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Title	Targeting the toll-like receptor pathway as a therapeutic strategy for neonatal infection
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Publication date	2021-10-06
Original Citation	Dias, M. L., O'Connor, K. M., Dempsey, E., O'Halloran, K. D. and McDonald, F. B. (2021) 'Targeting the toll-like receptor pathway as a therapeutic strategy for neonatal infection', American Journal of Physiology - Regulatory, Integrative and Comparative Physiology. doi: 10.1152/ajpregu.00307.2020
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1152/ajpregu.00307.2020
Rights	© 2021, the Authors. Licensed under Creative Commons Attribution CC-BY 4.0.Published by the American Physiological Society.R879Am J Physiol Regul Integr Comp Physiol 321: R879–R902, 2021.First published October 6, 2021; doi:10.1152/ajpregu.00307.2020 - https://creativecommons.org/licenses/by/4.0/
Download date	2026-11-06 20:23:44
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REGULATORY, INTEGRATIVE AND COMPARATIVE PHYSIOLOGY

Am J Physiol Regul Integr Comp Physiol 321: R879–R902, 2021.
First published October 6, 2021; doi:10.1152/ajpregu.00307.2020

REVIEW

Respiration

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

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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 that 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 signaling 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.

cardiorespiratory; early life; inflammation; preterm; sepsis

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 who are born before 37 complete wk of gestation (1). There is an increasing number of infants born extremely preterm and with very low birth weight annually. This relatively new cohort of infants is at increased risk of infection, which is defined as a physiological reaction to a pathogenic microorganism (2). 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 signaling, and feedback regulation are 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 nonimmune 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 signaling pathways is a result of ex vivo experiments using primary cell culture or cell lines that 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 cardiorespira-

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Submitted 29 October 2020 / Revised 30 September 2021 / Accepted 30 September 2021

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Published by the American Physiological Society.

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tory control may modulate the performance of those systems. Most integrative physiological studies have used 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 (3). We present the current understanding of TLR signaling 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. Finally, we will discuss the opportunities of TLRs as novel therapeutic targets in diagnosis and treatment, limiting the devastating short-term and long-term outcomes of sepsis and identifying future avenues that need to be explored.

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-organized 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 before microbial exposure (4). The innate immune system forms the front-line physiological defense against microbes. The integrity of the innate immune system during the first 3 mo of life is critical at a time when the baby lacks immunological memory important in mature adaptive immune responses (5–7). Passive immunity transferred to the offspring from the mother is one component of innate immunity that confers protection against microbes (8–10). Maternal neutralizing 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 (11, 12). The innate immune system initiates an immunocompetent response to many microorganisms including Gram-negative (GN) and Gram-positive (GP) bacteria, fungi, and viruses (13). Innate immune protection is dependent on the identification of conserved molecular patterns by pattern recognition receptors PRRs such as TLRs (14). Pathogen or microbe-associated molecular patterns (PAMPs) are expressed by invading pathogens, whereas damage/danger-associated molecular patterns (DAMPs) are released during sterile 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 (Fig. 1). There is likely cooperation between PAMPs and DAMPs and significant cross talk between members of the TLR family (15, 16). In newborns, TLR insufficiency increases the predisposition to infectious diseases such as sepsis, highlighting the significant reliance on innate immunity (17).

Neonatal Pathogenic and Sterile Inflammation

Increased inflammation is considered a risk factor and a promoter of preterm birth. Activation of the innate immune system in utero changes the expression of PRRs and consequently shapes the immune system (18, 19). Infants exposed to in utero infection such as chorioamnionitis, or exposed to chronic maternal infection, have distinct innate immunity compared with control infants (20, 21). A recent study in a large animal model of fetal growth restriction recapitulated neonatal vulnerability to infection (22). Infants born prematurely after hypertensive disorders with fetal growth restriction (FGR) or after FGR alone are more likely to be diagnosed with late-onset infection (23), pointing to not only microbial-mediated inflammation but also potentially sterile inflammation changing the immune landscape. Sterile inflammation is classified as inflammation in the absence of infection; immune cells and their signaling molecules are responsible for this inflammation and can impact 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 (24). 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 (25–27). Neonatal dendritic cells from term and preterm infants require increased stimulation for efficient activation. Impaired innate immune activity has been characterized by reduced innate cytokine and monocyte response, impaired neutrophil activity, decreased lung epithelial cell repair, and altered response to endotoxin lipopolysaccharide (LPS) (26, 28). 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 adults (29, 30). However, it is likely that the neonatal immune system, though functionally capable of mounting an immune response, is primed toward a stronger anti-inflammatory response (31). Studies that examined cytokine release in plasma, point toward a humoral agent such as adenosine that modulates the immune response in favor of anti-inflammatory factors (32). For example, very preterm infants with late-onset infection have a significant inflammatory response but it may be dominated by an anti-inflammatory response, as an increase in IL-10-to-TNF-α ratio is reported (33).

