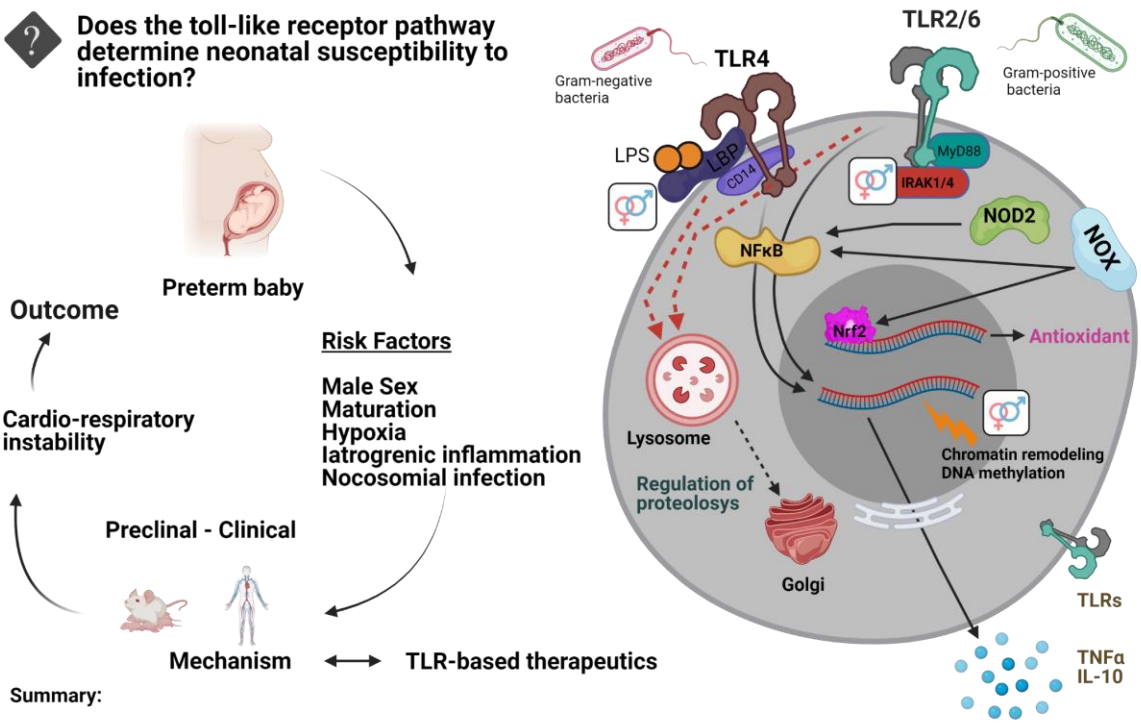


Summary:

Toll-like receptor expression is developmentally regulated. TLR signalling pathway exhibits sex-specific modulation. TLR activation and consequential cellular signalling influences the response to early life infection. Downstream pathways of TLRs signalling are potential therapeutic targets in the treatment of neonatal diseases

Targeting the toll-like receptor pathway as a therapeutic strategy for neonatal infection.

Maria L. Dias[1], Karen M. O'Connor[1], Eugene M. Dempsey[2,3], Ken D. O'Halloran[1,2] and Fiona B. McDonald[1,2]

1 Department of Physiology, School of Medicine, College of Medicine & Health, University College Cork, Cork, Ireland.
2 Irish Centre for Maternal and Child Health Research (INFANT), University College Cork, Cork, Ireland.
3. Department of Pediatrics & Child Health, School of Medicine, College of Medicine & Health, University College Cork, Cork, Ireland.

Corresponding Author

Dr. Fiona McDonald, BSc. PhD,
Department of Physiology, Room 3.82,
School of Medicine,
College of Medicine and Health,
University College Cork,
Western Gateway Building,
Western Road,
Cork, Ireland.
Tel: +353(0)21 4205432
Email: Fiona.mcdonald@ucc.ie

Contribution of the review to literature:

This review investigates available data on the role of toll-like receptors (TLRs) in the innate immune response in the neonate. We examine the control of TLR expression, aspects of sexual dimorphisms as well as activation during bacterial infection, focusing on early life data. We consider the consequences of TLR activation on redox status and cardiorespiratory function. Finally, we review existing therapeutic strategies that modulate pattern recognition receptor pathways. Novel effective treatment strategies for neonatal infection would positively impact the outcomes in new-borns most at risk. Thus, we highlight the role of TLRs as vital receptors of the innate immune system and future therapies that will likely target TLR signalling networks.

Short summary of review:

Toll-like receptors (TLRs) modulate the response to infection from Gram-positive and -negative bacteria and participate in a complex signalling network that involves accessory and adaptor proteins and ultimately the production of proinflammatory cytokines. New-borns, especially premature babies, are highly vulnerable to infection, which in some cases leads to a severe systemic inflammation. Herein, we present studies that provide evidence of time- and sex-dependent TLR-mediated responses in neonates.

Abstract

Toll-like receptors (TLRs) are crucial transmembrane receptors that form part of the innate immune response. They play a role in the recognition of various microorganisms and their elimination from the host. TLRs have been proposed as vital immunomodulators in the regulation of multiple neonatal stressors that extend beyond infection such as oxidative stress and pain. The immune system is immature at birth and takes some time to become fully established. As such, babies are especially vulnerable to sepsis at this early stage of life. Findings suggest a gestational age-dependent increase in TLR expression. TLRs engage with accessory and adaptor proteins to facilitate recognition of pathogens and their activation of the receptor. TLRs are generally upregulated during infection and promote the transcription and release of proinflammatory cytokines. Several studies report that TLRs are epigenetically modulated by chromatin changes and promoter methylation upon bacterial infection which have long-term influences on immune responses. TLR activation is reported to modulate cardiorespiratory responses during infection and may play a key role in driving homeostatic instability observed during sepsis. Although complex, TLR signalling and downstream pathways are potential therapeutic targets in the treatment of neonatal diseases. By reviewing the expression and function of key toll-like receptors**,** we aim to provide an important framework to understand the functional role of these receptors in response to stress and infection in premature infants.

KEY WORDS: Inflammation, sepsis, early life, preterm, cardiorespiratory.

1. Introduction

Early life requires complex physiological integration and adaptation to maintain homeostasis. It is now apparent that the migration, growth and maturation of our cells and the connections and networks that they form, are fine-tuned during early life. Preterm birth is defined by the World Health Organization (WHO) as babies that are born before 37 complete weeks of gestation (Vogel *et al.*, 2018). There is an increasing number of infants born extremely preterm and with very low birth weight annually. This relatively new cohort of infants are at increased risk of infection, which is defined as a physiological reaction to a pathogenic microorganism (Steiner *et al.*, 2019). Infection in extremely preterm infants threatens every element of physiological homeostasis. Pathogen recognition receptors (PRRs) are a group of receptors present on a wide variety of cell types that appear to have important roles in establishing, defending and maintaining a homeostatic environment. Pathogen recognition receptors form part of the innate immune system and the neonatal period is a time during which there is a heavy reliance on the innate immune system to defend the host. These receptors are potential therapeutic targets for immune modulation. Establishing an understanding of their activation, downstream signalling and feedback regulation is important within that context. Pathogen recognition receptors exhibit dynamic expression during development and in response to both pathogenic infection and endogenous damage associated molecular patterns. Uncovering the full extent of pathogen recognition receptor expression, their non-immune functions during early life and their contribution to innate immunity might enable patient stratification and/or targeted drug therapy in the preterm infant population. Much of what we know about the PRR signalling pathways is a result of ex vivo experiments using primary cell culture or cell lines which have provided a wealth of information but have inherent limitations due to the artificial experimental conditions. Stimulation of PRRs in microenvironments that are important for cardiorespiratory control may modulate the performance of those systems. Most integrative physiological studies have utilised animals to examine the physiological response when challenged with various bacterial and viral-derived molecular patterns such as lipopolysaccharide, peptidoglycan and polyinosinic-polycytidylic acid.

In this review, aimed at both clinical scientists and integrative physiologists, we reflect on the unmet clinical problem of neonatal infection. We focus on one of the four main families of PRRs, the toll-like receptors (TLRs) and discuss their function in health and disease (Kawai & Akira, 2009). We present current understanding of TLR signalling pathways and regulation of their expression. We examine the implications for activation of TLRs on cardiorespiratory function, systems primarily responsible for maintenance of acid-base balance and tissue perfusion and oxygenation. Lastly, we will discuss the opportunities of TLRs as novel therapeutic targets in diagnosis and treatment,

limiting the devastating short- and long-term outcomes of sepsis and identify future avenues that need to be explored.

2. Innate Immune System

The innate immune system is identified as a collection of factors, both cell-associated and cell-free, that comprise an impressively effective and well-organised network capable of immediate recognition of an extensive array of microbial components. During a normal pregnancy innate immunity matures with gestational age and is present at birth prior to microbial exposure (Yu *et al.*, 2018). The innate immune system forms the front-line physiological defence against microbes. The integrity of the innate immune system during the first 3 months of life is critical at a time when the baby lacks immunological memory important in mature adaptive immune responses (Olin *et al.*, 2018) (Ygberg & Nilsson, 2012; Idzikowski & Connors, 2020). Passive immunity transferred to the off-spring from the mother is one component of innate immunity that confers protection against microbes (Bussel *et al.*, 1988; Imler & Hoffmann, 2001; Molès et al., 2016). Maternal neutralising antibodies and other beneficial proteins such as human β-defensin (hBD-1) are transferred passively to the baby *via* breastfeeding. Maternal vaccination schemes such as influenza and whooping cough vaccinations are encouraged during pregnancy to confer further protection to the baby initially after birth (Gkentzi *et al.*, 2017; Barrett *et al.*, 2018). The innate immune system initiates an immunocompetent response to many microorganisms including Gram-negative (GN) and Gram-positive (GP) bacteria, fungi and viruses (Wynn & Levy, 2010). Innate immune protection is dependent on the identification of conserved molecular patterns by pattern recognition receptors PRRs such as TLRs (Fitzgerald & Kagan, 2020). Pathogen or microbe-associated molecular patterns (PAMPs) are expressed by invading pathogens whilst damage/danger associated molecular patterns (DAMPs) are released during sterile infection/injury. DAMPs constitute the components produced by dying or damaged cells such as cytokines and intracellular proteins whereas PAMPs represent the bacterial cell wall molecules, flagellin and nucleic acids from invading microbes (Figure 1). There is likely co-operation between PAMPs and DAMPs and significant cross talk between members of the TLR family (Takeda & Akira, 2015; Sharma & Naidu, 2016). In new-borns, TLR insufficiency increases the predisposition to infectious diseases such as sepsis, highlighting the significant reliance on innate immunity (Kollmann *et al.*, 2017).

2.1 Neonatal pathogenic and sterile inflammation

Increased inflammation is considered a risk factor and a promotor of preterm birth. Activation of the innate immune system in utero changes the expression of PRRs and consequently shapes the

immune system (Kirsten *et al.*, 2013; Yan *et al.*, 2019). Infants exposed to in utero infection such as chorioamnionitis, or exposed to chronic maternal infection, have distinct innate immunity compared to control infants (Dauby *et al.*, 2012; Bermick *et al.*, 2019). A recent study in a large animal model of fetal growth restriction recapitulated neonatal vulnerability to infection (Bæk *et al.*, 2020). Infants born premature after hypertensive disorders with fetal growth restriction (FGR) or after FGR alone, are more likely to be diagnosed with late onset infection (Letouzey *et al.*, 2021), pointing to not only microbial-mediated inflammation but potentially sterile inflammation changing the immune landscape. Sterile inflammation is classified as inflammation in the absence of infection; immune cells and their signalling molecules are responsible for this inflammation and can impact in the onset of preterm labor or birth. Important endogenous molecules such as alarmins (HMGB1, HSP) released by damaged cells and PRRs induce sterile inflammation and may play important roles in the development of innate immunity during early life (Negishi *et al.*, 2021). The nature of the immune stimulation and the timing, severity, and duration of TLR activation will determine the response to infection and the long-term outcomes.

Compared with term babies, premature infants have reduced levels of immunoglobulin (Ig) G, poor presentation of foreign antigens to cells of the adaptive system, which is dependent on cellular activation promoted by production of cytokines and chemokines (Cuenca *et al.*, 2013; M. Rueda et al., 2015; Ozdemir *et al.*, 2015). Neonatal dendritic cells from term and preterm infants require increased stimulation for efficient activation. Impaired innate immune activity has been characterised by reduced innate cytokine and monocyte response, impaired neutrophil activity, decreased lung epithelial cell repair and altered response to endotoxin lipopolysaccharide (LPS) (Bizzarro *et al.*, 2008; Ozdemir *et al.*, 2015;). Inflammation participates in the pathogenesis of neonatal brain damage, which may be precipitated by several deficiencies in the neonatal immune response, including decreased phagocytes, T cells, B cells and antigen-presenting cells (APCs) compared with adult (Kumral *et al.*, 2012; Cho *et al.*, 2019). However, it is likely that the neonatal immune system, though functionally capable of mounting an immune response, is primed towards a stronger anti-inflammatory response (Dong & Speer, 2015). Studies that examined cytokine release in plasma, point toward a humoral agent such as adenosine that modulates the immune response in favour of anti-inflammatory factors (Uematsu *et al.*, 2005). For example, very preterm infants with late onset infection have a significant inflammatory response but may be dominated by anti-inflammatory response as an increase in IL-10/TNF-α ratio (Hibbert *et al.*, 2020).

Early life infection claims the lives of nearly three thousand neonates worldwide every day (E Lawn , S. Cousens, 2005). Preterm and very low birth weight new-borns are at increased risk of developing significant infections compared to term new-borns (Escalante *et al.*, 2018; Steiner *et al.*, 2019, Viemann *et al.*, 2005). Premature infants often endure a difficult transition after birth as they are physiologically maladapted to the extrauterine environment and lack stable physiological integration that maintains homeostasis. Premature babies therefore often require extended hospital care with the need for invasive procedures that put them at risk of sepsis such as central venous cannulation, arterial line placement and endotracheal intubation (Bion *et al.*, 2013; Dong & Speer, 2015). A neonate with suspected infection will typically initially present clinically with signs of unstable cardio-respiratory function such as respiratory distress (tachypnea or apnea), hypotension and bradycardia or tachycardia. Other features may include lethargy, or feeding intolerance and these clinical presentations require prompt evaluation and treatment (Bizzarro *et al.*, 2008; Ohlin *et al.*, 2010; Escribano García *et al.*, 2018). Elevated C-reactive protein associated with positive blood culture results often confirm cases of infection (Ohlin *et al.*, 2010) but other biomarkers have been proposed by others and discussed in previous reviews (Ng & Lam, 2010; O'Connor *et al.*, 2021).

Sepsis is typically described as life-threatening organ dysfunction caused by a dysregulated response to infection, though there is no consensus on a neonatal-specific definition (Molloy *et al.*, 2020). Differential definitions of neonatal sepsis amongst leading medical organisations makes epidemiological interpretation of acute and chronic implications difficult (McGovern *et al.*, 2020). Neonatal sepsis is categorised into either early onset sepsis (EOS) or late onset sepsis (LOS). EOS occurs during the first 24-72 hours of life in the preterm infant and is often a fulminant multisystem infection acquired by vertical transmission from the mother (Meier & Engstrom, 2007). There are two major bacteria that are responsible for EONS: G*roup B streptococcus* (GBS) and *Escherichia coli* confirmed in 41% and 17% respectively from a total of 408 samples taken from infected infants within the first week of life (Hyde *et al.*, 2011, Cailes et al 2018). LOS is one of the most common causes of morbidity and mortality in preterm infants. In a recent UK study, *Escherichia coli accounted for 32% of the* Gram negative (GN) bacteria identified in LOS whereas *Staphylococcus aureus* (S. aureus) was the most common Gram positive (GP) bacteria causing LOS accounting for 31% of GP cases (Cailes *et al.*, 2018). It has been reported that GN infections are more likely to result in death within 72 hours after birth (29%) compared with GP infections (6%) (Stoll *et al.*, 2002). The differential outcomes from neonatal infection derived from GN versus GP infections may reflect the distinct TLR mediated recognition of these bacteria and the cell and tissue expression of those TLRs.

Inflammation in the neonate is not only driven by microbial-induced TLR activation but can be activated by endogenous molecular patterns such as neutrophil elastase and serum amyloid A. Redox homeostasis is essential for normal function and survival of cells. Imbalance between pro-oxidant and antioxidant abundance generates a toxic cell environment, leading to tissue damage, which has adverse consequences for the 'normal' development of a new-born, especially preterm infants. An infant's first breath is metabolically characterised by a swift change to an oxidant environment, the partial pressure of oxygen changes significantly from 35 mmHg (umbilical vein) to 85 mmHg (arterial) (Dear, 1987). Preterm infants commonly have an altered redox state in the hours following birth frequently explained by metabolic immaturity or clinical treatments, including oxygen therapy and parenteral nutrition (Mohamed *et al.*, 2015). New-born infants, and preterm infants in particular, have weak antioxidant defences and excessive production of free radicals can result in higher levels of oxidative stress. Activation of TLR mediated inflammation in response to ischemia/reperfusion injury or haemorrhagic shock/resuscitation has been reviewed by Gill et al., (2010) in which they propose both direct activation of TLRs by redox stress or indirectly via DAMPs (Gill et al., 2010).

2.2 Male sex as a risk-factor for late onset infection

Male sex is a risk factor for late onset sepsis (Benjamin *et al.*, 2006; Cortese *et al.*, 2016; Rafi *et al.*, 2020). It is widely acknowledged that male neonates have poorer disease prognosis and unfavourable outcomes upon microbial infection compared to females (Bentsen *et al.*, 2017; Bairam *et al.*, 2018). Androgens are suggested to enhance susceptibility whilst female gonadal hormones oestrogen and progesterone are reported to offer resilience as reflected in animal studies (Angele et all, 1997; Boukari *et al.*, 2017; El-Lakany *et al.*, 2018; Di Florio *et al.*, 2020). The role of sex hormones in modulating the immune response has been recently reviewed by Di Florio et al, (2020) and they propose hormonal modulation of TLR signalling is mediated by the TNF pathway. Sexual dimorphisms within the immune system of preterm neonates may account for the sex disparity in the incidence of several morbidities observed in this population, including bronchopulmonary dysplasia (BPD), Necrotising enterocolitis (NEC), retinopathy of prematurity and cerebral haemorrhage (Lavoie & Tremblay, 2018). These epidemiological findings of prenatal and sex-associated risk factors suggest that the neonatal population is already a heterogeneous population at the time of birth and subsequent environmental stressors such as hypoxia further shape the patient's development and intrinsic risk of severe infection or sepsis.

Ng and colleagues have reported upregulation of PRR pathways in patients with late onset sepsis that were born very preterm (Ng *et al.*, 2020). Preterm infants often have sterile infection as a result

of oxidative stress and local injury that activates the innate immune system (Melville & Moss, 2013). This population have a low reserve of antioxidants and are particularly at risk of oxidative stress (Ozsurekci & Aykac, 2016). There is significant interplay between cell redox state and immune regulation that will be discussed later in the review. The release of endogenous ligands for PRRs can modulate the innate immune system with ramifications for homeostatic balance and developmental growth and maturation (Yu *et al.*, 2018; Idzikowski & Connors, 2020). The activation of the innate immune system is complex and requires multiple levels of crosstalk between membrane receptors and effector proteins in order to produce a normal immune response (Cuenca *et al.*, 2013). Uncovering the expression and functional responsiveness of toll-like receptors during the neonatal period as well as their role in initiating and potential resolve of infection is an important step in understanding the pathophysiology of infection in preterm infants. Moreover, activation of TLRs is most closely associated with the induction of pro-inflammatory gene expression, however, has also been described to be modulated following TLR engagement (Sharma *et al.*, 2012). An additional level of complexity is thus associated with TLR transcriptional regulation, and the participation of a variety of accessory proteins and acting factors contribute to the innate immune system response to infections. Elucidating the immune responses related to TLR signalling by bacterial infection and indeed endogenous DAMPs may provide the key to understanding the reasons for preterm infant disease susceptibility.

