitis and leukocytosis in very-low-birth-weight infants.* J Perinatol, 2010. **30**(1): p. 58-62.

269. Hendson, L., et al., *Neonatal and Neurodevelopmental Outcomes of Very Low Birth Weight Infants with Histologic Chorioamnionitis.* J Pediatr, 2010.

270. Strunk, T., et al., *Innate immunity in human newborn infants: prematurity means more than immaturity.* J Matern Fetal Neonatal Med, 2011. **24**(1): p. 25-31.

271. Ragouilliaux, C.J., et al., *Maternal factors in extremely low birth weight infants who develop spontaneous intestinal perforation.* Pediatrics, 2007. **120**(6): p. e1458-64.

272. Liljedahl, M., L. Bodin, and J. Schollin, *Coagulase-negative staphylococcal sepsis as a predictor of bronchopulmonary dysplasia.* Acta Paediatr, 2004. **93**(2): p. 211-5.

273. Manami, R., et al., *Clinical sepsis in neonates is responsible for the lower prevalence of developing allergy.* Pediatrics International, 2007. **49**(1): p. 15-18.

274. Redline, R.W., *Placental inflammation.* Semin Neonatol, 2004. **9**(4): p. 265-74.

275. Allanson, B., et al., *Infection and fetal loss in the mid-second trimester of pregnancy.* Aust N Z J Obstet Gynaecol, 2010. **50**(3): p. 221-5.

276. Kramer, B.W., et al., *Intra-amniotic LPS modulation of TLR signaling in lung and blood monocytes of fetal sheep.* Innate Immun, 2009. **15**(2): p. 101-7.

277. Kim, S.K., et al., *Evidence of changes in the immunophenotype and metabolic characteristics (intracellular reactive oxygen radicals) of fetal, but not maternal, monocytes and granulocytes in the fetal inflammatory response syndrome.* J Perinat Med, 2009. **37**(5): p. 543-52.

278. Macaubas, C., et al., *Association between antenatal cytokine production and the development of atopy and asthma at age 6 years.* Lancet, 2003. **362**(9391): p. 1192-7.

279. Getahun, D., et al., *Effect of chorioamnionitis on early childhood asthma.* Arch Pediatr Adolesc Med, 2010. **164**(2): p. 187-92.

280. Kumar, R., et al., *Prematurity, chorioamnionitis, and the development of recurrent wheezing: a prospective birth cohort study.* J Allergy Clin Immunol, 2008. **121**(4): p. 878-84 e6.

281. Hecht, J.L., et al., *The relationship between neonatal blood protein profiles and placenta histologic characteristics in extremely low gestation age newborns.* Pediatr Res, 2010.

282. Schultz, C., et al., *Immature anti-inflammatory response in neonates.* Clin Exp Immunol, 2004. **135**(1): p. 130-6.

283. Schultz, C., et al., *Reduced IL-10 production and -receptor expression in neonatal T lymphocytes.* Acta Paediatr, 2007. **96**(8): p. 1122-5.

284. Nupponen, I., et al., *Extracellular release of bactericidal/permeability-increasing protein in newborn infants.* Pediatr Res, 2002. **51**(6): p. 670-4.

285. Carrieri, M.P., I. Stolfi, and M.L. Moro, *Intercenter variability and time of onset: two crucial issues in the analysis of risk factors for nosocomial sepsis.* Pediatr Infect Dis J, 2003. **22**(7): p. 599-609.

286. Kallman, J., et al., *Increase of staphylococci in neonatal septicaemia: a fourteen-year study.* Acta Paediatr, 1997. **86**(5): p. 533-8.

287. Stoll, B.J., et al., *Late-onset sepsis in very low birth weight neonates: the experience of the NICHD Neonatal Research Network.* Pediatrics, 2002. **110**(2 Pt 1): p. 285-91.

288. Wynn, J.L. and O. Levy, *Role of innate host defenses in susceptibility to early-onset neonatal sepsis.* Clin Perinatol, 2010. **37**(2): p. 307-37.