Early life infection claims the lives of nearly 3,000 neonates worldwide every day (34). Preterm and very-low-birthweight newborns are at increased risk of developing significant infections compared with term newborns (2, 35, 36). 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

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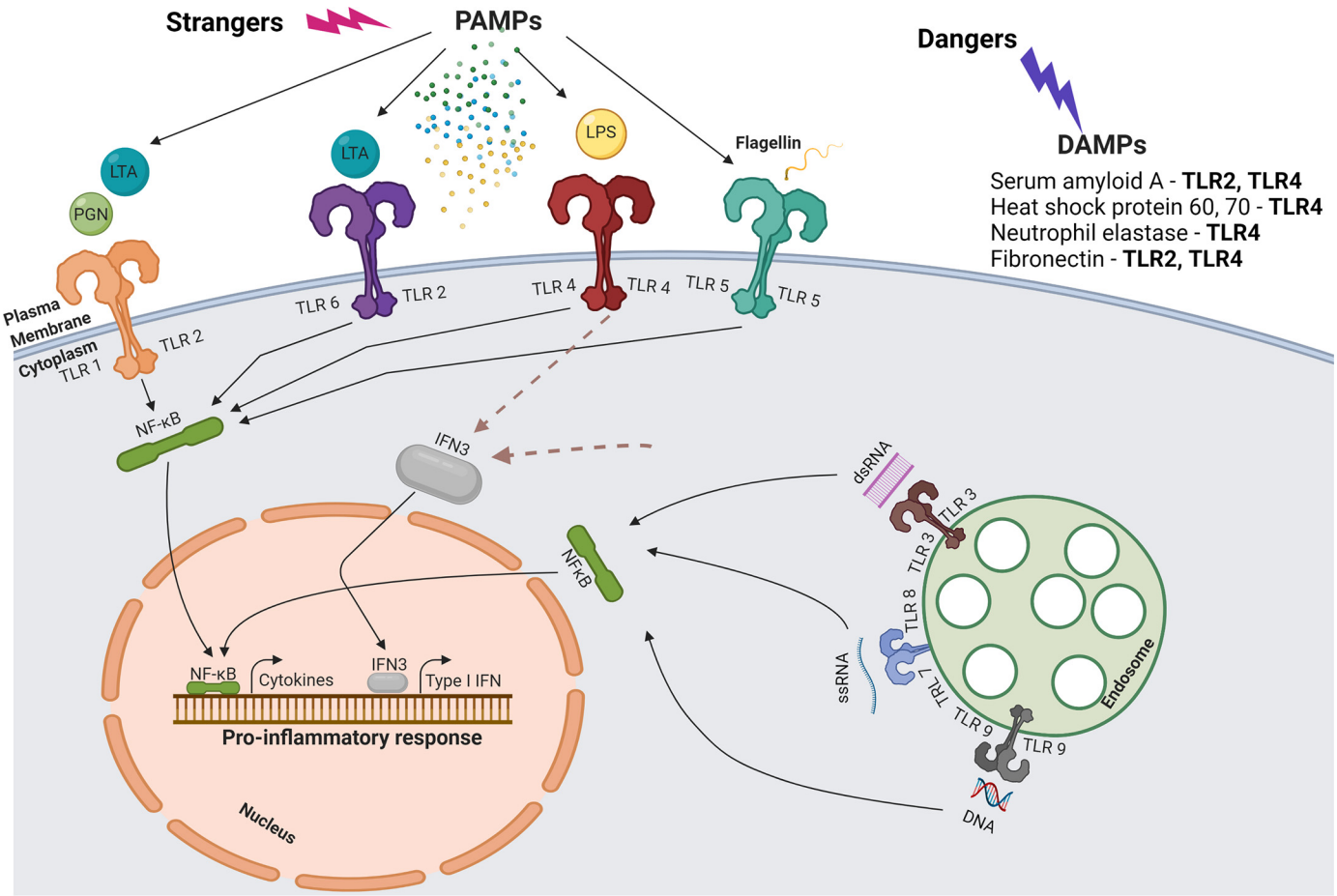


Figure 1. Localization of TLR expression and intracellular signaling. TLRs are mainly transported to their final destination in the plasma membrane; however, receptors such as TLR3, 7, 8, and 9 are located in the endosome membranes. All these receptors activate NF-κB to upregulate the expression of proinflammatory 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. DAMPs, damage/danger-associated molecular patterns; TLRs, Toll-like receptors. Created with BioRender.com.

venous cannulation, arterial line placement, and endotracheal intubation (31, 37). A neonate with suspected infection will typically initially present clinically with signs of unstable cardiorespiratory 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 (28, 38, 39). Elevated C-reactive protein associated with positive blood culture results often confirm cases of infection (39), but other biomarkers have been proposed by others and discussed in previous reviews (40, 41).