3. Toll-Like Receptor Signalling

In 1996, TLRs were first identified as essential receptors for dorsoventral patterning that were demonstrated to improve the immune response in the developing embryo of *Drosophila* (Lemaitre *et al.*, 1996). Research in the *Drosophila* continues to reveal important functional roles for TLRs such as modulating cell survival and proliferation (Li *et al.*, 2020). The first mammalian homolog receptor of the *Drosophila* was identified in 1997 and named hToll, now termed TLR4 (Medzhitov *et al.*, 1997). There are now ten TLR subtypes identified in humans (TLR1-TLR10) and twelve TLR subtypes identified in mice and rats (TLR1-TLR9 and TLR11-TLR13) (Takeda & Akira, 2015). These receptors enable multi-layered protection and are located either externally on the surface membrane and/or internally in the cytosol and endosomes depending on the specific subtype (Figure 1) (Fleer & Krediet, 2007; Kawai & Akira, 2009; Mielcarska *et al.*, 2021) TLRs have been identified on multiple cell types, including endothelial and epithelial cells, and on immune cells such as macrophages, monocytes, dendritic cells, neutrophils and microglia. TLR expression is not limited to immune cells but are also expressed on endothelial, epithelial cells, neuronal cells, neuronal progenitor cells, astrocytes, oligodendrocytes, satellite cells, myocytes and platelets (Figure 2) (Zong et al., 2013; Bauer et al., 2014; Jung et al., 2018).

3.1 Toll-like receptor formation

The C-terminal cytoplasmatic domain of all mammalian TLRs shows high similarity to that of the interleukin-1 (IL-1) receptor family and is known as the Toll/IL-1 receptor (TIR) domain, which is required to initiate downstream signalling through homo- or heterotypic dimerisation (Sanchez, 2013). The extracellular domains are responsible for binding PAMPs and each TLR subtype recognises specific patterns of microbial components upon infection (Figure 1) (Frasca & Lande, 2020). X-ray crystallography supports the hypothesis that ligand-binding induces dimerisation of the extracellular domains, which leads to juxtaposition that activates the intracellular TIR domain and induces the activation of downstream signalling cascades (Sanchez, 2013). TLR1-6, 9 and 13 present as monomers in solution and dimerisation takes place upon ligand-binding; conversely, TLR7 and 8 exist as preformed dimers that subsequently change conformation (Takeda & Akira, 2015), though recent studies suggest that TLR1/2 and TLR2/6 are also present as preformed dimers (Jin *et al.*, 2007; Cheng *et al.*, 2015). Thus, the ligand-recognition and activation mechanism upon ligand binding are dependent on the structural rearrangement and availability of TLRs on the cell membrane. Toll-like Receptor 4 recognises GN bacterial protein LPS and has been the most extensively studied TLR subtype, while GP bacterial infection is primarily mediated through TLR2 by its recognition of lipotechoic acid (LTA) and peptidoglycan (PGN) (Figure 1). Toll-like receptor 4 and TLR2 will be prominent in our discussion but we will also extend the discussion to other subtypes where appropriate.

3.2 Toll-like receptor accessory proteins

Optimal TLR-mediated pathogen recognition depends on vital accessory proteins such as lipopolysaccharide-binding protein (LBP), which extracts LPS monomers from GN bacteria and delivers them to the endotoxin receptor on the monocyte surface. The specific receptor that recognises LPS is then activated by formation of a cluster of differentiation 14 (CD14)/Myeloid differentiation primary response 88 (MyD88)/TLR4 complex. CD14 is generally localised to lipid rafts in the plasma membrane. This complex is required for LPS-mediated signalling through TLR4 and is upregulated during neonatal bacterial sepsis (Behrendt *et al.*, 2004). Common polymorphisms in the LBP gene expression are associated with an increased risk of sepsis from GN infection in adult males compared to females (Hubacek *et al.*, 2001). Novel variants in LBP have also been identified in preterm infants with bronchopulmonary dysplasia (Carrera *et al.*, 2015) .

Co-operation of TLRs with other receptors has also been reported. TLR2 is crucial for recognition of PGN and is known to form heterodimers with TLR1 and TLR6 that bind lipoproteins depending on whether they are tri- or diacylated, respectively (Triantafilou *et al.*, 2006). However, the detection of

GP bacteria relies on both TLR2 and the nucleotide oligomerisation domain (NOD) leucine-rich repeat containing family 2. NOD2 are mainly intracellular innate immune sensors involved in host defence with crucial roles in promoting specificity of protein recognition, enhancing antigen binding and maintaining intracellular crosstalk. Nucleotide oligomerisation domain 2 (NOD2) has been reported to be an essential sensor for PGN through muropeptide detection (Volz *et al.*, 2010). Both TLR2 and NOD2 co-operate to upregulate signalling pathways that promote inflammation and are non-redundant pathways (Ferwerda *et al.*, 2005; Chen *et al.*, 2008). Many studies suggest NOD2 plays a role in regulating inflammation in trophoblasts, astrocytes and intestinal epithelial cells (Girardin *et al.*, 2003; de Souza *et al.*, 2018; Singh *et al.*, 2018; Mulla *et al.*, 2019). NOD2 may also act downstream of TLR2, along the TLR2-NOD2-RIPK2 pathway, whereby NOD2 plays a key role in increasing IL-10 expression in response to *Streptococcus pneumonia* (Moreira *et al.*, 2008). In support of a role for NOD2 as a negative regulator of inflammation, Udden et al (2017) report that $NOD2^{-/-}$ mice are highly susceptible to colorectal tumorigenesis with significantly higher expression of inflammatory genes and greater proliferation of intestinal epithelial cells, suggesting that NOD2 supresses colorectal tumorigenesis by downregulation of NFκB and mitogen-activated protein kinase, blunting the proliferation of cancerous cells (Udden et al., 2017).

Crosstalk between NOD2 and another TLR subtype, TLR4, has been reported in the regulation of intestinal inflammation. It is suggested that NOD2 senses the intensity of TLR4-mediated signalling, resulting in either synergistic stimulation of IL-12, a cytokine crucial for stimulating adaptive immunity when TLR4 signalling intensity is low, or in contrast inhibition of IL-12 synthesis when signalling intensifies maintaining intestinal mucosal homeostasis (Kim *et al.*, 2015; Khader & Thirunavukkarasu, 2019). A recent study by Goethel *et al*. (2019) in neonatal mice demonstrated that antibiotic treatment started one day after birth and maintained until weaning, significantly alters the microbiota at weaning in *wild-type* (WT) and $NOD2^{-/-}$ littermates. However, the neonatal treated $NOD2^{-/-}$ mice had increased susceptibility to colitis compared with the WT mice and a sustained reduction in microbial diversity 14 days after cessation of antibiotics. That phenotype was associated with changes in intestinal T cells and the cytokine milieu following inflammation. This study demonstrates NOD2, is a potent microbial sensor in the gut, is functionally protective against mucosal damage, and influences the resilience to perturbations (Goethel *et al.*, 2019). Also interesting, is the fact that NOD2 activation has been demonstrated to inhibit serotonin transport through the RIP2/RICK intracellular pathway (Layunta *et al.*, 2018). Data from Layunta et al (2018) infers that NOD2 could modulate intestinal infection by interacting with TLRs and modulating the intestinal serotonergic system. As reviewed above, TLRs are dependent on accessory proteins and perform their functions in parallel with co-stimulatory receptor pathways such as NOD2. There are

329 mixed reports in regard to the expression of NOD2 in preterm infants. Chen *et al.,* (2019b) report a
330 decrease in NOD2 expression as well as decreased release of pro-inflammatory mediators compared
331 to adults using peripheral blood samples whereas Granland *et al* 2014 report no differences but
332 blood was sampled from both peripheral and cord blood (Granland *et al.*, 2014). It is also reported
333 that ≥2 NOD2 loss-of-function mutations is associated with an increased risk of NEC in preterm
334 infants (Härtel *et al.*, 2016; Chen *et al.*, 2019*b*). It is important for us to identify each of the players
335 within the innate immune system of the newborn infant as loss of function in accessory proteins or
336 co-stimulatory pathways will significantly impinge on neonatal outcome.

3.3 Toll-like receptor signalling cascades

The molecular mechanisms underlying TLR signalling cascades are still under investigation. However, one of the major signalling pathways of TLRs is the MyD88-dependent pathway, which utilises the adaptor protein MyD88 and IL1R-associated kinases (IRAKs). With the exception of TLR3, this pathway is activated by all TLRs and results in the activation of the transcription factor NFκB, expression of inflammatory genes and subsequent production of inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-1β and cyclooxygenase-2 (COX-2). LPS exposure activates TNFα synthesis and COX-2 expression in a MyD88-dependent manner (Pathak *et al.*, 2004). Moreover, previous *in vivo* studies using Irak-$1^{-/-}$ mouse fibroblast cells have confirmed that IRAK-1 is essential for IL-1 mediated IL-6 cytokine production as well as activation of NFκB (Kanakaraj *et al.*, 1998). Later, *in vitro* rodent studies demonstrated that IRAK-1 participates in NFκB activation of IL1R(TIR)-TLR dimerization (Janssens & Beyaert, 2003) and was subsequently shown to be an essential regulator for activation of TLR7- and TLR9-signalling pathways (Uematsu *et al.*, 2005). Studies have suggested that increased expression of IRAK-1 could, in part, be responsible for sex-specific responses to infection and subsequent immune advantage in female neonates. Females exhibit a significantly higher relative expression of IRAK-1 in human umbilical cord blood (O'Driscoll *et al.*, 2017). IRAK-1 and many other genes that are involved in immunity are encoded on the X chromosome at Xq21 and there is evidence that protein-coding genes in this chromosome are susceptible to X chromosome inactivation, which leads to a sex bias in gene expression that might confer disadvantages in males, in responding and resolving acute infections (Libert *et al.*, 2010). Important research has demonstrated that polymorphisms or mutations in TLRs and downstream signalling molecules such as MyD88 and IRAK-4 are associated with increased risk of infection in children (Ku *et al.*, 2007) and adults (Gern, 2009). In humans, IRAK-1 haplotype is associated with poorer outcome in sepsis and increase in NFκB expression. However, animal studies have shown that heterozygous females for the wild-type IRAK-1 haplotypes show no adverse clinical effects (Arcaroli *et al.*, 2006). Regulation of host defence during exposure to LPS is also dependent on TLR4-driven induction of a signalling adapter p62, crucial in selective autophagy and ubiquitination of protein aggregates (Fujita *et al.*, 2011). Once TLR receptors bind to their ligands they are incorporated into endosomes whereby they stimulate a MyD88 independent pathway as discussed in detail in another review (Aldrich *et al.*, 2020). The basal level of TLR expression and the activation of their downstream signalling cascades will influence the neonatal response to infection (Viemann *et al.*, 2005; Glaser & Speer, 2013).

3.4 Cooperation of toll-like receptors and antioxidants

The neonatal transition to a relatively high oxygen environment can activate several transcription factors, including NFκB that can promote inflammation but also nuclear factor erythroid 2-related factor 2 (Nrf2) that initiates the activation of endogenous antioxidant defence mechanisms (Pall & Levine, 2015). In human tracheal smooth muscle cells, ROS generation has been reported to be mediated by TLR2 inflammatory responses upon LTA administration, *via* the TLR2/MyD88/TRAF6/c-Src NADPH oxidase pathway, which in turn initiates the activation of Nrf2 conferring protection against oxidative stress injury (Lee *et al.*, 2008). This crosstalk between toll-like receptor signalling and the Nrf2 pathway is important in the regulation of inflammation (Mohan & Gupta, 2018*a*). The Nrf2 signalling pathway is a major regulator of antioxidant production and cooperates with TLRs to suppress chronic inflammation (Mohan & Gupta, 2018*b*). In mice, LPS induced activation of proinflammatory cytokines, IL-6 and IL-1-β in macrophages, has been demonstrated to be inhibited by treatment with diethyl maleate, a Nrf2-inducer molecule (Kobayashi *et al.*, 2016). In a murine model of allergic asthma, administration of a TLR7 agonist (Resiquimod), was beneficial in attenuating airway reactivity and inflammation through Nfr2-mediated antioxidant pathway, concomitant with a decrease in ROS, associated with induction of the Cu/Zn superoxide dismutase antioxidant (Nadeem *et al.*, 2016).

4. Regulation of Toll-Like Receptor

4.1 Feedback regulation

TLR expression is under feedback regulation (Figure 3). Positive feedback control is mediated through the transcription factor NFκB, a master regulator of inflammation and the immune response (Brenner & Bruserud, 2019). Many studies demonstrate cooperativity between several transcription factors in the regulation of TLR transcription. For example, NFκB cooperates with stimulation factor 1 (Sp1) and signal transducer and activator of transcription (STAT) 5 elements for maximal activation of TLR2 transcription in murine macrophages after GP bacterial infection (Musikacharoen *et al.*, 2001; Wang *et al.*, 2001). Moreover, TLR2 transcription has been reported to require TNF-α and androgen receptor activation function-1 cooperativity to promote activation of the TLR2 promotor and stimulate transcription (Hermoso *et al.*, 2004). In this regard, TLR2 has been suggested to be upregulated at the mRNA and protein levels in response to inflammatory mediators, such as TNF-α and other cytokines.

In contrast, naturally occurring soluble forms of TLR were previously reported to negatively modulate TLR expression. There is evidence that these soluble (s) receptors, sTLR2 and sTRL4, are released into the circulation from peripheral blood mononuclear cells upon exposure to microbial ligands (ten Oever *et al.*, 2014). In this study sTLR2 correlated positively with TNF-α, however, it was previously reported that sTLR2 is capable of modulating TLR2 signalling in human plasma and breast milk by interacting with CD14 (membrane or soluble forms) and decreasing the bacterial response (LeBouder *et al.*, 2003). Interestingly, sTLR4 did not activate production of cytokines (ten Oever *et al.*, 2014). Furthermore, Iwanmi *et al* (2000) report that in mice, sTLR4 inhibits LPS-mediated TNF-α signalling, thus operating as negative feedback agents. Downregulation of the immune system is mediated by decreasing type-I IFN-dependent cytokine production in macrophages for example (Gottschalk *et al.*, 2019). An *in vitro* study demonstrated that NFκB and cis-regulatory elements (CRE) of the COX-2 promoter are critical in regulating downstream pathogen-driven response associated with TLR2, upon GP bacterial infection in murine cells (Pathak *et al.*, 2004). In addition, microRNA mediated-silencing of gene expression, such as the repression of TLR2 gene by miRNA-146a in GP induced macrophage activation, was reported to prevent excessive inflammation (Griss et al., 2015). Unravelling the physiological effects of breast milk is still an area of active enquiry. Bioactive components such as sTLRs and miRNAs contained within exosomes potentially modulate the immune response in early life similar to feedback regulation in adulthood and it is essential that while we delve into its therapeutic potential. We should strive to ensure we optimise breast milk delivery to the preterm infant, whether that be mums milk or where there are challenges we use donor milk for preterm infants.

4.2 Epigenetic and chromatin modifications

Human and murine TLR2 gene expression is controlled by distinct, non-conserved regulatory elements, with some regions located in a cytosine-phosphate-guanine (CpG) island that modulates TLR2 gene repression (Figure 4.). These sites contain putative binding sites for several transcription factors, including Sp1 (Haehnel *et al.*, 2002). It is well established that hypermethylation of the gene promoter silences gene expression, while hypomethylation of the promoter region activates transcription. Upregulation of TLR gene expression caused by a hypomethylated regulatory region might aggravate cell inflammation and damage by excessive increases in cytokine concentrations. In a different study, hypomethylation of TLR2 gene was associated with increased proinflammatory response toward bacterial PGN in human cystic fibrosis bronchial epithelial cells suggesting that TLR2 is epigenetically regulated by CpG methylation (Shuto *et al.*, 2006; Ahangarani *et al.*, 2011). Some studies also suggest an association between decreased DNA methylation and reduced white matter

integrity in premature newborns, which has implications for the phenotype that often becomes apparent in these babies as they develop (Sparrow *et al.*, 2016). Altered methylation of TLR genes in neonatal sepsis has not been identified in pilot studies, but other inflammatory genes have been identified (Tendl *et al.*, 2013; Lorente-Pozo *et al.*, 2021).

In a murine alveolar macrophage cell line, the TLR2 promoter is reported to undergo chromatin remodelling following infection with GP bacteria. This chromatin remodelling was coupled with a rapid increase in both DNase I sensitivity and restriction enzyme accessibility at the promoter region. This resulted in enhanced TLR2 expression at the mRNA level and accessibility to transcription factors to initiate further transcription of TLR2; however, the persistence of this regulation was not explored further (Wang *et al.*, 2002). Additionally, the use of viral vectors for gene manipulation has been widely used to induce antigen-specific tolerance in animal studies. In mice, murine B cell integration of a retroviral vector, induced epigenetic chromatin modifications, upregulation of an immunosuppressive cytokine (IL-10) and inhibition of the translocation of the NFκB complex to the nucleus downstream of TLR2, thereby preventing the production of proinflammatory cytokines (Ahangarani *et al.*, 2011). A study on infants with confirmed prenatal chorioamnionitis revealed gain in histone tail modification H3K4me3 in monocytes distinct from non-infected infants, but it is clear their contribution to neonatal sepsis risk will depend on the location of the epigenetic changes, be they at promoter, intron, exon, and intergenic regions (Bermick *et al.*, 2019). In preterm infants, there is a paucity of large cohort studies concerning developmental epigenetic modifications in TLRs and TLR pathways and how those changes influence the future development of the immune system of the child.

4.3 Half-life and degradation of TLRs

It is crucial to navigate how TLRs are negatively regulated and ultimately degraded. During infection, surface levels of TLRs are maintained via continuous replenishment of TLR proteins from intracellular compartments like Golgi and endosomes; prolonged expression of TLRs may result in excessive inflammation. Studies in adipocytes demonstrated that TLR2 undergoes a long intracellular maturation process with a half-life of approximately three hours, more specifically, TLR2 protein induction is reached within one hour and subsequently decreases, suggesting the activation of proteolysis (Lin *et al.*, 2000). In lung tissue, TLR4 has been shown to present a half-life between 2-3 hours (Fan *et al.*, 2002). TLR3 has a half-life of 3 to 12 hours in human embryonic kidney cells (HEK) 293 (Pohar *et al.*, 2013). However, the length of the TLR3 protein critically impacts on its half-life: in the same cell cultures, TLR3 full length protein has a half-life of 3 hours and the cleaved protein fragments present with half-lives of over 7 hours (Qi *et al.*, 2012).

Uncontrolled TLR expression and activation will contribute to pathogenesis of inflammatory diseases therefore TLR stability is modulated by E3 ubiquitin-protein ligases, such as TRIM21 and RING finger proteins, such as 216 (RNF216) and PPP1R11 (Ghimire & Jeyaseelan, 2020). TLR2 is directly ubiquitinated by RING finger E3 ligase, PPP1R11 both *in vitro* and *in vivo* in white blood cell samples isolated form patients with Gram-positive infections and mouse lungs, respectively. This signalling cascade leads to degradation of TLR2 which greatly impacts Gram-positive bacterial clearance (McKelvey *et al.*, 2016). In macrophages, a lysosome-associated small guanosine triphosphatase (GTPase) Rab7b has been reported to negatively regulate LPS induced production of TNF-alpha and IL-6 by promoting the degradation of TLR3 protein.

TLR4, a crucial receptor for Gram-negative bacteria recognition receptor, has been reported to be transported from the cell plasma membrane to the cell endosome for ubiquitination and onward to the lysosome for degradation. Research regarding this receptor suggests that Rab7b negatively regulates TLR4 signalling, by establishing the translocation of TLR4 into lysosomes (Wang *et al.*, 2007). Under LPS exposure, blockade of a small GTPase Rab10 has been shown to decrease membrane TLR4 expression and diminish the production of proinflammatory cytokines by regulating the trafficking rate of TLR4 onto the plasma membrane (Wang *et al.*, 2010). (Evankovich *et al.*, 2020). TLR9 response to inflammation in macrophages has been also demonstrated to be negatively regulated by Rab7b as it promotes TLR9 protein degradation (Yao *et al.*, 2009). Small RNAs, such as microRNAs can activate the proteolysis of TLRs by interaction with zinc finger proteins. One example is TLR8, that has been reported to be marked for ubiquitination in a RN216-dependent pathway in response to circulating microRNAs. Negative regulation of TLR expression and promotion of TLR degradation are important steps for regulation the inflammatory response. Thus, understanding the mechanisms governing the dynamic turnover of TLR protein is an important element of understanding the sequence of events underlying immune response and capacity to resolve infection in early life.