289. Cheung, G.Y. and M. Otto, *Understanding the significance of Staphylococcus epidermidis bacteremia in babies and children.* Curr Opin Infect Dis. **23**(3): p. 208-16.

290. Kenzel, S. and P. Henneke, *The innate immune system and its relevance to neonatal sepsis.* Curr Opin Infect Dis, 2006. **19**(3): p. 264-70.

291. Currie, A.J., et al., *Preterm infants have deficient monocyte and lymphocyte cytokine responses to group B streptococcus.* Infect Immun, 2011.

292. Macaubas, C., et al., *Regulation of T-helper cell responses to inhalant allergen during early childhood.* Clin Exp Allergy, 1999. **29**(9): p. 1223-31.

293. Rowe, J., et al., *High IFN-gamma production by CD8+ T cells and early sensitization among infants at high risk of atopy.* J Allergy Clin Immunol, 2004. **113**(4): p. 710-6.

294. Schmittgen, T.D. and K.J. Livak, *Analyzing real-time PCR data by the comparative C(T) method.* Nat Protoc, 2008. **3**(6): p. 1101-8.

295. Currie, A., Curtis, S., Strunk, T., Riley, K., Liyanage, K., Prescott, S., Doherty, D., Simmer, K., Richmond, P., Burgner, D., *Preterm infants have deficient monocyte and lymphocyte cytokine responses to group B streptocuccus.* Infect Immun, 2011. **in press**.

296. Watanabe, I., et al., *TLR2-mediated survival of Staphylococcus aureus in macrophages: a novel bacterial strategy against host innate immunity.* J Immunol, 2007. **178**(8): p. 4917-25.

297. Carr JA, H.J., Freedmann RI, Levan NE., *Relationship between toxic erythema and infant maturity.* Am J Dis Child, 1966. **112**(2): p. 129-134.

298. Akira Hidano, R.P., Kumiko Jitsukawa,, *Statistical Survey of Skin Changes in Japanese Neonates.* Pediatric Dermatology, 1986. **3**(2): p. 140-144.

299. Speer, C.P., et al., *Phagocytosis-associated functions in neonatal monocyte-derived macrophages.* Pediatr Res, 1988. **24**(2): p. 213-6.

300. Speer, C.P., et al., *Oxidative metabolism in cord blood monocytes and monocyte-derived macrophages.* Infect Immun, 1985. **50**(3): p. 919-21.

301. Speer, C.P., et al., *Phagocytic activities in neonatal monocytes.* Eur J Pediatr, 1986. **145**(5): p. 418-21.

302. Conly, M.E. and D.P. Speert, *Human neonatal monocyte-derived macrophages and neutrophils exhibit normal nonopsonic and opsonic*

receptor-mediated phagocytosis and superoxide anion production. Biol Neonate, 1991. **60**(6): p. 361-6.

303. Pepys, M.B. and G.M. Hirschfield, *C-reactive protein: a critical update.* J Clin Invest, 2003. **111**(12): p. 1805-12.

304. Strunk, T., et al., *TLR2 mediates recognition of live Staphylococcus epidermidis and clearance of bacteremia.* PLoS One, 2010. **5**(4): p. e10111.

305. Karchmer, A.W. and A.S. Bayer, *Methicillin-resistant Staphylococcus aureus: an evolving clinical challenge.* Clin Infect Dis, 2008. **46 Suppl 5**: p. S342-3.

306. Otto, M., *Staphylococcal biofilms.* Curr Top Microbiol Immunol, 2008. **322**: p. 207-28.

307. Gill, S.R., et al., *Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant Staphylococcus aureus strain and a biofilm-producing methicillin-resistant Staphylococcus epidermidis strain.* J Bacteriol, 2005. **187**(7): p. 2426-38.

308. Kristian, S.A., et al., *Biofilm formation induces C3a release and protects Staphylococcus epidermidis from IgG and complement deposition and from neutrophil-dependent killing.* J Infect Dis, 2008. **197**(7): p. 1028-35.

309. Wetzler, L.M., *The role of Toll-like receptor 2 in microbial disease and immunity.* Vaccine, 2003. **21 Suppl 2**: p. S55-60.