5. Implications of Early Life Immune Activation

5.1 Sensitisation

Stimulating the immune system can change physiological responses to subsequent stressors. Several studies have examined the role of TLRs as putative mediators in such adapted responses. TLR activation by TLR ligands have the capacity to either increase the response to subsequent stimulation or dampen the inflammatory response via preconditioning or tolerance. Sensitization has been mainly reported with LPS exposure, the most widely studied TLR4 agonist. In a study by

Schletter *et al*. (1995) TLR4 activation by the binding of its ligand LPS on mononuclear phagocytes is responsible for the production of proinflammatory cytokines (TNF-α, IL1, IL-6), bioactive lipids (prostaglandins) and ROS (Schletter *et al.*, 1995). In the brain, TLRs sensitization has been the focus of intense investigation. For example, LPS dramatically increases vulnerability to hypoxic-ischaemic (HI) injury of the immature brain accompanied by an altered gene expression of CD14 and TLR4 in the brain and marked expression of TNF-α in the cerebral cortex (Coumans *et al.*, 2003). Vulnerability to HI has also been reported to increase with TLR3 activation in mice at postnatal day 9 which markedly increases both white and grey matter injury in a TLR3/TRIF dependent pathway (Stridh *et al.*, 2013). The impact of TLRs in the central nervous system (CNS), has also been reported in microglia cultures from rat cerebral cortex, spinal cord and cerebellum where activation of multiple TLRs (2, 3 and 4) prime microglia coupled with elevated extracellular ATP levels, (Facci *et al.*, 2014). At the cellular level, TLRs ligands have been reported to sensitize B-cell receptor signalling by increasing actin turnover dynamics such that the mobility of the B-cell antigen receptor (BCR) is increased. This suggests that cooperation between TLR and BCR signalling is present and allows discrimination between microbial and inert antigens by controlling the threshold for BCR activation (Freeman *et al.*, 2015). One study using a $TLR3^{-/-}$ mice demonstrated that TLR3 enhances antigen-independent skin inflammation in allergic contact dermatitis with increased expression of TNF-α (Nakamura *et al.*, 2015). Lastly, in adults, the TLR5 ligand flagellin promotes asthma symptoms by sensitizing the allergic response to indoor allergens (Wilson *et al.*, 2013).

5.2 Tolerance

Prior exposure or preconditioning to LPS is proposed to contribute to a state of hypo-responsiveness or endotoxin tolerance, which may limit inflammation-induced tissue injury (Glaser & Speer, 2013). Preterm sheep exposure to repeated prenatal GN bacterial protein LPS, reported that TLR4 signalling exhibited decreased expression of positive intracellular regulator IRAK-4 and increased expression of negative regulator IRAK-M consistent with the formation of endotoxin tolerance and attenuated subsequent inflammatory response, however single exposure to LPS did not induce the same response (Kallapur *et al.*, 2007). In premature infants that developed NEC and GN bacterial infection, exposure of primary enterocytes to LPS after birth leads to TLR tolerance, such that NFkB is not stabilised and it is thought that this hypo-responsiveness plays a role in host-immune stabilization of microbial colonisation (Lotz *et al.*, 2006). In a study by Dhillon *et al.,* (2015) LPS administration for 5 days in preterm fetal sheep decreased cellular apoptosis, microglial activation and reactive astrogliosis in response to subsequent hypoxia-ischemia (HI) injury. Compared with a negative control, the phenotype was associated with an upregulation of TLR4, TLR7 and IFN-β mRNA in the

brain that may suggest a role of TLR in mediating endogenous neuroprotection during neuronal development. This provides evidence that the LPS preconditioning effect is associated with upregulation of TLRs. In particular, TLR4 has a considerable influence on plasma IFN-β concentrations which is responsible for the activation of transcription factors that drive the expression of type I and III interferons that are crucial to counteract inflammation (Dhillon *et al.*, 2015). A novel TLR2 and TLR4 antagonist, OxPAPC, administrated to rats 24 hours prior to an LPS challenge has been reported to prevent the stress-induced potentiation of hippocampal pro-inflammatory response from isolated microglial (Weber *et al.*, 2013). A study by Kumral *et al*. (2012) performed in neonatal rats, suggests the number of apoptotic cell deaths and hypomyelination in the periventricular white matter region is significantly reduced with administration of LPS (preconditioning) 24 hours before acute LPS exposure (Kumral *et al.*, 2012). In the latter report, analysis of antioxidant gene expression revealed an upregulation of superoxide dismutase (SOD) 1,2 and 3 as well as TNFα expression levels, suggesting that low-dose LPS given one day before administration of a potent dose protects against endotoxin-induced white matter injury in cooperation with antioxidant enzymes (Kumral *et al.*, 2012); however, no assessment of TLR expression was made in this study. Interestingly, neural anti-inflammatory pathways have been demonstrated to be reprogramed by a single postnatal exposure to LPS by priming peripheral tissues to create a prostaglandin-mediated activation of the hypothalamic-pituitary-adrenal (HPA) axis and increased expression of TLR4 (Mouihate *et al.*, 2010). In LPS-exposed rats, administration of dobutamine (a beta-1-adrenergic agonist), in hippocampal slices from six day old mice, upregulates the expression of SOD3 and survival genes, and decreases the inflammatory response by modifying TLR4 signalling pathways, including IFN-β and NFκB (Markus *et al.*, 2018). Additionally, upregulation of SOD3 has been proposed to play a role in the activation of signalling cascades downstream from the beta-1-adrenoceptor providing strong protection of the neonatal brain against hypoxia. The precise underlying molecular mechanisms of ligand-induced sensitization and preconditioning remain unclear. However, evidence suggests that the priming effect of LPS is reliant on TLR4 and MyD88 adaptor protein (Tanga *et al.*, 2005; Dean *et al.*, 2010). Overall, the presence of a complex and dynamic immune response upon exposure to TLRs ligands can occur within the first period of life and can modulate the inflammatory response. Additionally, it one could infer that based on the distinct responses from early and later life, the molecular mechanisms that mediate both sensitization and precondition during the postnatal age would likely differ from those observed in adult life.

6. Expression of TLRs and Developmental Patterns

6.1 Mucosal barriers

Two physiological barriers protect the body from microbial invasion: the mucosa (respiratory and intestinal) and the skin. These barriers are in constant communication with the external environment. Developmental expression of TLRs have been reported in humans and rodents. Basal TLR expression fluctuates from early to adult life. The early and dynamic expression of TLRs and related factors reflect their protective role but also point toward their functional role in neurodevelopment outcome such as neurogenesis (Barak *et al.*, 2014; Chen *et al.*, 2019*a*; Yarandi *et al.*, 2020). The mucosal barriers contain multiple components that prevent infection, such as the overlying host-secreted mucous layer in the gut and epithelial cells spanning the respiratory tract, that offer the first line of defence against pathogenic, non-pathogenic microorganisms and endogenous DAMPs (Zanin *et al.*, 2016; Martens *et al.*, 2018).

6.1.1 Intestinal tract

After birth, the external environment of the gut lumen is quickly colonised and contains a significant repository of diverse microorganisms that contribute positively to physiological homeostasis (Martin & Walker, 2008). In preterm infants, aberrant gut microbiota and intestinal barrier disruption can develop as a result of caesarean delivery, parenteral feeding, antibiotic treatment and hypoxic stress (Neu, 2007; Zou *et al.*, 2018; Shao *et al.*, 2019). A study by Zou *et al.* (2018) provides evidence that both prenatal and postnatal antibiotic exposure influences the gut microbiota of preterm infants in the neonatal intensive care unit. They demonstrated a significantly diminished population of *Bifidobacteria*, a genus regarded as beneficial bacteria and one of the first to colonise the gut (Zou *et al.*, 2018). In humans, it has been reported that probiotic supplementation results in a significantly lower incidence of LOS in preterm new-borns exclusively fed with breast milk with great benefit in the reduction of necrotizing enterocolitis (NEC) (Repa *et al.*, 2015). Benefits of probiotic supplementation in a mouse model of NEC was shown to be dependent on TLR 9, in animals aged 7-10 postnatal days (Good et al., 2014). TLR expression along the gut has recently been described in mice between 6-12 weeks of age (Price *et al.*, 2018). TLR9 expression was not observed in the gut epithelium in these mice however. Nonetheless, the expression other TLRs varied dramatically along the length of the intestine. For example, expression of TLR5 (a receptor that recognizes flagellin and has been shown to cooperate with TLR4) is restricted to Paneth cells of the small intestine and is gradually decreased during the postnatal period. Whereas, TLR4 expression was detected in the small intestine and colon, functional responses to the TLR4-ligand LPS, was not detected in colonic

organoids (Price *et al.*, 2018; Hussain *et al.*, 2020). This suggests that there may be signalling mechanisms that specifically blunt TLR4 responses.

TLRs are implicated in mediating local intestinal inflammation and selective activation of mucosal endothelial and mesenchymal cells to promote angiogenic responses (Schirbel *et al.*, 2013). In the latter report, human intestinal microvascular endothelial cells and human intestinal fibroblast were exposed to bacterial ligands for TLR2/6 and 4 and both were reported to induce proliferation of blood vessels, suggesting that this innate immune response may expand the mucosal microvascular network and contribute to chronic intestinal inflammation. Hypoxia is a common stimulus in preterm infants that can compromise the intestinal barrier (Xing *et al.*, 2018). The primary stimulus for neuronal regulation of intestinal microvasculature is mediated by intrinsic enteric neurons which under hypoxic conditions, loss of prolyl hydroxylase enzymes (PHDs) has been implicated in detrimental gut phenotypes (Zheng *et al.*, 2015). An *ex vivo* study in humans showed that a hypoxic insult of 1-hour stabilized hypoxia inducible factor (HIF-1α) expression and disrupted the intestinal epithelial layer mimicking *bona fide* NEC histology in preterm neonates. Hypoxia induced disruption was associated with a downregulation of specific PRRs expression, suggesting that hypoxia can increase vulnerability, as PRRs likely play an important role in controlling intestinal permeability and specific tight junction functionality (Bein *et al.*, 2018).

6.1.2 Respiratory tract

In the lung, mechanisms that regulate mucosal inflammation impact lung development and depend on TLR signalling (Harju *et al.*, 2001; Jiang *et al.*, 2005; Petrikin *et al.*, 2010). The respiratory mucosa present along the length of the conducting airway is composed of multiple cell types. The conducting zone of the airway plays an important role in filtering the air entering from the external environment and maintenance of airway patency, which is critical in facilitating breathing, reducing the cost of breathing and in that way contributing to acid-base homeostasis. The preterm neonatal respiratory mucosa is at particular risk of both sterile and pathogenic induced inflammation. Cellular stress and trauma as a result of clinical interventions such as supplemental oxygen, adverse tracheal intubation–associated events and aspiration as a result of poor oral co-ordination have long been identified as complications of preterm birth (Dreyfuss & Saumon, 1998; Lee *et al.*, 2011; Madurga *et al.*, 2013; Foglia *et al.*, 2019; Kamity *et al.*, 2020). TLRs are evident length of the respiratory tract (Figure 6). TLR 9 is expressed in the nasal mucosa of healthy adult humans on mast cells, dendritic cells and fibroblast-like cells (Fransson *et al.*, 2007). Sha et al (2004) demonstrated mRNA expression of all TLRs in BEAS-2b cell line (human bronchial epithelium transformed by an adenovirus) and PBEC (human primary bronchial epithelial cells) (Sha *et al.*, 2004). Bronchial epithelial cells express functional TLR1–6 and TLR9 in adults, though the responsiveness of TLR 2 was shown to be relatively

subdued (Mayer *et al.*, 2007; Jung *et al.*, 2018). TLR2 appears to have an apical surface location whereas TLR4 is expressed more abundantly in the basolateral region of the epithelial cell (Muir *et al.*, 2004). Expression of both TLR2 and TLR4 receptors are also evident in alveolar epithelium cells in human adults (Go *et al.*, 2014). Similar to the intestinal mucosa the developmental expression of TLRs is also apparent in the lung. In mice, TLR2 and TLR4 mRNA expression was reported to be very low in the fetal lung at early gestation (embryonic day 14) increasing by eightfold in late mouse lung development from pseudoglandular to saccular phase (Harju *et al.*, 2001). In preterm lambs, both TLR4 and CLR-family PRRs in respiratory epithelia, increased from late gestation to term birth (Meyerholz et al., 2006). This was consistent with a study from Hillman *et al* (2008), which reported that TLR2 and TLR4 mRNAs increased throughout late gestation in full-term newborn lambs. In neonatal guinea pigs, mRNA expression levels of TLR2,3,6,7,8,9 and 10 were significantly increased in the lungs at 45 days of gestation, while TLR1 and 4 were significantly increased at 52 days suggesting a dynamic and developmental pattern of expression between members of the TLR family (Ma *et al.*, 2017). In the guinea pig, all TLRs had significantly higher expression in lung during later gestational age compared to postnatal expression (Ma *et al.*, 2017). A report by Awasthi *et al* (2008) performed in premature baboons show that TLR2 and TLR4 gene expression in lung tissue was developmentally regulated. The authors studied samples from animals delivered at 125, 140 and 175 days of gestational age (dGA) (term = 185±2 days) and reported a significant increase of TLR proteins at 175 dGA compared with protein expression at both 125 and 140 dGA in healthy baboons. TLR expression at 175dGA was equivalent to adult baboons. The dynamic developmental expression of the TLRs may be consistent with the rapid development of the lung during late gestation but they may also play a functional role in that maturation (Awasthi *et al.*, 2008). Hillman *et al*., (2008) provide evidence of LPS-induced TLR2 and TLR4 mRNA expression in the fetal sheep lung. In this study administration of a TLR4 agonist (LPS) by intra-amniotic injection 2 or 7 days before operative delivery of the preterm lambs, induced lung inflammation and interestingly maturation, although a TLR2 agonist (PAMCysK4) evoked less consistent responses (Hillman *et al.*, 2008).

6.2 Central nervous system

Developmental expression of TLRs in the central nervous system is important in understanding neonatal susceptibility to infection and the long-term consequences of TLR activation on functional outcome. A measured response to PAMPS and DAMPS is required to limit potential harm in network development during the neonatal period. A subtype specific pattern of TLR mRNA expression in the murine brain was evident across early development (embryonic day 13 to postnatal day 12); the expression of TLR 1, TLR7 and TLR9 increased more than 10-fold by postnatal day 12. In the same

study TLR 2, TLR4 and TLR6 mRNA expression was higher in the postnatal period compared to the embryonic phase, however MyD88 expression did not follow the same trend over this period (Kaul *et al.*, 2012). The expression of TLRs is evident on multiple cells expressed in the central nervous system neuroprogenitor cells, neurons, astrocytes and microglia (Lathia *et al.*, 2008; McCarthy *et al.*, 2017).

The microglia first became known as the primary immune effector cells within the brain. Microglia are limited in number at birth and increase rapidly in the first week of life in rodents (Alliot *et al.*, 1999). A study by Scheffel *et al.,* (2012) in mice, demonstrated differential protein inductions and functional outcomes upon TLR activation in postnatal microglia compared to adult, suggesting specialisation in clearance tasks, synthesis of inflammatory mediators and antigen presentation. In neurons, the process of myelin incorporation by microglia does not require TLRs, however myelination is supressed by TLR activation in a MyD88-dependent pathway (van Rossum et al., 2008). Poor myelination is associated with cognitive and motor deficits. Scheffel *et al.* (2012) demonstrated that exposure to LPS inhibited myelin phagocytosis by microglia in neonatal mice, a process important in repair and regeneration of nerves. In this study, microglia were isolated at various postnatal ages from mice infected with GN bacteria. The authors reported that TLR4-mediated LPS response underwent transient alterations and hyporeactivity around postnatal day 21, a phase matching critical immune and central nervous system maturation; moreover by postnatal day 49, TLR4 regained sensitivity to the agonist (Scheffel *et al.*, 2012).

TLR-mediated neuroimmune inflammation may be reduced by depletion and repopulation of microglia. Newly differentiated microglia in primary organotypic hippocampal slice cultures (OHSCs) after microglia depletion had a blunted TLR response. Thus, microglial repopulation is suggested to promote an anti-inflammatory response, trophic neuroenvironment as well as a normalised proinflammatory gene expression (Coleman *et al.*, 2020). Adenosine system is a potential regulator of this response in microglia. These neuronal cells are highly sensitive to subtle changes of adenosine triphosphate (ATP) which belongs to the purinergic signalling system. Receptors that recognise purines such as P2X, P2Y and P1 receptors mediate the generation of intracellular adenosine by degradation of ATP contributing to the microglial anti- and pro-inflammatory shift (Calovi *et al.*, 2019). Adenosine signals the downstream increase of cyclic adenosine monophosphate (cAMP) by binding G-protein coupled adenosine receptors in the cytoplasm impairing the TLR-mediated signalling system (Herington *et al.*, 2012). Given the susceptibility of newborns to infection, a study using human neonatal cord blood monocytes has reported that the adenosine system selectively inhibits TNF-α production in human neonates, and reported relatively high adenosine concentrations in neonatal blood plasma (Herington *et al.*, 2012). A large body of evidence suggests that the

purinergic system might constitute a fundamental signalling network that establishes microglial response to infection and inflammation as well as mediating nerve repair by promoting myelination. Caffeine therapy utilised in the amelioration of the symptoms of apnea of prematurity mediates some of its effects through its action as an antagonist of A2a receptors and a non-selective antagonist of A1 receptors (Abdel-Hady, 2015). Apnea of prematurity are recurrent clinical events that are accompanied by intermittent hypoxia and bradycardia, suggested to present worse outcomes in the male sex (O'Halloran & McDonald, 2018). Benefits of caffeine may extend beyond stabilising breathing and may play a role in immune modulation and neuroprotection (Potter *et al.*, 2018).

7. The Influence of Toll-Like Receptors on the Cardiorespiratory System

There is evidence of TLR expression across tissues such as the intestinal epithelium, mesenteric lymph nodes, spleen, lung, heart, adrenal glands and brainstem (Zarember & Godowski, 2002; Price *et al.*, 2018). Cardiorespiratory dysfunction is one of the primary symptoms of infection with requirement for increased ventilatory support and vasopressor administration in the days prior to LOS-induced death (Wynn *et al.*, 2017). The role of TLRs in cardiorespiratory control is poorly understood. Cardiorespiratory dysfunction is likely mediated by local release of inflammatory cytokines and oxidative stress in addition to the action of cytokines on distant targets involved in homeostatic control.

7.1 Toll-like receptors and cardiorespiratory control

The autonomic nervous system is comprised of two arms, the parasympathetic and the sympathetic pathways which have afferent sensory input to the brainstem and efferent motor output that regulate cardiorespiratory function. As discussed in section 2.1, neonatal infection impinges on cardiorespiratory function destabilising breathing and modulating heart rate. Toll-like receptors have been identified in the peripheral autonomic neural network such as the vagal ganglion and the carotid body.

The activation of vagal afferents has been demonstrated to play a key role in airway defences and are extremely sensitive to a wide range of proinflammatory molecules, such as IL-1, IL-6 and TNF-α (Prescott et al., 2020). During infection, the release of such molecules stimulates vagal afferent signalling, which can be traced to crucial regions that regulate breathing such as the nucleus of the solitary tract (NTS) (Marvel *et al.*, 2004). TLR4 mRNA and protein is expressed in the rat nodose ganglion, another potential site of inflammatory-induced afferent vagal nerve stimulation (Hosoi *et*

al., 2005). A recent study in mice has reported that jugular ganglia express TLR4 gene and protein and TLR4 is selectively involved in stimulating the release of calcitonin gene-related peptide CGRP from a subset of vagal afferents (Jia *et al.*, 2021). TLR4 has also been identified in the carotid body in adult human, rat and mice and the rat petrosal ganglia which signals directly to the NTS (Fernández *et al.*, 2011; Mkrtchian *et al.*, 2012).