310. Royet, J. and R. Dziarski, *Peptidoglycan recognition proteins: pleiotropic sensors and effectors of antimicrobial defences.* Nat Rev Microbiol, 2007. **5**(4): p. 264-77.

311. Akira, S., *TLR signaling.* Curr Top Microbiol Immunol, 2006. **311**: p. 1-16.

312. O'Neill, L.A., *When signaling pathways collide: positive and negative regulation of toll-like receptor signal transduction.* Immunity, 2008. **29**(1): p. 12-20.

313. Henneke, P., et al., *Lipoproteins are critical TLR2 activating toxins in group B streptococcal sepsis.* J Immunol, 2008. **180**(9): p. 6149-58.

314. Cuzzola, M., et al., *Beta 2 integrins are involved in cytokine responses to whole Gram-positive bacteria.* Journal of Immunology, 2000. **164**(11): p. 5871-6.

315. Levy, O., et al., *Critical role of the complement system in Group B streptococcus-induced tumor necrosis factor alpha release.* Infect Immun, 2003. **71**: p. 6344-6353.

316. Albanyan, E.A. and M.S. Edwards, *Lectin site interaction with capsular polysaccharide mediates nonimmune phagocytosis of type III group B streptococci.* Infect Immun, 2000. **68**(10): p. 5794-802.

317. Stuart, L.M., et al., *Response to Staphylococcus aureus requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain.* J Cell Biol, 2005. **170**(3): p. 477-85.

318. Kapetanovic, R., et al., *Contribution of phagocytosis and intracellular sensing for cytokine production by Staphylococcus aureus-activated macrophages.* Infect Immun, 2007. **75**(2): p. 830-7.

319. Shaw, M.H., et al., *NOD-like receptors (NLRs): bona fide intracellular microbial sensors.* Curr Opin Immunol, 2008. **20**(4): p. 377-82.

320. Munoz-Planillo, R., et al., *A critical role for hemolysins and bacterial lipoproteins in Staphylococcus aureus-induced activation of the Nlrp3 inflammasome.* J Immunol, 2009. **183**(6): p. 3942-8.

321. Hruz, P., et al., *NOD2 contributes to cutaneous defense against Staphylococcus aureus through alpha-toxin-dependent innate immune activation.* Proc Natl Acad Sci U S A, 2009. **106**(31): p. 12873-8.

322. Skerrett, S.J., et al., *Cutting edge: myeloid differentiation factor 88 is essential for pulmonary host defense against Pseudomonas aeruginosa but not Staphylococcus aureus.* J Immunol, 2004. **172**(6): p. 3377-81.

323. Knuefermann, P., et al., *Toll-like receptor 2 mediates Staphylococcus aureus-induced myocardial dysfunction and cytokine production in the heart.* Circulation, 2004. **110**(24): p. 3693-8.

324. Takeuchi, O., K. Hoshino, and S. Akira, *Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection.* Journal of Immunology., 2000. **165**(10): p. 5392-6.

325. Mestas, J. and C.C. Hughes, *Of mice and not men: differences between mouse and human immunology.* J Immunol, 2004. **172**(5): p. 2731-8.

326. Robinson, W.A., et al., *Bacterial stimulation and granulocyte inhibition of granulopoietic factor production.* N Engl J Med, 1977. **297**(21): p. 1129-34.

327. Stuyt, R.J., et al., *Regulation of Staphylococcus epidermidis-induced IFN-gamma in whole human blood: the role of endogenous IL-18, IL-12, IL-1, and TNF.* Cytokine, 2003. **21**(2): p. 65-73.

328. Hajjar, A.M., et al., *Cutting edge: functional interactions between toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin.* Journal of Immunology., 2001. **166**(1): p. 15-9.

329. Raby, A.C., et al., *Soluble TLR2 reduces inflammation without compromising bacterial clearance by disrupting TLR2 triggering.* J Immunol, 2009. **183**(1): p. 506-17.