Whilst the brain was originally proposed to have immune privilege there is evidence of TLR expression in glia cells within the central nervous system but also direct entry of cytokines from the periphery into the brain across the BBB by a saturable transport mechanism such as RAGE, the interaction of peripheral cytokines with the circumventricular organs which lack the BBB and peripheral immune-mediated activation of afferent neurons within the vagus nerve (Katsuura *et al.*, 1990; Ek *et al.*, 1998). Toll-like receptors have also been identified centrally in the nucleus of the solitary tract and parventricular nucleus (PVN); these receptors have the capacity to modulate cardio-respiratory control. In the paraventricular nucleus of the hypothalamus, regulation of TLR1 and 2 in neonatal mice brains after hypoxia has been proposed to play a crucial role in neonatal brain injury (Stridh *et al.*, 2011). The function of TLR2 immunoreactive neurons in PVN is unknown however it is speculated that they may participate in the neuroendocrine response of the hypothalamus and may act as sensors for incoming inflammatory signals.

7.2 Toll-like receptors and inflammatory lung disease

Alterations in the TLR pathway has been associated with many neonatal respiratory diseases. Polymorphisms in the TIRAP gene have been suggested to be associated with severity of BPD among preterm neonates (Malash *et al.*, 2016). Furthermore, a study performed in children aged 2-4 years old, demonstrated that single-nucleotide polymorphism within TIRAP adaptor protein S180L was associated with susceptibility to recurrent pneumococcal lower respiratory tract infections, suggesting that carriage of TIRAP S180L heterozygous trait decreases the likelihood of recurrent infection (Siebert *et al.*, 2018). A study conducted in infants with severe respiratory syncytial virus (RSV) bronchiolitis reported that TLR4 expression in neutrophils from bronchoalveolar lavage fluid and blood was higher in preterm babies compared to term babies. Conversely, preterm and term infants suffering from RSV had diminished TLR 4 expression compared with healthy control infants. The inverse association of severe respiratory bronchiolitis with TLR4 expression, supports an important role of TLR4 in eliminating the virus (Halfhide *et al.*, 2009), though other TLRs likely play a role. Preconditioning in mice pre-exposed to CpG oligodeoxynucleotides (TLR9 ligand) was associated with increased circulating APCs, which protected the animal against subsequent neonatal RSV infection (Yamaguchi *et al.*, 2012). TLR expression in the respiratory mucosa has been reported

to be associated with several diseases such as lung cancer and chronic obstructive pulmonary disease (COPD). In human non-small cell lung carcinoma, higher mRNA expression of TLR1-3 and 5-8 were significantly associated with increased cell survival and thus improved disease outcomes (Bauer *et al.*, 2017). TLR4 is negatively associated with COPD outcome as the mRNA expression is reported to increase in the bronchial mucosa of patients with severe COPD associated with increased bronchial inflammation and airflow obstruction (Di Stefano *et al.*, 2017). Both the upregulation of TLRs in the lungs under septic states, together with the suggested protective function of TLRs during inflammation, reveals the complexity of these receptor signalling pathways and their control of inflammation. However, further research of their role in respiratory inflammation and repair will offer opportunities for novel therapeutic strategies to combat sepsis-induced respiratory failure in premature infants.

7.3 Toll-Like receptors and cardiovascular dysfunction

There is evidence that TLR signalling is implicated in cardiovascular dysfunction in the neonate and many adult cardiovascular inflammatory diseases. As previously discussed, TLR expression occurs along the vasculature, in cardiac tissue and at levels of the brainstem involved in cardiovascular control. In preterm fetal sheep systemic *in utero* GN infection induced myocardial inflammation (hypotension, tachycardia and cardiac hypoxia) within 72 hours of exposure, by activation of TLR2 and TLR4 on cardiomyocytes (Seehase *et al.*, 2011). This study implicates cardiomyocyte TLR2 and TLR4 dysregulation in the fetal cardiac dysfunction. Alternatively, an *in vitro* study in neonatal rat ventricular myocytes showed that TLR2 inhibited hydrogen peroxide-induced nuclear translocation of NF-κB and activator protein 1 (AP1) suggesting that TLR2 contributes to the increased survival of cardiac myocytes during oxidative stress (Frantz *et al.*, 2001). Remodelling of cardiomyocytes has been associated with an upregulation of PRRs that trigger host inflammation through inflammasome activation (Wu *et al.*, 2017). A study by Li *et al.* (2018) utilised a model of cardiac hypertrophy, neonatal mouse cardiomyocytes stimulated with phenylephrine (α1 adrenoceptor agonist), mouse hearts with deficiency in NLRP3 (a member of the NOD-like receptor family) demonstrated an upregulation of TLR4 mRNA expression. In NLRP3 knockout mice, TLR4 inhibition ameliorated cardiac dysfunction, suggesting that NLRP3 is a negative regulator of cardiomyocyte remodelling and might have a role as a therapeutic target for heart failure (Li *et al.*, 2018). A study conducted in human coronary artery endothelial cells treated with atorvastatin (ATV; an inhibitor of enzyme hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, critical in lipid metabolism) demonstrated inhibition of TLR2 protein expression in a time- and dose-dependent manner by a mechanism that involves casein kinase-2 and SP1 phosphorylation. This suggests that ATV administration is

atheroprotective by controlling the over-expression of proinflammatory endothelial TLR2 protein expression (Bertocchi *et al.*, 2011). In preterm fetal sheep, gardiquimod (GDQ) a TLR7 agonist, modulates fetal physiological adaptation after severe asphyxia and was associated with transient tachycardia followed by sustained hypertension that persisted well after GDQ (Cho *et al.*, 2019). Conversely, GDQ was previously reported to protect against hepatotoxicity in liver injury induced by sepsis in mice through suppression of oxidative stress and IL-6 (Guo *et al.*, 2017).

Overall, the studies presented reveal early life expression of TLRs in cardiac tissue and such expression is upregulated during infection. In the present review, suppression of the TLR4 pathway was discussed as crucial in preventing an exacerbation of the inflammatory state. These studies also indicate that expression of TLR2 and TLR4 in cardiomyocytes induces oxidative stress mediated damage but protects against infection.

Local inflammation mediated by TLR expression might be an important factor in sepsis progression, particularly if DAMPs act as either endogenous ligands or signalling enhancing complexes (Tsan & Gao, 2004). TLR4 has been proposed to bind endogenous ligands or so called 'alarmins', including heat shock proteins (HSP) and high mobility group box 1 protein of fibronectins, though further research is required in this area (Yang *et al.*, 2016). Nonetheless, it is known that alarmins released in a hyperoxic environment modulate TLR4 signalling. In a recent study by Mian *et al* (2019), the authors demonstrated that administration of a TLR4 antagonist to rats exposed to hyperoxia (80% oxygen from postnatal day 3 to 10) prevented neonatal hyperoxia-induced left ventricular hypertrophy and dysfunction (Mian *et al.*, 2019). Moreover, in this study TLR4 was reported to be upregulated in cardiomyocytes at day 10 in hyperoxia exposed animals, suggesting that neonatal exposure to high oxygen activates TLR4 and protects against cardiorespiratory dysfunction. In addition, extracellular HSP (ex-HSP) is released from damaged cells and stimulate APCs and the release of cytokines. These molecules can bind TLR2, 3, 4 and 9. One example is ex-HSP90 that mediates the ability of associated innate ligands such as LPS to initiate a signalling network (Taha *et al.*, 2019). In summary, there is evidence that TLRs are vital for cardiorespiratory homeostasis in neonates and alteration in TLR expression in the heart could severely affect cardiovascular function and contribute to increased morbidity and mortality associated with neonatal infection.

8. Translational Opportunities in Neonatal Immunology

The mechanisms by which the innate immune system recognises and responds to infection has been the focus of an extensive body of research. It is now clear that many PAMPs activate TLRs which through intracellular signalling and effective crosstalk, induce immune responses and rapid feedback control to fight invading pathogens and facilitate repair and regeneration in a variety of tissues

(Figure 5). Sufficient functional expression of TLRs is required to quickly curb the infection and elicit a number of protective functions. TLR-mediated induction of cytokines to mount a rapid recruitment of immune cells to quickly identify, sequester and eradicate the pathogen. Cytokines help to raise the alarm and isolate the pathogen, and inflammation is generally contained within the local environment. As previously discussed, TLR expression and cytokine production differs between newborn infants and adults (Pettengill et al., 2016). Simultaneous increases in both pro-inflammatory cytokines and importantly, anti-inflammatory cytokine production such as, IL-4, IL-10, IL-11 and IL-13 help to control inflammation by countering the actions of the pro-inflammatory cytokines and promoting resolution of the inflammatory response (Ng & Lam, 2012). However, an overproduction and over activation of immune cells result in exaggerated cytokine response or "cytokine storm" and increased mortality in humans. While cytokine storms are often associated with viral infections, very high cytokine concentrations in serum and cerebrospinal fluid samples after development of bacterial-induced sepsis with *S. aureus* and *Staphylococcal* endotoxins was reported to be associated with sudden infant death (Vennemann *et al.*, 2012). In neonates, the reduction of excessive inflammation could minimise mortality and decrease the frequency and intensity of devastating post-sepsis damage, including chronic lung disease (Philip, 2009) and white matter damage (Falck *et al.*, 2018), which may enhance longer term outcome. The risk of death following infection is highly increased in the preterm neonate potentially due to specific immune impairments as previously highlighted. In this section, we will review the evidence for putative therapeutics in the treatment of neonatal infection and illustrate examples of vaccine therapy, clinical trials, administration of surfactant proteins, and the potential role of cytokines as neonatal biomarkers.

8.1 TLR-based therapeutics

Modulation of the TLRs and associated pathways offer opportunities to improve pathogen recognition and/or temper the inflammatory response to improve neonatal outcomes. To date, two allergy vaccines containing TLR agonists have been investigated in clinical trials: Pollinex Quattro and AIC, which contain a TLR4 agonist and a CpG motif activating the TLR9 cascade, respectively (Aryan & Rezaei, 2015). This immunotherapy is known to increase allergen-specific IgG antibody levels and dampen the IgE response during allergen exposure (Rosewich *et al.*, 2013). Another TLR stimulating vaccine is the Bacillus Calmette-Guérin (BCG), a live attenuated strain of *Mycobacterium bovis* with TLR2/4 agonist activity. A study performed in Guinea-Bissau has reported an association with the improvement in survival in very low body weight neonates compared to those that did not receive

863 the vaccine (Roth *et al.*, 2004); unfortunately, some developed countries have now excluded BCG's
864 from their vaccination schemes which likely imparts non-specific immune benefits.

865 Benefits of administration immune adjuvants that stimulate specific TLR (e.g. Resiquimod and LPS)
866 have been demonstrated in a neonatal mouse model predisposed to poor sepsis outcome (Wynn *et*
867 *al.*, 2008). A recent study by Yang *et al*. (2019) has reported that monophosphoryl lipid A (MPLA), a
868 TLR9 neutrophil agonist, protects against fatal bacterial sepsis in mice through the process of
869 inflammatory preconditioning, which occurred in the setting of a low-dose bacterial challenge by
870 enabling TLR9 membrane translocation and signalling. Moreover, this study suggested that depletion
871 of TLR9 in $tlr9^{-/-}$ mice largely eliminated the neutrophil-mediated inflammatory preconditioning
872 effect in this sepsis model (Yang *et al.*, 2019). VTX-294, a novel TLR8 agonist, was demonstrated to
873 induce the expression of TNF-α and IL-1β by leukocytes by acting in combination with MPLA
874 (Dowling *et al.*, 2013). Morris & Surendran, (2016) have advocated the need to develop novel
875 adjuvants and the key may be to stimulate multiple TLR pathways, particularly TLR7 & 8 which are
876 active in neonatal infants (Krumbiegel *et al.*, 2007; Philbin et al., 2012).

877 Whilst stimulation of TLR9 has been shown to act via effective preconditioning, stimulating TLR2 and
878 TLR4 do not work in a similar manner. Injection of PAM (a TLR2 agonist) or LPS (a TLR4 agonist) alone
879 results in neuro-inflammatory alterations that have been reported to sensitise the immature brain to
880 brain HI injury when injected systemically (Falck *et al.*, 2017). Teasing apart the different
881 mechanisms and environments in which tolerance versus sensitisation occurs will be important to
882 advance TLR-based therapies. Interestingly in the study by Falck et al., (2017), hypothermia
883 treatment was highly neuroprotective after PAM induced brain injury, potentially highlighting the
884 usefulness of neonatal therapeutic hypothermia, but this therapy is currently limited to term infants
885 only with moderate to severe hypoxic ischemic encephalopathy.

886 A clinical trial using a single dosage of 180mg of Ticagrelor (a $P2Y_{12}$ receptor antagonist) in healthy
887 individuals, reported reduced platelet-monocyte complex formation associated with an increase in
888 pro-inflammatory cytokines in blood exposed to a TLR2 agonist, but a decrease in these cytokines in
889 blood exposed to LPS, TLR 4 agonist (Tunjungputri *et al.*, 2015). These finding suggest that it is
890 important to examine the therapeutic strategies to a variety of PAMPS as they may reveal opposing
891 effects depending on the stimulus.

892 Biopharmaceutical research should consider immune development status during TLRs-based drug
893 discovery. Variations likely exist in both the nature and intensity of simulated infection in different

894 populations. For novel drug therapies it will be important to demonstrate both improved survival in
895 preclinical models of acute neonatal sepsis and positive long-term outcomes.

896 *8.2 Antioxidants*

897 Neonatal response to infection is influenced by an altered redox environment. Diminished
898 production of antioxidants during neonatal development in the presence of infection may lack the
899 inflammatory suppression necessary to keep the system in check. Supplemental antioxidants have
900 been studied as a means of preventing the accumulation of ROS in premature babies. In the brain,
901 oxidative stress is a primary risk factor for neuronal damage in premature newborns and therefore
902 of high clinical relevance (Panfoli *et al.*, 2018).
903 A study by Sevastiadou *et al.* (2011) showed that glutamine, an amino acid with immunomodulatory
904 properties acts to mediate inflammation by decreasing the expression of oxidative stress-related
905 genes and has suggested beneficial effects on intestinal integrity and the overall incidence of NEC
906 and septicemia in preterm infants (Sevastiadou *et al.*, 2011). Several additional studies have
907 reported the benefits of glutamine supplementation in preterm babies, such as early improvement
908 in liver function and increase in head circumference growth during the first year of life (Wang *et al.*,
909 2013; Kieviet *et al.*, 2014). To determine if there is an effect of glutamine on TLRs, a study performed
910 in adult patients in critical care demonstrated that daily glutamine supplementation lasting 5 days
911 did not increase the expression of TLR2 and TLR4 in peripheral blood monocytes (Pérez-Bárcena *et*
912 *al.*, 2008). However, glutamine mediates a decrease in proinflammatory cytokines (Aosasa *et al.*,
913 1999) and an increase in the levels of glutathione and the oxidative capacity (Amores-Sánchez &
914 Medina, 1999). However, studies still cannot infer if this modulation involves the TLR pathway.
915 Vitamin D is a potent modulator of both innate and adaptive immune system by activation of
916 monocytes/macrophages and is involved in the clearance of pro-oxidants. A study conducted in
917 healthy adults shows that regulation of vitamin D receptor (VDR)-mediated TLR2/1 signalling is
918 multifactorial, involving epigenetic modulation by the methylation of VDR key regulatory sites and
919 genetic variation by the regulated translation of TIRAP proteins (Meyer *et al.*, 2017). In a review,
920 Pludowshi *et al.,* (2018) discuss the emergence of Vitamin D deficiency in recent years and the
921 establishment of dietary supplementation in various countries such as Ireland, Canada, United
922 Kingdom and USA. 5 micrograms of vitamin D is the recommended daily dose for new-borns during
923 the first 12 months, however premature infants may require higher doses (Pludowski *et al.*, 2018).
924 There is a dearth of studies focusing on the cooperation between TLRs and antioxidants during early
925 life and this area should be explored in future studies.

8.3 Breast milk

Breast milk has been championed in the last 20 years for its extensive benefits in disease prevention. Studies by deBouder (2003, 2006) illustrate the bioactive components of breastmilk on immune modulation in the first few days after birth. Others have provided evidence of enhanced microbiota diversity and richness in the gut interacting with TLRs and is protective of conditions such as NEC. A major avenue of research at the moment is unpacking the bioactive cargo within exosomes present in colostrum including coding RNA (mRNA) and non-coding RNA (ncRNA), such as miRNA and long non-coding RNA (lncRNA) (LeBouder *et al.*, 2006; Jiang *et al.*, 2021). These miRNAs contained within porcine milk have been demonstrated to attenuate TLR4 response to LPS (Xie *et al.*, 2019). Further research on this rich fluid holds promise for novel therapeutic strategies aimed at modulating early life immunity. However, the interference from maternal antibodies carried to the baby via breast milk is one of the challenges of the administration of vaccines in neonates as well as the use of novel vaccines (Morris & Surendran, 2016).

8.4 Probiotic supplementation

Colonisation of the neonatal gut can be severely compromised in the neonatal intensive care setting. Probiotic administration decreases the risk of severe NEC in preterm infants (Morgan et al., 2020). Examining probiotic supplementation from pregnancy week 36, Forsberg and colleagues collected postnatal mononuclear cells at birth, 2, 12 and 24 months and examined TLR2 signalling. mRNA expression of TLR2 was unaltered in the probiotic supplementation cohort, at 12 months infants had decreased LTA induced IL-6 response. This suggests that decreased responses to TLR2 in probiotic-treated babies seem to be dependent on factors downstream of TLR mRNA expression (Forsberg *et al.*, 2014). This strategy of maternal probiotic administration that has not yet been explored in humans (Grev J, 2018). Clinical studies and interventional trials are key to explore novel ways to prevent and treat infection and related morbidity in the neonatal population. A trial testing the efficacy of a probiotic containing a mix of 8 strains of bacteria in sepsis prevention in neonates is currently recruiting participants (Sinha *et al.*, 2021). Another new trial is assessing the benefits of multi-strain probiotic product in the prevention of multi-drug resistant bacterial infection. Moreover, previous trials using synbiotics (mixture of probiotics and prebiotics) report significant beneficial effects in the treatment arm in the prevention of neonatal sepsis (Panigrahi *et al.*, 2017).

Further investigations are required to understand the tissue, organ, cellular patterns of TLR expression and receptor turnover during the neonatal period and in response to infection. We need to investigate how common drugs utilised in the NICU such as supplemental oxygen and caffeine

modify the immune response. We need to clarify to what extent neonatal infection is modulated by TLR-mediated cytokine release, establish if preconditioning lessons mortality, and if immunological patterns persist into later life. Cytokines bind to their specific receptor and can activate the intrinsic apoptotic pathway (such as IL-1β) or promote cell survival (such as IL-2) (Kelly *et al.*, 2002; Grunnet *et al.*, 2009). Furthermore, novel biomarkers of sepsis and those at risk of sepsis need to be identified and validated to allow for timely stratification and identification of vulnerable infants (Buhimschi *et al.*, 2007).