330. Equils, O., et al., *Toll-like receptor 2 (TLR2) and TLR9 signaling results in HIV-long terminal repeat trans-activation and HIV replication in HIV-1 transgenic mouse spleen cells: implications of simultaneous activation of TLRs on HIV replication.* J Immunol, 2003. **170**(10): p. 5159-64.

331. Otto, M., et al., *Activity of Staphylococcus epidermidis phenol-soluble modulin peptides expressed in Staphylococcus carnosus.* J Infect Dis, 2004. **190**(4): p. 748-55.

332. Stevens, N.T., et al., *Staphylococcus epidermidis Polysaccharide Intercellular Adhesin induces IL-8 expression in human astrocytes via a mechanism involving TLR2.* Cell Microbiol, 2008.

333. Natsuka, M., et al., *A polymer-type water-soluble peptidoglycan exhibited both Toll-like receptor 2- and NOD2-agonistic activities, resulting in synergistic activation of human monocytic cells.* Innate Immun, 2008. **14**(5): p. 298-308.

334. Gekara, N.O., et al., *Signals triggered by a bacterial pore-forming toxin contribute to toll-like receptor redundancy in gram-positive bacterial recognition.* J Infect Dis, 2009. **199**(1): p. 124-33.

335. Mack, D., N. Siemssen, and R. Laufs, *Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic-adherent Staphylococcus epidermidis: evidence for functional relation to intercellular adhesion.* Infect Immun, 1992. **60**(5): p. 2048-57.

336. Latz, E., et al., *Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction.* Journal of Biological Chemistry., 2002. **277**(49): p. 47834-43.

337. McLoughlin, R.M., et al., *Interplay between IFN-gamma and IL-6 signaling governs neutrophil trafficking and apoptosis during acute inflammation.* J Clin Invest, 2003. **112**(4): p. 598-607.

338. McCurdy, J.D., et al., *Cutting edge: distinct Toll-like receptor 2 activators selectively induce different classes of mediator production from human mast cells.* J Immunol, 2003. **170**(4): p. 1625-9.

339. Cole, L.E., et al., *Macrophage proinflammatory response to Francisella tularensis live vaccine strain requires coordination of multiple signaling pathways.* J Immunol, 2008. **180**(10): p. 6885-91.

340. Puel, A., et al., *Recurrent staphylococcal cellulitis and subcutaneous abscesses in a child with autoantibodies against IL-6.* J Immunol, 2008. **180**(1): p. 647-54.

341. Schaffer, A.C. and J.C. Lee, *Staphylococcal vaccines and immunotherapies.* Infect Dis Clin North Am, 2009. **23**(1): p. 153-71.

342. Ferwerda, B., et al., *Functional and genetic evidence that the Mal/TIRAP allele variant 180L has been selected by providing protection against septic shock.* Proc Natl Acad Sci U S A, 2009. **106**(25): p. 10272-7.

343. Charrel-Dennis, M., et al., *TLR-independent type I interferon induction in response to an extracellular bacterial pathogen via intracellular recognition of its DNA.* Cell Host Microbe, 2008. **4**(6): p. 543-54.

344. Levy, O., et al., *Critical role of the complement system in group B streptococcus-induced tumor necrosis factor alpha release.* Infect Immun, 2003. **71**(11): p. 6344-53.

345. Henneke, P., et al., *Novel engagement of CD14 and multiple toll-like receptors by group B streptococci.* J Immunol, 2001. **167**(12): p. 7069-76.

346. Malley, R., et al., *Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection.* Proc Natl Acad Sci U S A, 2003. **100**(4): p. 1966-71.

347. Mogensen, T.H., et al., *Live Streptococcus pneumoniae, Haemophilus influenzae, and Neisseria meningitidis activate the inflammatory response through Toll-like receptors 2, 4, and 9 in species-specific patterns.* J Leukoc Biol, 2006. **80**(2): p. 267-277.

348. Opitz, B., et al., *Nucleotide-binding Oligomerization Domain Proteins Are Innate Immune Receptors for Internalized Streptococcus pneumoniae.* J. Biol. Chem., 2004. **279**(35): p. 36426-36432.