Perspectives and Significance

The TLR superfamily recognises conserved molecular patterns expressed by bacteria and amplifies the downstream inflammatory and defensive response of the host to aid elimination of the pathogen. Evidence shows that TLRs are constitutively expressed on many cell types across tissues such as brain, lung, heart, and gut, in addition to circulating immune cells. TLR signalling cascades include accessory, adaptor proteins and second messengers that promote intracellular stabilisation of NFκB, and sequential release of TNF-α, a cytokine that contributes to feedback regulation of the receptor. As basal TLR expression fluctuates from early to adult life, in new-borns, TLR2 and 4 expression have been reported to increase from early life to late development, thus being developmentally regulated. An understanding of this regulation is important to better appreciate the vulnerability of new-borns to infection, which is also modulated in a sex-dependent manner. We have described that TLR expression can be positively or negatively regulated and can affect the expression of antioxidants influencing cell damage and inflammation. Furthermore, the importance of controlling the redox state in early life supports the development of antioxidant-based therapies, especially during the neonatal period where the prevention of the formation of dangerous ROS is critical. In conclusion, the immune response to bacterial infection depends on the prompt recognition by TLRs and subsequent upregulation of their expression as these influence cardiorespiratory function and impact on the severity of many neonatal conditions. Future studies should focus on expanding the current understanding of neonatal immunity, bringing insight into the molecular mechanisms underpinning the development of neonatal sepsis and neonatal cellular damage and delineating signalling networks. In addition, we should focus not only on the development of novel therapeutic strategies but also the identification of early biomarkers to facilitate protection with a consideration of personalised sex-specific approaches.

992

993 **References**

994 Abdel-Hady H (2015). Caffeine therapy in preterm infants. *World J Clin Pediatr* **4,** 81.

995 Ahangarani RR, Janssens W, Carlier V, Vanderelst L, Vandendriessche T, Chuah M, Jacquemin M &
996 Saint-Remy JM (2011). Retroviral vectors induce epigenetic chromatin modifications and IL-10
997 production in transduced B cells via activation of toll-like receptor 2. *Mol Ther* **19,** 711–722.

998 Aldrich AL, Heim CE, Shi W, Fallet RW, Duan B & Kielian T (2020). TLR2 and caspase-1 signaling are
999 critical for bacterial containment but not clearance during craniotomy-associated biofilm
1000 infection. *J Neuroinflammation* **17,** 1–17.

1001 Alliot F, Godin I & Pessac B (1999). Microglia derive from progenitors, originating from the yolk sac,
1002 and which proliferate in the brain. *Dev Brain Res* **117,** 145–152.

1003 Amores-Sánchez MI & Medina MÁ (1999). Glutamine, as a precursor of glutathione, and oxidative
1004 stress. *Mol Genet Metab* **67,** 100–105.

1005 Angele et all (1997). Testosterone receptor blockade after hemorrhage in males. **73,** 2016.

1006 Aosasa S, Mochizuki H, Yamamoto T, Ono S & Ichikura T (1999). A clinical study of the effectiveness
1007 of oral glutamine supplementation during total parenteral nutrition: Influence on mesenteric
1008 mononuclear cells. *J Parenter Enter Nutr* **23,** S41–S44.

1009 Arcaroli J, Silva E, Maloney JP, He Q, Svetkauskaite D, Murphy JR & Abraham E (2006). Variant IRAK-1
1010 haplotype is associated with increased nuclear factor-κB activation and worse outcomes in
1011 sepsis. *Am J Respir Crit Care Med* **173,** 1335–1341.

1012 Aryan Z & Rezaei N (2015). Toll-like receptors as targets for allergen immunotherapy. *Curr Opin*
1013 *Allergy Clin Immunol* **15,** 568–574.

1014 Awasthi S, Cropper J & Brown KM (2008). Developmental expression of Toll-like receptors-2 and -4 in
1015 preterm baboon lung. *Dev Comp Immunol* **32,** 1088–1098.

1016 Bæk O, Ren S, Brunse A, Sangild PT & Nguyen DN (2020). Impaired Neonatal Immunity and Infection
1017 Resistance Following Fetal Growth Restriction in Preterm Pigs. *Front Immunol* **11,** 1–13.

1018 Bairam A, Laflamme N, Drolet C, Piedboeuf B, Shah PS & Kinkead R (2018). Sex-based differences in
1019 apnoea of prematurity: A retrospective cohort study. *Exp Physiol* **103,** 1403–1411.

1020 Barak B, Feldman N & Okun E (2014). Toll-like receptors as developmental tools that regulate
1021 neurogenesis during development: an update. *Front Neurosci* **8,** 1–6.

1022 Barrett T, McEntee E, Drew R, O'Reilly F, O'Carroll A, O'Shea A & Cleary B (2018). Influenza
1023 vaccination in pregnancy: vaccine uptake, maternal and healthcare providers' knowledge and
1024 attitudes. A quantitative study. *BJGP Open*bjgpopen18X101599.

1025 Bauer AK, Upham BL, Rondini EA, Tennis MA, Velmuragan K & Wiese D (2017). Toll-like receptor
1026 expression in human non-small cell lung carcinoma: Potential prognostic indicators of disease.

Oncotarget **8,** 91860–91875.

Bauer et al. (2014). Genetic Deletion of TLR4 on Platelets Attenuates Experimental Pulmonary Hypertension. *Circ Res* **23,** 1–7.

Behrendt D, Dembinski J, Heep A & Bartmann P (2004). Lipopolysaccharide binding protein in preterm infants. *Arch Dis Child Fetal Neonatal Ed* **89,** 551–554.

Bein A, Eventov-Friedman S, Arbell D & Schwartz B (2018). Intestinal tight junctions are severely altered in NEC preterm neonates. *Pediatr Neonatol* **59,** 464–473.

Benjamin DK, Stoll BJ, Fanaroff AA, McDonald SA, Oh W, Higgins RD, Duara S, Poole K, Laptook A & Goldberg R (2006). Neonatal candidiasis among extremely low birth weight infants: Risk factors, mortality rates, and neurodevelopmental outcomes at 18 to 22 months. *Pediatrics* **117,** 84–92.

Bentsen MH, Markestad T, Øymar K & Halvorsen T (2017). Lung function at term in extremely preterm-born infants: A regional prospective cohort study. *BMJ Open* **7,** 1–9.

Bermick J, Gallagher K, denDekker A, Kunkel S, Lukacs N & Schaller M (2019). Chorioamnionitis exposure remodels the unique histone modification landscape of neonatal monocytes and alters the expression of immune pathway genes. *FEBS J* **286,** 82–109.

Bertocchi C, Traunwieser M, Dörler J, Hasslacher J, Joannidis M & Dunzendorfer S (2011). Atorvastatin inhibits functional expression of proatherogenic TLR2 in arterial endothelial cells. *Cell Physiol Biochem* **28,** 625–630.

Bion J, Richardson A, Hibbert P, Beer J, Abrusci T, McCutcheon M, Cassidy J, Eddleston J, Gunning K, Bellingan G, Patten M & Harrison D (2013). Matching Michigan: A 2-year stepped interventional programme to minimise central venous catheterblood stream infections in intensive care units in England. *BMJ Qual Saf* **22,** 110–123.

Bizzarro MJ, Dembry LM, Baltimore RS & Gallagher PG (2008). Changing patterns in neonatal escherichia coli sepsis and ampicillin resistance in the era of intrapartum antibiotic prophylaxis. *Pediatrics* **121,** 689–696.

Boukari R, Laouafa S, Ribon-Demars A, Bairam A & Joseph V (2017). Ovarian steroids act as respiratory stimulant and antioxidant against the causes and consequences of sleep-apnea in women. *Respir Physiol Neurobiol* **239,** 46–54.

Brenner & Bruserud (2019). Functional Toll-Like Receptors (TLRs) Are Expressed by a Majority of Primary Human Acute Myeloid Leukemia Cells and Inducibility of the TLR Signaling Pathway Is Associated with a More.

Buhimschi CS, Bhandari V, Hamar BD, Bahtiyar MO, Zhao G, Sfakianaki AK, Pettker CM, Magloire L, Funai E, Norwitz ER, Paidas M, Copel JA, Weiner CP, Lockwood CJ & Buhimschi IA (2007).

Proteomic profiling of the amniotic fluid to detect inflammation, infection, and neonatal sepsis. *PLoS Med* **4,** 0084–0094.

Bussel JB, Berkowitz RL, McFarland JG, Lynch L & Chitkara U (1988). Protection against cholera in breast-fed children by antibodies in breast milk. *N Engl J Med* **319,** 1374–1378.

Butcher SK, O'Carroll CE, Wells CA & Carmody RJ (2018). Toll-like receptors drive specific patterns of tolerance and training on restimulation of macrophages. *Front Immunol*; DOI: 10.3389/fimmu.2018.00933.

Cailes B, Kortsalioudaki C, Buttery J, Pattnayak S, Greenough A, Matthes J, Bedford Russell A, Kennea N & Heath PT (2018). Epidemiology of UK neonatal infections: The neonIN infection surveillance network. *Arch Dis Child Fetal Neonatal Ed* **103,** 1–8.

Calovi S, Mut-Arbona P & Sperlágh B (2019). Microglia and the Purinergic Signaling System. *Neuroscience* **405,** 137–147.

Carrera P et al. (2015). Exome sequencing and pathway analysis for identification of genetic variability relevant for bronchopulmonary dysplasia (BPD) in preterm newborns: A pilot study. *Clin Chim Acta* **451,** 39–45.

Castro-Manrreza ME & Montesinos JJ (2015). Immunoregulation by mesenchymal stem cells: Biological aspects and clinical applications. *J Immunol Res*; DOI: 10.1155/2015/394917.

Chen CY, Shih YC, Hung YF & Hsueh YP (2019*a*). Beyond defense: Regulation of neuronal morphogenesis and brain functions via Toll-like receptors. *J Biomed Sci* **26,** 1–13.

Chen K, Zhang L, Huang J, Gong W, Dunlop NM & Wang JM (2008). Cooperation between NOD2 and Toll-like receptor 2 ligands in the up-regulation of mouse mFPR2, a G-protein-coupled A 42 peptide receptor, in microglial cells. *J Leukoc Biol* **83,** 1467–1475.

Chen Y, Yu SL, Li YP & Zhang MM (2019*b*). Nucleotide-binding oligomerization domain (NOD) plays an important role in neonatal infection. *Int J Biol Macromol* **121,** 686–690.

Cheng K, Gao M, Godfroy JI, Brown PN, Kastelowitz N & Yin H (2015). Specific activation of the TLR1-TLR2 heterodimer by small-molecule agonists. *Sci Adv*; DOI: 10.1126/sciadv.1400139.

Cho KHT, Wassink G, Galinsky R, Xu B, Mathai S, Dhillon SK, van den Heuij LG, Davidson JO, Weaver-Mikaere L, Bennet L, Gunn AJ & Fraser M (2019). Protective effects of delayed intraventricular TLR7 agonist administration on cerebral white and gray matter following asphyxia in the preterm fetal sheep. *Sci Rep* **9,** 1–17.

Coleman LG, Zou J & Crews FT (2020). Microglial depletion and repopulation in brain slice culture normalizes sensitized proinflammatory signaling. *J Neuroinflammation* **17,** 1–20.

Cortese F, Scicchitano P, Gesualdo M, Filaninno A, De Giorgi E, Schettini F, Laforgia N & Ciccone MM (2016). Early and Late Infections in Newborns: Where Do We Stand? A Review. *Pediatr*

Neonatol **57,** 265–273.

Costa D & Castelo R (2016). Umbilical cord gene expression reveals the molecular architecture of the fetal inflammatory response in extremely preterm newborns. *Pediatr Res* **79,** 473–481.

Coumans ABC, Middelanis J, Garnier Y, Vaihinger HM, Leib SL, Von Duering MU, Hasaart THM, Jensen A & Berger R (2003). Intracisternal application of endotoxin enhances the susceptibility to subsequent hypoxic-ischemic brain damage in neonatal rats. *Pediatr Res* **53,** 770–775.

Cuenca AG, Wynn JL, Moldawer LL & Levy O (2013). Role of innate immunity in neonatal infection. *Am J Perinatol* **30,** 105–112.

Dauby N, Goetghebuer T, Kollmann TR, Levy J & Marchant A (2012). Uninfected but not unaffected: Chronic maternal infections during pregnancy, fetal immunity, and susceptibility to postnatal infections. *Lancet Infect Dis* **12,** 330–340.

Dean JM, Wang X, Kaindl AM, Gressens P, Fleiss B, Hagberg H & Mallard C (2010). Microglial MyD88 signaling regulates acute neuronal toxicity of LPS-stimulated microglia in vitro. *Brain Behav Immun* **24,** 776–783.

Dear PRF (1987). Monitoring oxygen in the newborn: Saturation or partial pressure? *Arch Dis Child* **62,** 879–881.

Dhillon SK, Gunn AJ, Jung Y, Mathai S, Bennet L & Fraser M (2015). Lipopolysaccharide-induced preconditioning attenuates apoptosis and differentially regulates TLR4 and TLR7 gene expression after ischemia in the preterm ovine fetal brain. *Dev Neurosci* **37,** 497–514.

Dong Y & Speer CP (2015). Late-onset neonatal sepsis:Recent developments. *Arch Dis Child Fetal Neonatal Ed* **100,** F257–F263.

Doreste B, Torelli S & Morgan J (2020). Irradiation dependent inflammatory response may enhance satellite cell engraftment. *Sci Rep* **10,** 1–12.

Dowling DJ, Tan Z, Prokopowicz ZM, Palmer CD, Matthews MAH, Dietsch GN, Hershberg RM & Levy O (2013). The Ultra-Potent and Selective TLR8 Agonist VTX-294 Activates Human Newborn and Adult Leukocytes. *PLoS One*; DOI: 10.1371/journal.pone.0058164.

Dreyfuss D & Saumon G (1998). State of the Art Ventilator-induced Lung Injury.

E Lawn , S. Cousens JZ (2005). 4 million neonatal deaths: When? Where? Why? *Lancet* **365,** 891–900.

Ek M, Kurosawa M, Lundeberg T & Ericsson A (1998). Activation of vagal afferents after intravenous injection of interleukin-1β: Role of endogenous prostaglandins. *J Neurosci* **18,** 9471–9479.

El-Lakany MA, Fouda MA, El-Gowelli HM, El-Gowilly SM & El-Mas MM (2018). Gonadal hormone receptors underlie the resistance of female rats to inflammatory and cardiovascular complications of endotoxemia. *Eur J Pharmacol* **823,** 41–48.

Escalante MJ, Ceriani-Cernadas JM, D'Apremont I, Bancalari A, Webb V, Genes L, Villarroel L, Munoz

E & Tapia JL (2018). Late Onset Sepsis in Very Low Birth Weight Infants in the South American NEOCOSUR Network. *Pediatr Infect Dis J* **37,** 1022–1027.

Escribano García C, Montejo Vicente M del M, Izquierdo Caballero R, Samaniego Fernández CM, Marín Urueña SI, Infante López ME & Caserío Carbonero S (2018). Clinical observation of newborns with infection risk: A safe practice. *An Pediatr* **88,** 239–245.

Evankovich J, Lear T, Baldwin C, Chen Y, White V, Villandre J, Londino J, Liu Y, McVerry B, Kitsios GD, Mallampalli RK & Chen BB (2020). Toll-like Receptor 8 Stability Is Regulated by Ring Finger 216 in Response to Circulating MicroRNAs. *Am J Respir Cell Mol Biol* **62,** 157–167.

Facci L, Barbierato M, Marinelli C, Argentini C, Skaper SD & Giusti P (2014). Toll-like receptors 2, -3 and -4 prime microglia but not astrocytes across central nervous system regions for ATP-dependent interleukin-1β release. *Sci Rep* **4,** 1–9.

Faksh A, Britt RD, Vogel ER, Thompson MA, Pandya HC, Martin RJ, Pabelick CM & Prakash YS (2016). TLR3 activation increases chemokine expression in human fetal airway smooth muscle cells. *Am J Physiol - Lung Cell Mol Physiol* **310,** L202–L211.

Falck M, Osredkar D, Maes E, Flatebø T, Wood TR, Sabir H & Thoresen M (2017). Hypothermic Neuronal Rescue from Infection-Sensitised Hypoxic-Ischaemic Brain Injury Is Pathogen Dependent. *Dev Neurosci* **39,** 238–247.

Falck M, Osredkar D, Wood TR, Maes E, Flatebø T, Sabir H & Thoresen M (2018). Neonatal Systemic Inflammation Induces Inflammatory Reactions and Brain Apoptosis in a Pathogen-Specific Manner. *Neonatology* **113,** 212–220.

Fan J, Kapus A, Marsden PA, Li YH, Oreopoulos G, Marshall JC, Frantz S, Kelly RA, Medzhitov R & Rotstein OD (2002). Regulation of Toll-Like Receptor 4 Expression in the Lung Following Hemorrhagic Shock and Lipopolysaccharide. *J Immunol* **168,** 5252–5259.

Fernández R, Nardocci G, Simon F, Martin A, Becerra A, Rodríguez-Tirado C, Maisey KR, Acuña-Castillo C & Cortes PP (2011). Lipopolysaccharide signaling in the carotid chemoreceptor pathway of rats with sepsis syndrome. *Respir Physiol Neurobiol* **175,** 336–348.

Ferwerda G, Girardin SE, Kullberg BJ, Le Bourhis L, De Jong DJ, Langenberg DML, Van Crevel R, Adema GJ, Ottenhoff THM, Van Der Meer JWM & Netea MG (2005). NOD2 and toll-like receptors are nonredundant recognition systems of Mycobacterium tuberculosis. *PLoS Pathog* **1,** 0279–0285.

Fitzgerald KA & Kagan JC (2020). Toll-like Receptors and the Control of Immunity. *Cell*1–23.

Fleer A & Krediet TG (2007). Innate immunity: Toll-like receptors and some more - A brief history, basic organization and relevance for the human newborn. *Neonatology* **92,** 145–157.

Di Florio DN, Sin J, Coronado MJ, Atwal PS & Fairweather D (2020). Sex differences in inflammation,

redox biology, mitochondria and autoimmunity. *Redox Biol*101482.

Foglia EE, Ades A, Sawyer T, Glass KM, Singh N, Jung P, Quek BH, Johnston LC, Barry J, Zenge J, Moussa A, Kim JH, DeMeo SD, Napolitano N, Nadkarni V & Nishisaki A (2019). Neonatal intubation practice and outcomes: An international registry study. *Pediatrics*; DOI: 10.1542/peds.2018-0902.

Forsberg A, Abrahamsson TR, Björkstén B & Jenmalm MC (2014). Pre- and postnatal administration of Lactobacillus reuteri decreases TLR2 responses in infants. *Clin Transl Allergy* **4,** 1–7.

Fransson M, Benson M, Erjefält JS, Jansson L, Uddman R, Björnsson S, Cardell LO & Adner M (2007). Expression of Toll-like Receptor 9 in nose, peripheral blood and bone marrow during symptomatic allergic rhinitis. *Respir Res* **8,** 1–13.

Frantz S, Kelly RA & Bourcier T (2001). Role of TLR-2 in the Activation of Nuclear Factor κB by Oxidative Stress in Cardiac Myocytes. *J Biol Chem* **276,** 5197–5203.

Frasca L & Lande R (2020). Toll-like receptors in mediating pathogenesis in systemic sclerosis. *Clin Exp Immunol*1–11.

Freeman SA, Jaumouillé V, Choi K, Hsu BE, Wong HS, Abraham L, Graves ML, Coombs D, Roskelley CD, Das R, Grinstein S & Gold MR (2015). Toll-like receptor ligands sensitize B-cell receptor signalling by reducing actin-dependent spatial confinement of the receptor. *Nat Commun*; DOI: 10.1038/ncomms7168.

Fujita KI, Maeda D, Xiao Q & Srinivasula SM (2011). Nrf2-mediated induction of p62 controls Toll-like receptor-4-driven aggresome-like induced structure formation and autophagic degradation. *Proc Natl Acad Sci U S A* **108,** 1427–1432.

Gern JE (2009). Pyogenic bacterial infections in humans with MyD88 deficiency. *Pediatrics* **124,** 2076–2079.

Ghimire L & Jeyaseelan S (2020). Kiss of death: Ring Finger 216 Regulates Toll-like Receptor 8 Stability through Ubiquitination. *Am J Respir Cell Mol Biol* **62,** 125–127.

Gill et al. (2010). Linking oxidative stress to inflammation: Toll-like receptors. *Free Radic Biol Med* **23,** 1–7.

Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, Philpott DJ & Sansonetti PJ (2003). Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem* **278,** 8869–8872.

Gkentzi D, Katsakiori P, Marangos M, Hsia Y, Amirthalingam G, Heath PT & Ladhani S (2017). Maternal vaccination against pertussis: A systematic review of the recent literature. *Arch Dis Child Fetal Neonatal Ed* **102,** F456–F463.

Glaser K & Speer CP (2013). Toll-like receptor signaling in neonatal sepsis and inflammation: A

matter of orchestration and conditioning. *Expert Rev Clin Immunol* **9,** 1239–1252.

Go H, Koh J, Kim HS, Jeon YK & Chung DH (2014). Expression of toll-like receptor 2 and 4 is increased in the respiratory epithelial cells of chronic idiopathic interstitial pneumonia patients. *Respir Med* **108,** 783–792.

Goethel A, Turpin W, Rouquier S, Zanello G, Robertson SJ, Streutker CJ, Philpott DJ & Croitoru K (2019). Nod2 influences microbial resilience and susceptibility to colitis following antibiotic exposure. *Mucosal Immunol* **12,** 720–732.

Good M, Sodhi CP, Ozolek JA, Buck RH, Goehring KC, Thomas DL, Vikram A, Bibby K, Morowitz MJ, Firek B, Lu P & Hackam DJ (2014). Lactobacillus rhamnosus HN001 decreases the severity of necrotizing enterocolitis in neonatal mice and preterm piglets: Evidence in mice for a role of TLR9. *Am J Physiol - Gastrointest Liver Physiol*; DOI: 10.1152/ajpgi.00452.2013.

Gottschalk RA, Dorrington MG, Dutta B, Krauss KS, Martins AJ, Uderhardt S, Chan W, Tsang JS, Torabi-Parizi P, Fraser ID & Germain RN (2019). IFN-mediated negative feedback supports bacteria class-specific macrophage inflammatory responses. *Elife* **8,** 1–23.

Granland C, Strunk T, Hibbert J, Prosser A, Simmer K, Burgner D, Richmond P & Currie AJ (2014). NOD1 and NOD2 expression and function in very preterm infant mononuclear cells. *Acta Paediatr Int J Paediatr* **103,** 212–218.

Grev J SR (2018). Maternal probiotic supplementation for prevention of morbidity and mortality in preterm infants (Review). ; DOI: 10.1002/14651858.CD012519.pub2.www.cochranelibrary.com.

Griss et al. (2015). microRNAs constitute a negative feedback loop in Streptococcus pneumoniae induced macrophage activation. 1–26.

Grunnet LG, Aikin R, Tonnesen MF, Paraskevas S, Blaabjerg L, Størling J, Rosenberg L, Billestrup N, Maysinger D & Mandrup-Poulsen T (2009). Proinflammatory cytokines activate the intrinsic apoptotic pathway in β-cells. *Diabetes* **58,** 1807–1815.

Guo S, Zhang Y, Wang Z, Yu Y & Wang G (2017). Intraperitoneal gardiquimod protects against hepatotoxicity through inhibition of oxidative stress and inflammation in mice with sepsis. *J Biochem Mol Toxicol* **31,** 2–7.

Haehnel V, Schwarzfischer L, Fenton MJ & Rehli M (2002). Transcriptional Regulation of the Human Toll-Like Receptor 2 Gene in Monocytes and Macrophages. *J Immunol* **168,** 5629–5637.

Halfhide CP, Brearey SP, Flanagan BF, Hunt JA, Howarth D, Cummerson J, Edwards S, Hart CA & Smyth RL (2009). Neutrophil TLR4 expression is reduced in the airways of infants with severe bronchiolitis. *Thorax* **64,** 798–805.

Harju K, Glumoff V & Hallman M (2001). Ontogeny of toll-like receptors Tlr2 and Tlr4 in mice. *Pediatr*

Res **49,** 81–83.

Härtel C, Hartz A, Pagel J, Rupp J, Stein A, Kribs A, Müller A, Haase R, Gille C, Böttger R, Kittel J, Jensen R, Wieg C, Herting E & Göpel W (2016). NOD2 Loss-of-Function Mutations and Risks of Necrotizing Enterocolitis or Focal Intestinal Perforation in Very Low-birth-weight Infants. *Inflamm Bowel Dis* **22,** 249–256.

Herington JL, Crispens MA, Carvalho-macedo AC, Camargos AF, Lebovic DI, Bruner- KL & Osteen KG (2012). The Adenosine System Selectively Inhibits TLR-Mediated TNF-α Production in the Human Newborn. **95,** 1295–1301.

Hermoso MA, Matsuguchi T, Smoak K & Cidlowski JA (2004). Glucocorticoids and Tumor Necrosis Factor Alpha Cooperatively Regulate Toll-Like Receptor 2 Gene Expression. *Mol Cell Biol* **24,** 4743–4756.

Hibbert J, Strunk T, Simmer K, Richmond P, Burgner D & Currie A (2020). Plasma cytokine profiles in very preterm infants with late-onset sepsis. *PLoS One* **15,** 1–10.

Hillman NH, Moss TJM, Nitsos I, Kramer BW, Bachurski CJ, Ikegami M, Jobe AH & Kallapur SG (2008). Toll-like receptors and agonist responses in the developing fetal sheep lung. *Pediatr Res* **63,** 388–393.

Hosoi T, Okuma Y, Matsuda T & Nomura Y (2005). Novel pathway for LPS-induced afferent vagus nerve activation: Possible role of nodose ganglion. *Auton Neurosci Basic Clin* **120,** 104–107.

Hubacek JA, Book M, Wetegrove S, Ritter M, Rothe G & Schmitz G (2001). Gene variants of the bactericidal/permeability increasing protein and lipopolysaccharide binding protein in sepsis patients: Gender-specific genetic predisposition to sepsis. **29,** 557–561.

Hussain S et al. (2020). Tlr5 participates in the TLR4 receptor complex and promotes MyD88-dependent signaling in environmental lung injury. *Elife* **9,** 1–18.

Hyde TB, Hilger TM, Reingold A, Farley MM, Katherine L, Brien O, Schuchat A, Brien KLO & Bacterial A (2011). Trends in Incidence and Antimicrobial Resistance of Early-Onset Sepsis: Population-Based Surveillance in San Francisco and Atlanta. *Heal (San Fr* **110,** 1996–2003.

Idzikowski E & Connors TJ (2020). Impact and Clinical Implications of Prematurity on Adaptive Immune Development. *Curr Pediatr Rep* **8,** 194–201.

Imler JL & Hoffmann JA (2001). Toll receptors in innate immunity. *Trends Cell Biol* **11,** 304–311.

Janssens S & Beyaert R (2003). Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members. *Mol Cell* **11,** 293–302.

Jia L, Lee S, Tierney JA, Elmquist JK, Burton MD & Gautron L (2021). TLR4 signaling selectively and directly promotes CGRP release from Vagal afferents in the mouse. *eNeuro* **8,** 1–16.

Jiang D, Liang J, Fan J, Yu S, Chen S, Luo Y, Prestwich GD, Mascarenhas MM, Garg HG, Quinn DA,

1265 Homer RJ, Goldstein DR, Bucala R, Lee PJ, Medzhitov R & Noble PW (2005). Regulation of lung
1266 injury and repair by Toll-like receptors and hyaluronan. *Nat Med* **11,** 1173–1179.
1267 Jiang H, Wang HY, Wang JW, Lou DY, Niu N, Li GH & Qu P (2018). NF-κb inhibitor on toll-like receptor
1268 4 signal-induced expression of angiotensinogen and AT1a receptor in neonatal rat left
1269 ventricular myocytes. *Exp Ther Med* **16,** 3875–3882.
1270 Jiang X, You L, Zhang Z, Cui X, Zhong H, Sun X, Ji C & Chi X (2021). Biological Properties of Milk-
1271 Derived Extracellular Vesicles and Their Physiological Functions in Infant. *Front Cell Dev Biol* **9,**
1272 1–13.
1273 Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, Lee H & Lee JO (2007). Crystal Structure of the
1274 TLR1-TLR2 Heterodimer Induced by Binding of a Tri-Acylated Lipopeptide. *Cell* **130,** 1071–1082.
1275 Jung WJ, Lee SY, Choi SI, Kim BK, Lee EJ, In KH & Lee MG (2018). Toll-like receptor expression in
1276 pulmonary sensory neurons in the bleomycin-induced fibrosis model. *PLoS One* **13,** 1–13.
1277 Kallapur SG, Jobe AH, Ball MK, Nitsos I, Moss TJM, Hillman NH, Newnham JP & Kramer BW (2007).
1278 Pulmonary and Systemic Endotoxin Tolerance in Preterm Fetal Sheep Exposed to
1279 Chorioamnionitis. *J Immunol* **179,** 8491–8499.
1280 Kamity R, Ferrara L, Dumpa V, Reynolds J, Islam S & Hanna N (2020). Simultaneous Videofluoroscopy
1281 and Endoscopy for Dysphagia Evaluation in Preterm Infants—A Pilot Study. *Front Pediatr* **8,** 1–
1282 11.
1283 Kanakaraj P, Schafer PH, Cavender DE, Wu Y, Ngo K, Grealish PF, Wadsworth SA, Peterson PA,
1284 Siekierka JJ, Harris CA & Fung-Leung WP (1998). Interleukin (IL)-1 receptor-associated kinase
1285 (IRAK) requirement for optimal induction of multiple IL-1 signaling pathways and IL-6
1286 production. *J Exp Med* **187,** 2073–2079.
1287 Katsuura G, Arimura A, Koves K & Gottschall PE (1990). Involvement of organum vasculosum of
1288 lamina terminalis and preoptic area in interleukin 1β-induced ACTH release. *Am J Physiol -*
1289 *Endocrinol Metab*; DOI: 10.1152/ajpendo.1990.258.1.e163.
1290 Kaul D, Habbel P, Derkow K, Krüger C, Franzoni E, Wulczyn FG, Bereswill S, Nitsch R, Schott E, Veh R,
1291 Naumann T & Lehnardt S (2012). Expression of toll-like receptors in the developing brain. *PLoS*
1292 *One* **7,** 1–9.
1293 Kawai T & Akira S (2009). The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int Immunol* **21,**
1294 317–337.
1295 Kelly E, Won A, Refaeli Y & Van Parijs L (2002). IL-2 and Related Cytokines Can Promote T Cell
1296 Survival by Activating AKT. *J Immunol* **168,** 597–603.
1297 Khader SA & Thirunavukkarasu S (2019). The Tale of IL-12 and IL-23: A Paradigm Shift. *J Immunol*
1298 **202,** 629–630.

1299 Kieviet JF De, Vuijk PJ, Berg A Van Den, Lafeber HN, Oosterlaan J & Elburg RM Van (2014). Glutamine
1300 effects on brain growth in very preterm children in the fi rst year of life. *Clin Nutr* **33,** 69–74.
1301 Kim H, Zhao Q, Zheng H, Li X, Zhang T & Ma X (2015). A novel crosstalk between TLR4- and NOD2-
1302 mediated signaling in the regulation of intestinal inflammation. *Sci Rep* **5,** 1–17.
1303 Kirsten TB, Lippi LL, Bevilacqua E & Bernardi MM (2013). LPS exposure increases maternal
1304 corticosterone levels, causes placental injury and increases IL-1B levels in adult rat offspring:
1305 Relevance to autism. *PLoS One* **8,** 1–10.
1306 Kobayashi EH, Suzuki T, Funayama R, Nagashima T, Hayashi M, Sekine H, Tanaka N, Moriguchi T,
1307 Motohashi H, Nakayama K & Yamamoto M (2016). Nrf2 suppresses macrophage inflammatory
1308 response by blocking proinflammatory cytokine transcription. *Nat Commun* **7,** 1–14.
1309 Kollmann TR, Kampmann B, Mazmanian SK, Marchant A & Levy O (2017). Protecting the Newborn
1310 and Young Infant from Infectious Diseases: Lessons from Immune Ontogeny. *Immunity* **46,**
1311 350–363.
1312 Krumbiegel D, Zepp F & Meyer CU (2007). Combined Toll-like receptor agonists synergistically
1313 increase production of inflammatory cytokines in human neonatal dendritic cells. *Hum*
1314 *Immunol* **68,** 813–822.
1315 Ku CL et al. (2007). Selective predisposition to bacterial infections in IRAK-4-deficient children: IRAK-
1316 4-dependent TLRs are otherwise redundant in protective immunity. *J Exp Med* **204,** 2407–2422.
1317 Kumral A, Tuzun F, Ozbal S, Ugur Ergur B, Ylmaz O, Duman N & Ozkan H (2012). Lipopolysaccharide-
1318 preconditioning protects against endotoxin-induced white matter injury in the neonatal rat
1319 brain. *Brain Res* **1489,** 81–89.
1320 Lathia JD, Okun E, Tang SC, Griffioen K, Cheng A, Mughal MR, Laryea G, Selvaraj PK, Ffrench-Constant
1321 C, Magnus T, Arumugam T V. & Mattson MP (2008). Toll-like receptor 3 is a negative regulator
1322 of embryonic neural progenitor cell proliferation. *J Neurosci* **28,** 13978–13984.
1323 Lavoie JC & Tremblay A (2018). Sex-specificity of oxidative stress in newborns leading to a
1324 personalized antioxidant nutritive strategy. *Antioxidants*; DOI: 10.3390/antiox7040049.
1325 Layunta E, Latorre E, Forcén R, Grasa L, Castro M, Arias MA, Alcalde AI & Mesonero JE (2018). NOD2
1326 modulates serotonin transporter and interacts with TLR2 and TLR4 in intestinal epithelial cells.
1327 *Cell Physiol Biochem* **47,** 1217–1229.
1328 LeBouder E, Rey-Nores JE, Raby A-C, Affolter M, Vidal K, Thornton CA & Labéta MO (2006).
1329 Modulation of Neonatal Microbial Recognition: TLR-Mediated Innate Immune Responses Are
1330 Specifically and Differentially Modulated by Human Milk. *J Immunol* **176,** 3742–3752.
1331 LeBouder E, Rey-Nores JE, Rushmere NK, Grigorov M, Lawn SD, Affolter M, Griffin GE, Ferrara P,
1332 Schiffrin EJ, Morgan BP & Labéta MO (2003). Soluble Forms of Toll-Like Receptor (TLR)2

Capable of Modulating TLR2 Signaling Are Present in Human Plasma and Breast Milk. *J Immunol* **171,** 6680–6689.

Lee I-T, Wang S-W, Lee C-W, Chang C-C, Lin C-C, Luo S-F & Yang C-M (2008). Lipoteichoic Acid Induces HO-1 Expression via the TLR2/MyD88/c-Src/NADPH Oxidase Pathway and Nrf2 in Human Tracheal Smooth Muscle Cells. *J Immunol* **181,** 5098–5110.

Lee JH, Chang YS, Yoo HS, Ahn SY, Seo HJ, Choi SH, Jeon GW, Koo SH, Hwang JH & Park WS (2011). Swallowing dysfunction in very low birth weight infants with oral feeding desaturation. *World J Pediatr* **7,** 337–343.

Lemaitre B, Nicolas E, Michaut L, Reichhart JM & Hoffmann JA (1996). The dorsoventral regulatory gene cassette spatzle/Toll/Cactus controls the potent antifungal response in Drosophila adults. *Cell* **86,** 973–983.

Letouzey M et al. (2021). Cause of preterm birth and late-onset sepsis in very preterm infants: the EPIPAGE-2 cohort study. *Pediatr Res*1–9.

Li F, Zhang H, Yang L, Yong H, Qin Q, Tan M, Xu L, Liang K, Zong J & Qian W (2018). NLRP3 deficiency accelerates pressure overload-induced cardiac remodeling via increased TLR4 expression. *J Mol Med* **96,** 1189–1202.

Li G, Forero MG, Wentzell J, Durmus I, Wolf R, Anthoney N, Parker M, Jiang R, Hasenauer J, Strausfeld N, Heisenberg M & Hidalgo A (2020). A toll-receptor map underlies structural brain plasticity. *Elife* **9,** 1–32.

Libert C, Dejager L & Pinheiro I (2010). The X chromosome in immune functions: When a chromosome makes the difference. *Nat Rev Immunol* **10,** 594–604.

Lin Y, Lee H, Berg AH, Lisanti MP, Shapiro L & Scherer PE (2000). The lipopolysaccharide-activated Toll-like receptor (TLR)-4 induces synthesis of the closely related receptor TLR-2 in adipocytes. *J Biol Chem* **275,** 24255–24263.

Lorente-Pozo S, Navarrete P, Garzón MJ, Lara-Cantón I, Beltrán-García J, Osca-Verdegal R, Mena-Mollá S, García-López E, Vento M, Pallardó F V. & García-Giménez JL (2021). DNA Methylation Analysis to Unravel Altered Genetic Pathways Underlying Early Onset and Late Onset Neonatal Sepsis. A Pilot Study. *Front Immunol* **12,** 1–13.

Lotz M, Gütle D, Walther S, Ménard S, Bogdan C & Hornef MW (2006). Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells. *J Exp Med* **203,** 973–984.

M. Rueda et al. (2015). Neonatal regulatory T cells have reduced capacity to suppress dendritic cell function. *Physiol Behav* **176,** 139–148.

Ma L, Yang J, Yang L, Shi J, Xue J, Li Y & Liu X (2017). Developmental expression of Toll-like receptors in the Guinea pig lung. *Mol Med Rep* **15,** 1243–1251.

1367 Madurga A, Mižíková I, Ruiz-Camp J & Morty RE (2013). Recent advances in late lung development
1368 and the pathogenesis of bronchopulmonary dysplasia. *Am J Physiol - Lung Cell Mol Physiol*; DOI:
1369 10.1152/ajplung.00267.2013.
1370 Malash AH, Ali AA, Samy RM & Shamma RA (2016). Association of TLR polymorphisms with
1371 bronchopulmonary dysplasia. *Gene* **592,** 23–28.
1372 Markus T, Ley D, Hansson SR, Wieloch T & Ruscher K (2018). Neuroprotective dobutamine treatment
1373 upregulates superoxide dismutase 3, anti-oxidant and survival genes and attenuates genes
1374 mediating inflammation. *BMC Neurosci* **19,** 1–17.
1375 Martens EC, Neumann M & Desai MS (2018). Interactions of commensal and pathogenic
1376 microorganisms with the intestinal mucosal barrier. *Nat Rev Microbiol* **16,** 457–470.
1377 Martin CR & Walker WA (2008). Probiotics: Role in Pathophysiology and Prevention in Necrotizing
1378 Enterocolitis. *Semin Perinatol* **32,** 127–137.
1379 Marvel FA, Chen CC, Badr N, Gaykema RPA & Goehler LE (2004). Reversible inactivation of the dorsal
1380 vagal complex blocks lipopolysaccharide-induced social withdrawal and c-Fos expression in
1381 central autonomic nuclei. *Brain Behav Immun* **18,** 123–134.
1382 Mayer AK, Muehmer M, Mages J, Gueinzius K, Hess C, Heeg K, Bals R, Lang R & Dalpke AH (2007).
1383 Differential Recognition of TLR-Dependent Microbial Ligands in Human Bronchial Epithelial
1384 Cells. *J Immunol* **178,** 3134–3142.
1385 McCarthy GM, Harris RA, Bridges CR, Blednov YA & Herkenham M (2017). CNS cell-type localization
1386 and LPS response of TLR signaling pathways [version 1 ; referees : 2 approved] Referee Status :
1387 *F1000Research*1–24.
1388 McGovern M, Giannoni E, Kuester H, Turner MA, van den Hoogen A, Bliss JM, Koenig JM, Keij FM,
1389 Mazela J, Finnegan R, Degtyareva M, Simons SHP, de Boode WP, Strunk T, Reiss IKM, Wynn JL &
1390 Molloy EJ (2020). Challenges in developing a consensus definition of neonatal sepsis. *Pediatr*
1391 *Res* **88,** 14–26.
1392 McKelvey AC, Lear TB, Dunn SR, Evankovich J, Londino JD, Bednash JS, Zhang Y, McVerry BJ, Liu Y &
1393 Chen BB (2016). RING finger E3 ligase PPP1R11 regulates TLR2 signaling and innate immunity.
1394 *Elife* **5,** 1–15.
1395 Medzhitov R, Preston-Hurlburt P & Janeway CA (1997). A human homologue of the Drosophila toll
1396 protein signals activation of adaptive immunity. *Nature* **388,** 394–397.
1397 Meier PP & Engstrom JL (2007). Infectious diseases of the fetus and newborn infant. *Arch Dis Child*
1398 *Fetal Neonatal Ed* **92,** 2006.
1399 Melville JM & Moss TJM (2013). The immune consequences of preterm birth. *Front Neurosci* **7,** 1–9.
1400 Meyer V, Saccone DS, Tugizimana F, Asani FF, Jeffery TJ & Bornman L (2017). Methylation of the

Vitamin D Receptor (VDR) gene, together with genetic variation, race, and environment influence the signaling efficacy of the toll-like receptor 2/1-VDR pathway. *Front Immunol* **8,** 1–15.