349. Veckman, V., et al., *Streptococcus pyogenes and Lactobacillus rhamnosus differentially induce maturation and production of Th1-type cytokines and chemokines in human monocyte-derived dendritic cells.* J Leukoc Biol, 2004. **75**(5): p. 764-71.

350. Elson, G., et al., *Contribution of Toll-like receptors to the innate immune response to Gram-negative and Gram-positive bacteria.* Blood, 2007. **109**(4): p. 1574-1583.

351. Yang, J., et al., *Induction of Proinflammatory Cytokines in Human Lung Epithelial Cells during Chlamydia pneumoniae Infection.* Infect. Immun., 2003. **71**(2): p. 614-620.

352. Wong, C. and Z. Ustunol, *Mode of inactivation of probiotic bacteria affects interleukin 6 and interleukin 8 production in human intestinal epithelial-like Caco-2 cells.* J Food Prot, 2006. **69**(9): p. 2285-8.

353. Machata, S., et al., *Lipoproteins of Listeria monocytogenes are critical for virulence and TLR2-mediated immune activation.* J Immunol, 2008. **181**(3): p. 2028-35.

354. Flo, T.H., et al., *Human toll-like receptor 2 mediates monocyte activation by Listeria monocytogenes, but not by group B streptococci or lipopolysaccharide.* J Immunol, 2000. **164**(4): p. 2064-9.

355. Edelson, B.T. and E.R. Unanue, *MyD88-dependent but Toll-like receptor 2-independent innate immunity to Listeria: no role for either in macrophage listericidal activity.* J Immunol, 2002. **169**(7): p. 3869-75.

356. Salazar, J.C., et al., *Activation of human monocytes by live Borrelia burgdorferi generates TLR2-dependent and -independent responses which include induction of IFN-beta.* PLoS Pathog, 2009. **5**(5): p. e1000444.

357. Gervassi, A., et al., *Differential Regulation of Inflammatory Cytokine Secretion by Human Dendritic Cells upon Chlamydia trachomatis Infection.* Infect. Immun., 2004. **72**(12): p. 7231-7239.

358. Klein, M., et al., *Innate immunity to pneumococcal infection of the central nervous system depends on toll-like receptor (TLR) 2 and TLR4.* J Infect Dis, 2008. **198**(7): p. 1028-36.

359. Weisman, L.E., et al., *Intravenous immune globulin prophylaxis of late-onset sepsis in premature neonates.* J Pediatr, 1994. **125**(6 Pt 1): p. 922-30.

360. Strunk, T., et al., *Innate immunity in human newborn infants: prematurity means more than immaturity.* J Matern Fetal Neonatal Med, 2010.

361. Burgner, D., Howman, R., Strunk, T., Simmer, K., *CRP is a useful marker in preterm infants.* Arch Dis Child, 2010.

362. Bonhoeffer, J., C.A. Siegrist, and P.T. Heath, *Immunisation of premature infants.* Arch Dis Child, 2006. **91**(11): p. 929-35.

363. White, G.P., et al., *Differential patterns of methylation of the IFN-gamma promoter at CpG and non-CpG sites underlie differences in IFN-gamma gene expression between human neonatal and adult CD45RO- T cells.* J Immunol, 2002. **168**(6): p. 2820-7.

364. Cuzzola, M., et al., *Beta 2 integrins are involved in cytokine responses to whole Gram- positive bacteria.* J Immunol, 2000. **164**(11): p. 5871-6.

365. Albanyan, E.A. and M.S. Edwards, *Lectin site interaction with capsular polysaccharide mediates nonimmune phagocytosis of type III group B streptococci.* Infect Immun, 2000. **68**(10): p. 5794-802.

366. Kapetanovic, R., et al., *Contribution of Phagocytosis and Intracellular Sensing for Cytokine Production by Staphylococcus aureus-Activated Macrophages.* Infect. Immun., 2007. **75**(2): p. 830-837.

367. Strunk, T., et al., *Method of bacterial killing differentially affects the human innate immune response to Staphylococcus epidermidis.* Innate Immun, 2010.