Meyerholz et al. (2006). Expression of select immune genes (surfactant proteins A and D, sheep beta defensin 1, and toll-like receptor 4) by respiratory epithelia is developmentally regulated in the preterm neonatal lamb. *Dev Comp Immunol* **23,** 1–7.

Mian MOR, He Y, Bertagnolli M, Mai-Vo TA, Fernandes RO, Boudreau F, Cloutier A, Luu TM & Nuyt AM (2019). TLR (Toll-Like Receptor) 4 Antagonism Prevents Left Ventricular Hypertrophy and Dysfunction Caused by Neonatal Hyperoxia Exposure in Rats. *Hypertension* **74,** 843–853.

Mielcarska MB, Bossowska-Nowicka M & Toka FN (2021). Cell Surface Expression of Endosomal Toll-Like Receptors—A Necessity or a Superfluous Duplication? *Front Immunol* **11,** 1–13.

Mkrtchian S, Kåhlin J, Ebberyd A, Gonzalez C, Sanchez D, Balbir A, Kostuk EW, Shirahata M, Fagerlund MJ & Eriksson LI (2012). The human carotid body transcriptome with focus on oxygen sensing and inflammation - a comparative analysis. *J Physiol* **590,** 3807–3819.

Mohamed I, Elremaly W, Rouleau T & Lavoie JC (2015). Oxygen and parenteral nutrition two main oxidants for extremely preterm infants: "It all adds up." *J Neonatal Perinatal Med* **8,** 189–197.

Mohan S & Gupta D (2018*a*). Crosstalk of toll-like receptors signaling and Nrf2 pathway for regulation of inflammation. *Biomed Pharmacother* **108,** 1866–1878.

Mohan S & Gupta D (2018*b*). Crosstalk of toll-like receptors signaling and Nrf2 pathway for regulation of in fl ammation. *Biomed Pharmacother* **108,** 1866–1878.

Molès et al. (2016). Breast milk cells trafficking induces microchimerism-mediated immune system maturation in the infant. *Int J Lab Hematol* **38,** 42–49.

Molloy EJ, Wynn JL, Bliss J, Koenig JM, Keij FM, McGovern M, Kuester H, Turner MA, Giannoni E, Mazela J, Degtyaeva M, Strunk T, Simons SHP, Janota J, Plotz FB, van den Hoogen A, de Boode W, Schlapbach LJ & Reiss IKM (2020). Neonatal sepsis: need for consensus definition, collaboration and core outcomes. *Pediatr Res* **88,** 2–4.

Moreira LO, El Kasmi KC, Smith AM, Finkelstein D, Fillon S, Kim YG, Núñez G, Tuomanen E & Murray PJ (2008). The TLR2-MyD88-NOD2-RIPK2 signalling axis regulates a balanced pro-inflammatory and IL-10-mediated anti-inflammatory cytokine response to Gram-positive cell walls. *Cell Microbiol* **10,** 2067–2077.

Morgan et al. (2020). Probiotics reduce mortality and morbidity in preterm, low-birth-weight infants: A systematic review and network meta-analysis of randomized trials. *Physiol Behav* **176,** 139–148.

Morris MC & Surendran N (2016). Neonatal vaccination: Challenges and intervention strategies.

Neonatology **109,** 161–169.

Mouihate A, Galic MA, Ellis SL, Spencer SJ, Tsutsui S & Pittman QJ (2010). Early life activation of toll-like receptor 4 reprograms neural anti-inflammatory pathways. *J Neurosci* **30,** 7975–7983.

Muir A, Soong G, Sokol S, Reddy B, Gomez MI, Van Heeckeren A & Prince A (2004). Toll-like receptors in normal and cystic fibrosis airway epithelial cells. *Am J Respir Cell Mol Biol* **30,** 777–783.

Mulla MJ, Pasternak MC, Salmon JE, Chamley LW & Abrahams VM (2019). Role of NOD2 in antiphospholipid antibody-induced and bacterial MDP amplification of trophoblast inflammation. *J Autoimmun* **98,** 103–112.

Musikacharoen T, Matsuguchi T, Kikuchi T & Yoshikai Y (2001). NF-κB and STAT5 Play Important Roles in the Regulation of Mouse Toll-Like Receptor 2 Gene Expression. *J Immunol* **166,** 4516–4524.

Nadeem A, Siddiqui N, Al-Harbi NO, Al-Harbi MM & Ahmad SF (2016). TLR-7 agonist attenuates airway reactivity and inflammation through Nrf2-mediated antioxidant protection in a murine model of allergic asthma. *Int J Biochem Cell Biol* **73,** 53–62.

Nakamura N, Tamagawa-Mineoka R, Ueta M, Kinoshita S & Katoh N (2015). Toll-Like receptor 3 increases allergic and irritant contact dermatitis. *J Invest Dermatol* **135,** 411–417.

Nandi A & Bishayi B (2015). Host antioxidant enzymes and TLR-2 neutralization modulate intracellular survival of Staphylococcus aureus: Evidence of the effect of redox balance on host pathogen relationship during acute staphylococcal infection. *Microb Pathog* **89,** 114–127.

Negishi Y, Shima Y, Takeshita T & Morita R (2021). Harmful and beneficial effects of inflammatory response on reproduction: sterile and pathogen-associated inflammation. *Immunol Med* **44,** 98–115.

Neu J (2007). Gastrointestinal maturation and implications for infant feeding. *Early Hum Dev* **83,** 767–775.

Ng PC & Lam HS (2010). Biomarkers for Late-Onset Neonatal Sepsis: Cytokines and Beyond. *Clin Perinatol* **37,** 599–610.

Ng PC & Lam HS (2012). Biomarkers in neonatology: The next generation of tests. *Neonatology* **102,** 145–151.

Ng S, Strunk T, Lee AH, Gill EE, Falsafi R, Woodman T, Hibbert J, Hancock REW & Currie A (2020). Whole blood transcriptional responses of very preterm infants during late-onset sepsis. *PLoS One* **15,** 1–21.

O'Connor KM, Ashoori M, Dias ML, Dempsey E, O'Halloran KD & McDonald FB (2021). Influence of innate immune activation on endocrine and metabolic pathways in infancy. *Am J Physiol Metab*; DOI: 10.1152/ajpendo.00542.2020.

O'Driscoll DN, De Santi C, McKiernan PJ, McEneaney V, Molloy EJ & Greene CM (2017). Expression of X-linked Toll-like receptor 4 signaling genes in female vs. male neonates. *Pediatr Res* **81,** 831–837.

O'Halloran KD & McDonald FB (2018). Pharmacotherapies for apnoea of prematurity: time to pause and consider targeted sex-specific strategies? *Exp Physiol* **103,** 170–171.

ten Oever J, Kox M, van de Veerdonk FL, Mothapo KM, Slavcovici A, Jansen TL, Tweehuysen L, Giamarellos-Bourboulis EJ, Schneeberger PM, Wever PC, Stoffels M, Simon A, Simon A, Johnson MD, Kullberg BJ, Pickkers P, Pachot A, Pachot A & Netea MG (2014). The discriminative capacity of soluble Toll-like receptor (sTLR)2 and sTLR4 in inflammatory diseases. *BMC Immunol* **15,** 1–10.

Ohlin A, Björkqvist M, Montgomery SM & Schollin J (2010). Clinical signs and CRP values associated with blood culture results in neonates evaluated for suspected sepsis. *Acta Paediatr Int J Paediatr* **99,** 1635–1640.

Olin A, Henckel E, Chen Y, Lakshmikanth T, Pou C, Mikes J, Gustafsson A, Bernhardsson AK, Zhang C, Bohlin K & Brodin P (2018). Stereotypic Immune System Development in Newborn Children. *Cell* **174,** 1277-1292.e14.

Ozdemir SA, Ozer EA, Kose S, Ilhan O, Ozturk C & Sutcuoglu S (2015). Reference values of serum IgG and IgM levels in preterm and term newborns. *J Matern Neonatal Med* **00,** 1–5.

Ozsurekci Y & Aykac K (2016). Oxidative Stress Related Diseases in Newborns. *Oxid Med Cell Longev*; DOI: 10.1155/2016/2768365.

Pall ML & Levine S (2015). Nrf2, a master regulator of detoxification and also antioxidant, anti. *Acta Physiol Sin* **67,** 1–18.

Panfoli I, Candiano G, Malova M, De Angelis L, Cardiello V, Buonocore G & Ramenghi LA (2018). Oxidative stress as a primary risk factor for brain damage in preterm newborns. *Front Pediatr* **6,** 1–6.

Panigrahi P, Parida S, Nanda NC, Satpathy R, Pradhan L, Chandel DiS, Baccaglini L, Mohapatra A, Mohapatra SS, Misra PR, Chaudhry R, Chen HH, Johnson JA, Morris JG, Paneth N & Gewolb IH (2017). A randomized synbiotic trial to prevent sepsis among infants in rural India. *Nature* **548,** 407–412.

Pathak SK, Bhattacharyya A, Pathak S, Basak C, Mandal D, Kundu M & Basu J (2004). Toll-like receptor 2 and mitogen- and stress-activated kinase 1 are effectors of Mycobacterium avium-induced cyclooxygenase-2 expression in macrophages. *J Biol Chem* **279,** 55127–55136.

Pérez-Bárcena J, Regueiro V, Marsé P, Raurich JM, Rodríguez A, Ibáñez J, de Lorenzo Mateos AG & Bengoechea JA (2008). Glutamine as a modulator of the immune system of critical care

patients: Effect on Toll-like receptor expression. A preliminary study. *Nutrition* **24,** 522–527.

Petrikin JE, Gaedigk R, Leeder JS & Truog WE (2010). Selective toll-like receptor expression in human fetal lung. *Pediatr Res* **68,** 335–338.

Pettengill et al. (2016). Distinct Toll-like receptor-mediated cytokine production and immunoglobulin secretion in human newborn naïve B cells. *Physiol Behav* **176,** 139–148.

Philbin et al. (2012). Imidazoquinoline TLR8 agonists activate human newborn monocytes and dendritic cells via adenosine-refractory and caspase-1-dependent pathways. *Bone* **23,** 1–7.

Philip AGS (2009). Chronic lung disease of prematurity: A short history. *Semin Fetal Neonatal Med* **14,** 333–338.

Pludowski P et al. (2018). Vitamin D supplementation guidelines. *J Steroid Biochem Mol Biol* **175,** 125–135.

Pohar J, Pirher N, Benčina M, Manček-Keber M & Jerala R (2013). The role of UNC93B1 protein in surface localization of TLR3 receptor and in cell priming to nucleic acid agonists. *J Biol Chem* **288,** 442–454.

Potter M, Rosenkrantz T & Fitch RH (2018). Behavioral and neuroanatomical outcomes in a rat model of preterm hypoxic-ischemic brain Injury: Effects of caffeine and hypothermia. *Int J Dev Neurosci* **70,** 46–55.

Prescott et al. (2020). An airway protection program revealed by sweeping genetic control of vagal afferents. *Physiol Behav* **176,** 139–148.

Price AE, Shamardani K, Lugo KA, Deguine J, Roberts AW, Lee BL & Barton GM (2018). A Map of Toll-like Receptor Expression in the Intestinal Epithelium Reveals Distinct Spatial, Cell Type-Specific, and Temporal Patterns. *Immunity* **49,** 560-575.e6.

Qi R, Singh D & Kao CC (2012). Proteolytic processing regulates toll-like receptor 3 stability and endosomal localization. *J Biol Chem* **287,** 32617–32629.

Rafi MA, Miah MMZ, Wadood MA & Hossain MG (2020). Risk factors and etiology of neonatal sepsis after hospital delivery: A case-control study in a tertiary care hospital of Rajshahi, Bangladesh. *PLoS One* **15,** 1–14.

Repa A, Thanhaeuser M, Endress D, Weber M, Kreissl A, Binder C, Berger A & Haiden N (2015). Probiotics (Lactobacillus acidophilus and Bifidobacterium bifidum) prevent NEC in VLBW infants fed breast milk but not formula. *Pediatr Res* **77,** 381–388.

Ritter M, Mennerich D, Weith A & Seither P (2005). Characterization of Toll-like receptors in primary lung epithelial cells: Strong impact of the TLR3 ligand poly(I:C) on the regulation of Toll-like receptors, adaptor proteins and inflammatory response. *J Inflamm* **2,** 1–15.

Rosewich M, Lee D & Zielen S (2013). Pollinex Quattro: An innovative four injections immunotherapy

in allergic rhinitis. *Hum Vaccines Immunother* **9,** 1523–1531.

van Rossum et al. (2008). Myelin-Phagocytosing Macrophages in Isolated Sciatic and Optic Nerves Reveal a Unique Reactive Phenotype. *Sixth Rep World Nutr Situat Geneva* **479,** 132.

Roth A, Jensen H, Garly ML, Djana Q, Martins CL, Sodemann M, Rodrigues A & Aaby P (2004). Low birth weight infants and Calmette-Guérin bacillus vaccination at birth: Community study from Guinea-Bissau. *Pediatr Infect Dis J* **23,** 544–550.

Sanchez G (2013). Structures and Recognition Modes of Toll-Like Receptors. *El Sist argentino innovación Inst Empres y redes El desafío la creación y apropiación Conoc*; DOI: 10.1002/prot.

Scheffel J, Regen T, Van Rossum D, Seifert S, Ribes S, Nau R, Parsa R, Harris RA, Boddeke HWGM, Chuang HN, Pukrop T, Wessels JT, Jürgens T, Merkler D, Brück W, Schnaars M, Simons M, Kettenmann H & Hanisch UK (2012). Toll-like receptor activation reveals developmental reorganization and unmasks responder subsets of microglia. *Glia* **60,** 1930–1943.

Schirbel A, Kessler S, Rieder F, West G, Rebert N, Asosingh K, McDonald C & Fiocchi C (2013). Pro-angiogenic activity of TLRs and NLRs: A novel link between gut microbiota and intestinal angiogenesis. *Gastroenterology* **144,** 613-623.e9.

Schletter J, Heine H, Ulmer AJ & Rietschel ET (1995). Molecular mechanisms of endotoxin activity. *Arch Microbiol* **164,** 383–389.

Seehase M, Gantert M, Ladenburger A, Garnier Y, Kunzmann S, Thomas W, Wirbelauer J, Speer CP & Kramer BW (2011). Myocardial response in preterm fetal sheep exposed to systemic endotoxinaemia. *Pediatr Res* **70,** 242–246.

Sevastiadou S, Malamitsi-puchner A, Costalos C, Briana DD, Antsaklis A, Roma-giannikou E, Sevastiadou S, Malamitsi-puchner A, Costalos C, Briana DD, Antsaklis A, The ER, Sevastiadou S, Malamitsi-puchner A, Costalos C, Skouroliakou M & Briana DD (2011). The impact of oral glutamine supplementation on the intestinal permeability and incidence of necrotizing enterocolitis / septicemia in premature neonates The impact of oral glutamine supplementation on the intestinal permeability and incidence of necrotiz. ; DOI: 10.3109/14767058.2011.564240.

Sha Q, Truong-Tran AQ, Plitt JR, Beck LA & Schleimer RP (2004). Activation of airway epithelial cells by toll-like receptor agonists. *Am J Respir Cell Mol Biol* **31,** 358–364.

Shao Y, Forster SC, Tsaliki E, Vervier K, Strang A, Simpson N, Kumar N, Stares MD, Rodger A, Brocklehurst P, Field N & Lawley TD (2019). Stunted microbiota and opportunistic pathogen colonization in caesarean-section birth. *Nature* **574,** 117–121.

Sharma AA, Jen R, Butler A & Lavoie PM (2012). The developing human preterm neonatal immune system: A case for more research in this area. *Clin Immunol* **145,** 61–68.

1571 Sharma SK & Naidu G (2016). The role of danger-associated molecular patterns (DAMPs) in trauma
1572 and infections. *J Thorac Dis* **8,** 1406–1409.
1573 Shuto T, Furuta T, Oba M, Xu H, Li J-D, Cheung J, Gruenert DC, Uehara A, Suico MA, Okiyoneda T &
1574 Kai H (2006). Promoter hypomethylation of Toll-like receptor-2 gene is associated with
1575 increased proinflammatory response toward bacterial peptidoglycan in cystic fibrosis bronchial
1576 epithelial cells. *FASEB J* **20,** 782–784.
1577 Siebert JN, Hamann L, Verolet CM, Gameiro C, Grillet S, Siegrist CA & Posfay-Barbe KM (2018). Toll-
1578 interleukin 1 receptor domain-containing adaptor protein 180L single-nucleotide
1579 polymorphism is associated with susceptibility to recurrent pneumococcal lower respiratory
1580 tract infections in children. *Front Immunol* **9,** 1–15.
1581 Singh K, Han K, Tilve S, Wu K, Geller HM & Sack MN (2018). Parkin targets NOD2 to regulate
1582 astrocyte endoplasmic reticulum stress and inflammation. *Glia* **66,** 2427–2437.
1583 Sinha AP et al. (2021). Evaluating the efficacy of a multistrain probiotic supplementation for
1584 prevention of neonatal sepsis in 0–2-month-old low birth weight infants in India—the
1585 "ProSPoNS" Study protocol for a phase III, multicentric, randomized, double-blind, placebo-
1586 controll. *Trials* **22,** 1–12.
1587 de Souza PR, Guimarães FR, Sales-Campos H, Bonfá G, Nardini V, Chica JEL, Turato WM, Silva JS,
1588 Zamboni DS & Cardoso CR de B (2018). Absence of NOD2 receptor predisposes to intestinal
1589 inflammation by a deregulation in the immune response in hosts that are unable to control gut
1590 dysbiosis. *Immunobiology* **223,** 577–585.
1591 Sparrow S, Manning JR, Cartier J, Anblagan D, Bastin ME, Piyasena C, Pataky R, Moore EJ, Semple SI,
1592 Wilkinson AG, Evans M, Drake AJ & Boardman JP (2016). Epigenomic profiling of preterm
1593 infants reveals DNA methylation differences at sites associated with neural function. *Transl*
1594 *Psychiatry* **6,** e716.
1595 Di Stefano A, Ricciardolo FLM, Caramori G, Adcock IM, Chung KF, Barnes PJ, Brun P, Leonardi A, Andò
1596 F, Vallese D, Gnemmi I, Righi L, Cappello F & Balbi B (2017). Bronchial inflammation and
1597 bacterial load in stable COPD is associated with TLR4 overexpression. *Eur Respir J* **49,** 1–11.
1598 Steiner L, Diesner SC & Voitl P (2019). Risk of infection in the first year of life in preterm children: An
1599 Austrian observational study. *PLoS One* **14,** 1–11.
1600 Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, Ehrenkranz RA, Lemons JA, Donovan EF, Stark
1601 AR, Tyson JE, Oh W, Bauer CR, Korones SB, Shankaran S, Laptook AR, Stevenson DK, Papile LA &
1602 Kenneth Poole W (2002). Changes in pathogens causing early-onset sepsis in very-low-birth-
1603 weight infants. *N Engl J Med* **347,** 240–247.
1604 Stridh L, Mottahedin A, Johansson ME, Valdez RC, Northington F, Wang X & Mallard C (2013). Toll-

1605 Like Receptor-3 Activation Increases the Vulnerability of the Neonatal Brain to Hypoxia-
1606 Ischemia. *J Neurosci* **33,** 12041–12051.
1607 Stridh L, Smith PLP, Naylor AS, Wang X & Mallard C (2011). Regulation of Toll-like receptor 1 and -2
1608 in neonatal mice brains after hypoxia-ischemia. *J Neuroinflammation* **8,** 1–10.
1609 Taha EA, Ono K & Eguchi T (2019). Roles of extracellular HSPs as biomarkers in immune surveillance
1610 and immune evasion. *Int J Mol Sci*; DOI: 10.3390/ijms20184588.
1611 Takeda K & Akira S (2015). Toll-Like receptors. *Curr Protoc Immunol* **2015,** 14.12.1-14.12.10.
1612 Tanga FY, Nutile-McMenemy N & DeLeo JA (2005). The CNS role of Toll-like receptor 4 in innate
1613 neuroimmunity and painful neuropathy. *Proc Natl Acad Sci U S A* **102,** 5856–5861.
1614 Tendl KA, Schulz SMF, Mechtler TP, Bohn A, Metz T, Greber-Platzer S, Kasper DC, Herkner KR & Item
1615 CB (2013). DNA methylation pattern of CALCA in preterm neonates with bacterial sepsis as a
1616 putative epigenetic biomarker. *Epigenetics* **8,** 1261–1267.
1617 Triantafilou M, Gamper FGJ, Haston RM, Mouratis MA, Morath S, Hartung T & Triantafilou K (2006).
1618 Membrane sorting of toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface
1619 determines heterotypic associations with CD36 and intracellular targeting. *J Biol Chem* **281,**
1620 31002–31011.
1621 Tsan M & Gao B (2004). Endogenous ligands of Toll-like receptors.pdf. ; DOI: 10.1189/jlb.0304127.1.
1622 Tunjungputri RN, van der Ven AJ, Riksen N, Rongen G, Tacke S, van den Berg TNAD, Fijnheer R,
1623 Gomes ME, Dinarello CA, van de Veerdonk FL, Gasem MH, Netea MG, Joosten LAB, de Groot PG
1624 & de Mast Q (2015). Differential effects of platelets and platelet inhibition by ticagrelor on
1625 TLR2- and TLR4-mediated inflammatory responses. *Thromb Haemost* **113,** 1035–1045.
1626 Udden et al. (2017). NOD2 suppresses colorectal tumorigenesis via downregulation of the TLR
1627 pathways. *Physiol Behav* **176,** 139–148.
1628 Uematsu S, Sato S, Yamamoto M, Hirotani T, Kato H, Takeshita F, Matsuda M, Coban C, Ishii KJ,
1629 Kawai T, Takeuchi O & Akira S (2005). Interleukin-1 receptor-associated kinase-1 plays an
1630 essential role for Toll-like receptor (TLR)7- and TLR9-mediated interferon-α induction. *J Exp*
1631 *Med* **201,** 915–923.
1632 Vennemann MMT, Loddenkötter B, Fracasso T, Mitchell EA, Debertin AS, Larsch KP, Sperhake JP,
1633 Brinkmann B, Sauerland C, Lindemann M & Bajanowski T (2012). Cytokines and sudden infant
1634 death. *Int J Legal Med* **126,** 279–284.
1635 Viemann D, Dubbel G, Schleifenbaum S, Harms E, Sorg C & Roth J (2005). Expression of toll-like
1636 receptors in neonatal sepsis. *Pediatr Res* **58,** 654–659.
1637 Vogel JP, Chawanpaiboon S, Moller AB, Watananirun K, Bonet M & Lumbiganon P (2018). The global
1638 epidemiology of preterm birth. *Best Pract Res Clin Obstet Gynaecol* **52,** 3–12.

1639 Volz T, Nega M, Buschmann J, Kaesler S, Guenova E, Peschel A, Röcken M, Götz F & Biedermann T
1640 (2010). Natural Staphylococcus aureus-derived peptidoglycan fragments activate NOD2 and act
1641 as potent costimulators of the innate immune system exclusively in the presence of TLR signals.
1642 *FASEB J* **24,** 4089–4102.
1643 Wang D, Lou J, Ouyang C, Chen W, Liu Y, Liu X, Cao X, Wang J & Lu L (2010). Ras-related protein
1644 Rab10 facilitates TLR4 signaling by promoting replenishment of TLR4 onto the plasma
1645 membrane. *Proc Natl Acad Sci U S A* **107,** 13806–13811.
1646 Wang T, Lafuse WP, Takeda K, Akira S & Zwilling BS (2002). Rapid Chromatin Remodeling of Toll-Like
1647 Receptor 2 Promoter During Infection of Macrophages with Mycobacterium avium . *J Immunol*
1648 **169,** 795–801.
1649 Wang T, Lafuse WP & Zwilling BS (2001). NFκB and Sp1 Elements Are Necessary for Maximal
1650 Transcription of Toll-like Receptor 2 Induced by Mycobacterium avium . *J Immunol* **167,** 6924–
1651 6932.
1652 Wang Y, Cai W, Tao Y & Tang Q (2013). Glutamine supplementation in preterm infants receiving
1653 parenteral nutrition leads to an early improvement in liver function. **22,** 530–536.
1654 Wang Y, Chen T, Han C, He D, Liu H, An H, Cai Z & Cao X (2007). Lysosome-associated small Rab
1655 GTPase Rab7b negatively regulates TLR4 signaling in macrophages by promoting lysosomal
1656 degradation of TLR4. *Blood* **110,** 962–971.
1657 Weber MD, Frank MG, Sobesky JL, Watkins LR & Maier SF (2013). Blocking toll-like receptor 2 and 4
1658 signaling during a stressor prevents stress-induced priming of neuroinflammatory responses to
1659 a subsequent immune challenge. *Brain Behav Immun* **32,** 112–121.
1660 Wilson RH, Maruoka S, Whitehead GS, Foley JF, Flake GP, Sever ML, Zeldin DC, Kraft M & Carolina N
1661 (2013). The toll-like receptor 5 ligand flagellin promotes asthma by priming allergic responses
1662 to indoor allergens. **18,** 1705–1710.
1663 Wu QQ, Xiao Y, Yuan Y, Ma ZG, Liao HH, Liu C, Zhu JX, Yang Z, Deng W & Tang QZ (2017).
1664 Mechanisms contributing to cardiac remodelling. *Clin Sci* **131,** 2319–2345.
1665 Wynn JL, Kelly MS, Clark RH, Greenberg R, Benjamin DK & Smith PB (2017). Timing of Multiorgan
1666 Dysfunction among Hospitalized Infants with Fatal Fulminant Sepsis. *Am J Perinatol* **34,** 633–
1667 639.
1668 Wynn JL & Levy O (2010). Role of innate host defenses in susceptibility to early-onset neonatal
1669 sepsis. *Clin Perinatol* **37,** 307–337.
1670 Wynn JL, Seumpia PO, Winfield RD, Delano MJ, Kelly-Scumpia K, Barker T, Ungaro R, Levy O &
1671 Moldawer LL (2008). Defective innate immunity predisposes murine neonates to poor sepsis
1672 outcome but is reversed by TLR agonists. *Blood* **112,** 1750–1758.

1673 Xie MY, Hou LJ, Sun JJ, Zeng B, Xi QY, Luo JY, Chen T & Zhang YL (2019). Porcine Milk Exosome
1674 MiRNAs Attenuate LPS-Induced Apoptosis through Inhibiting TLR4/NF-κB and p53 Pathways in
1675 Intestinal Epithelial Cells. *J Agric Food Chem* **67,** 9477–9491.
1676 Xing J, Ying Y, Mao C, Liu Y, Wang T, Zhao Q, Zhang X, Yan F & Zhang H (2018). Hypoxia induces
1677 senescence of bone marrow mesenchymal stem cells via altered gut microbiota. *Nat Commun*;
1678 DOI: 10.1038/s41467-018-04453-9.
1679 Yamaguchi Y, Harker JA, Wang B, Openshaw PJ, Tregoning JS & Culley FJ (2012). Preexposure to CpG
1680 Protects against the Delayed Effects of Neonatal Respiratory Syncytial Virus Infection. *J Virol* **86,**
1681 10456–10461.
1682 Yan H, Li H, Zhu L, Gao J, Li P & Zhang Z (2019). Increased TLR4 and TREM-1 expression on monocytes
1683 and neutrophils in preterm birth: further evidence of a proinflammatory state. *J Matern*
1684 *Neonatal Med* **32,** 2961–2969.
1685 Yang Y, Lv J, Jiang S, Ma Z, Wang D, Hu W, Deng C, Fan C, Di S, Sun Y & Yi W (2016). The emerging
1686 role of toll-like receptor 4 in myocardial inflammation. *Cell Death Dis* **7,** 1–10.
1687 Yang Z, Wang L, Yu H, Wang R, Gou Y, Zhang MM, Kang C, Liu T, Lan Y, Wang X, Liu J, Cooper MA, Li
1688 X, Yue K, Yu Y, Wang L, Kim BYS, Jiang W & Sun W (2019). Membrane TLR9 positive neutrophil
1689 mediated MPLA protects against fatal bacterial sepsis. *Theranostics* **9,** 6269–6283.
1690 Yao M, Liu X, Li D, Chen T, Cai Z & Cao X (2009). Late Endosome/Lysosome-Localized Rab7b
1691 Suppresses TLR9-Initiated Proinflammatory Cytokine and Type I IFN Production in
1692 Macrophages. *J Immunol* **183,** 1751–1758.
1693 Yarandi SS, Kulkarni S, Saha M, Sylvia KE, Sears CL & Pasricha PJ (2020). Intestinal Bacteria Maintain
1694 Adult Enteric Nervous System and Nitrergic Neurons via Toll-like Receptor 2-induced
1695 Neurogenesis in Mice. *Gastroenterology* **159,** 200-213.e8.
1696 Ygberg S & Nilsson A (2012). The developing immune system - From foetus to toddler. *Acta Paediatr*
1697 *Int J Paediatr* **101,** 120–127.
1698 Yu JC, Khodadadi H, Malik A, Davidson B, Salles É da SL, Bhatia J, Hale VL & Baban B (2018). Innate
1699 Immunity of Neonates and Infants. *Front Immunol* **9,** 1759.
1700 Zanin M, Baviskar P, Webster R & Webby R (2016). The Interaction between Respiratory Pathogens
1701 and Mucus. *Cell Host Microbe* **19,** 159–168.
1702 Zarember KA & Godowski PJ (2002). Tissue Expression of Human Toll-Like Receptors and Differential
1703 Regulation of Toll-Like Receptor mRNAs in Leukocytes in Response to Microbes, Their Products,
1704 and Cytokines. *J Immunol* **168,** 554–561.
1705 Zheng L, Kelly CJ & Colgan SP (2015). Physiologic hypoxia and oxygen homeostasis in the healthy
1706 intestine. A review in the theme: Cellular responses to hypoxia. *Am J Physiol - Cell Physiol* **309,**

C350–C360.

Zong M, Bruton JD, Grundtman C, Yang H, Li JH, Alexanderson H, Palmblad K, Andersson U, Harris HE, Lundberg IE & Westerblad H (2013). TLR4 as receptor for HMGB1 induced muscle dysfunction in myositis. *Ann Rheum Dis* **72,** 1390–1399.

Zou ZH, Liu D, Li HD, Zhu DP, He Y, Hou T & Yu JL (2018). Prenatal and postnatal antibiotic exposure influences the gut microbiota of preterm infants in neonatal intensive care units. *Ann Clin Microbiol Antimicrob* **17,** 1–11.

Figure 1. Localization of TLR expression and intracellular signalling.

TLRs are mainly transported to their final destination in the plasma membrane, however receptors such as TLR3, 7, 8 and 9 are located in the endosome membranes. All these receptors activate NFKB to upregulate the expression of pro-inflammatory cytokines in the nucleus. IKN3 pathway is activated upon microbial infections that act on TLR4 and TLR3. Inflammation is also mediated by a class of proteins called DAMPs, endogenous danger molecules that are released from dying or damaged cells. Multiple damps such as serum amyloid A, heat shock protein 60 and 70, neutrophil elastase and fibronectin have been reported to interact with TLR4 and TLR2 to activate the innate immune system. *Created with BioRender.com.*

Figure 2. Toll-like receptors and their expression in multiple cell types

Activation of distinct signalling TLRs pathways is cell-type specific, ranging from neuroprogenitor to epithelial cells that are part of the central nervous system or respiratory mucosal immune system, respectively. For example, endothelial cells that constitute the mucosa barrier of the respiratory tract express both TLR4 and the co-receptor TLR2/6 that play a crucial role in defence against Gram-negative and Gram-positive bacteria. Neuronal cells are the only type of cells that express all 10 TLRs and both microglia and astrocytes have been described to express a wide range of these receptors.
(Frantz *et al.*, 2001; Lathia *et al.*, 2008; Petrikin *et al.*, 2010; Kaul *et al.*, 2012; Scheffel *et al.*, 2012; Castro-Manrreza & Montesinos, 2015; Yang *et al.*, 2016; Faksh *et al.*, 2016; Jiang *et al.*, 2018; Price *et al.*, 2018; Doreste *et al.*, 2020). *Created with BioRender.com.*

Figure 3. TLRs feedback regulation during sepsis. TLRs positive feedback control is mediated through the transcription factor NFκB that interacts with Sp1 and STAT5 elements to upregulate mRNA and protein levels of TLRs. In contrast, soluble(s) receptors correlated positively with TNF-α and inhibit mediated TNF-α and CD14 signalling, modulating negatively TLRs expression. *Created with BioRender.com.*

Figure 4. TLR2 transcriptional regulation is mediated through methylation of CpG islands.

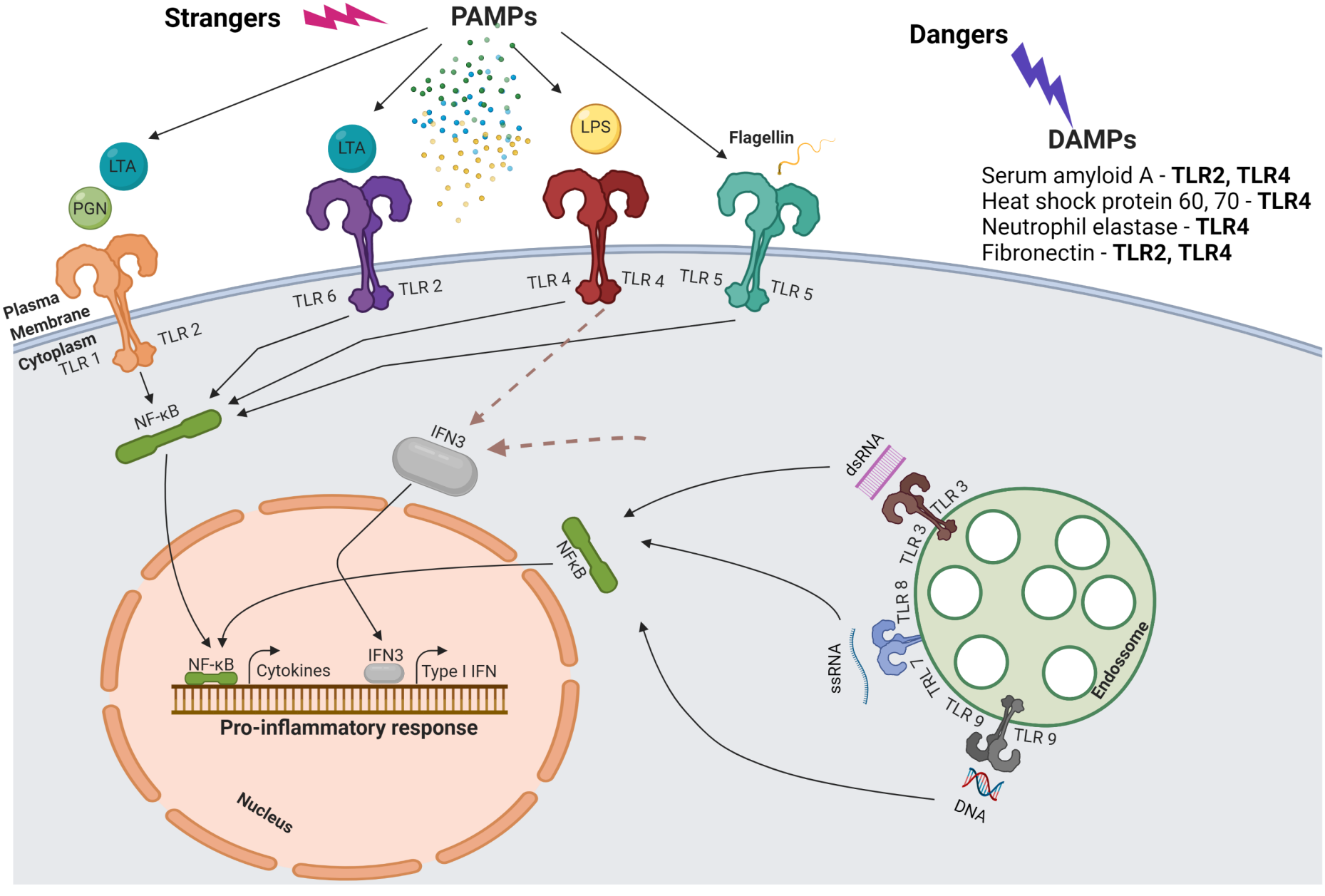
TLR2 is epigenetically regulated by hypermethylation of the promoter that silences gene expression, while hypomethylation activates transcription with aggravation of the proinflammatory response. *Created with BioRender.com.*

Figure 5. Overview of the development of neonatal sepsis and TLRs cooperation in infants.

Effective mediated crosstalk by TLRs is responsible for priming the innate immune response in many tissues upon infection. It is now clear that many PAMPs activate TLRs that actively modulate crucial body functions in neonates, such as respiratory, intestinal and cardiovascular through effective regulation of the antioxidant response and gene expression. *Created with BioRender.com.*

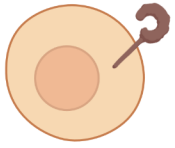
Figure 6. Toll-like receptors on the lung mucosa

Studies have reported the expression of TLRs in a variety of cells present in the lung mucosa and alveoli (Type and II pneumocystis and macrophages). However, there is a paucity of investigation in specific cells present in the respiratory epithelium, such as the secretory, goblet and stem cells. (Ritter *et al.*, 2005; Go *et al.*, 2014; Butcher *et al.*, 2018). *Created with BioRender.com.*



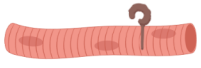
Toll-like receptors cell specificity

Satellite Cell



TLR4

Striated Myocyte



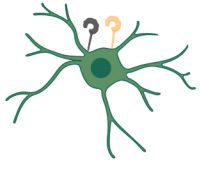
TLR4

Neuroprogenitor Cell

TLR4

TLR3

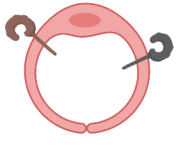
Oligodendrocyte



TLR2

TLR3

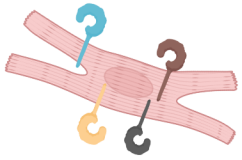
Endothelial Cell



TLR4

TLR2

Cardiac Myocyte



TLR2

TLR4

TLR3

TLR9

Mesenchymal Stem Cell

TLR2

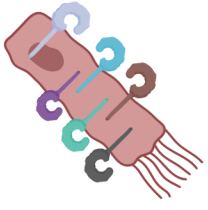
TLR4

TLR3

TLR7

TLR9

Epithelial Cell



TLR1

TLR2

TLR4

TLR5

TLR6

TLR9

Astrocyte

TLR1

TLR2

TLR3

TLR4

TLR5

TLR9

Microglia Cell

TLR1

TLR6

TLR2

TLR7

TLR3

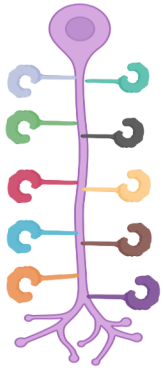
TLR8

TLR4

TLR9

TLR5

Neuronal Cell



TLR1

TLR6

TLR2

TLR7

TLR3

TLR8

TLR4

TLR9

TLR5

TLR10