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 (42). Differential definitions of neonatal sepsis among leading medical organizations make epidemiological interpretation of acute and chronic implications difficult (43). Neonatal sepsis is categorized into either early onset sepsis (EOS) or late-onset sepsis (LOS). EOS occurs during the first 24–72 h of life in the preterm infant and is often a fulminant multisystem infection acquired by vertical transmission from the mother (44). There are two major bacteria that are responsible for EONS: Group B *Streptococcus* (GBS) and *Escherichia coli* were confirmed in 41% and 17%, respectively, from a total of 408 samples taken from infected infants within the first week of life (45, 46). LOS is one of the most common causes of morbidity and mortality in preterm infants. In a recent United Kingdom 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 (46). It has been reported that GN infections are more likely to result in death within 72 h after birth (29%) compared with GP infections (6%) (47). 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 prooxidant

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and antioxidant abundance generates a toxic cell environment, leading to tissue damage, which has adverse consequences for the "normal" development of a newborn, especially preterm infants. An infant's first breath is metabolically characterized by a swift change to an oxidant environment, the partial pressure of oxygen changes significantly from 35 mmHg (umbilical vein) to 85 mmHg (arterial) (48). 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 (49). Newborn infants, and preterm infants, in particular, have weak antioxidant defenses 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 hemorrhagic shock/resuscitation has been reviewed by Gill et al. (50), in which they propose both direct activation of TLRs by redox stress or indirectly via DAMPs.

Male Sex as a Risk Factor for Late-Onset Infection

Male sex is a risk factor for late-onset sepsis (51–53). It is widely acknowledged that male neonates have poorer disease prognosis and unfavorable outcomes upon microbial infection compared with females (54, 55). Androgens are suggested to enhance susceptibility, whereas female gonadal hormones estrogen and progesterone are reported to offer resilience as reflected in animal studies (56–59). The role of sex hormones in modulating the immune response has been recently reviewed by Di Florio et al. (56) and they propose that hormonal modulation of TLR signaling 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), necrotizing enterocolitis (NEC), retinopathy of prematurity, and cerebral hemorrhage (60). 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.

The Impact of Prematurity in PRR Signaling and Cell Redox State

Ng et al. (61) have reported upregulation of PRR pathways in patients with late-onset sepsis who were born very preterm. Preterm infants often have sterile inflammation as a result of oxidative stress and local injury that activates the innate immune system (62). This population has a low reserve of antioxidants and is particularly at risk of oxidative stress (63). There is a 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 (4, 6). The activation of the innate immune system is complex and requires multiple levels of cross talk between membrane receptors and effector proteins to produce a normal immune response (25). Uncovering the expression and functional responsiveness of Toll-like receptors during the neonatal period as well as their role in initiating and potentially resolving 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 proinflammatory gene expression, however, it has also been described to be modulated following TLR engagement (64). An additional level of complexity is thus associated with TLR transcriptional regulation, and the participation of a variety of accessory proteins and transcription factors contribute to the innate immune system response to infections. Elucidating the immune responses related to TLR signaling by bacterial infection and indeed endogenous DAMPs may provide the key to understanding the reasons for preterm infant disease susceptibility.

TOLL-LIKE RECEPTOR SIGNALING

In 1996, Toll receptors were first identified as essential receptors for dorsoventral patterning that were demonstrated to improve the immune response in the developing embryo of *Drosophila* (65). Research in *Drosophila* continues to reveal important functional roles for TLRs such as modulating cell survival and proliferation (66). The first mammalian homolog receptor of *Drosophila* was identified in 1997 and named hToll, now termed TLR4 (67). There are now 10 TLR subtypes identified in humans (TLR1–TLR10) and 12 TLR subtypes identified in mice and rats (TLR1–TLR9 and TLR11–TLR13) (16). These receptors enable multilayered protection and are located either externally on the surface membrane and/or internally in the cytosol and endosomes depending on the specific subtype (Fig. 1) (3, 68, 69) TLRs have been identified on multiple cell types, including endothelial and epithelial cells, and immune cells such as macrophages, monocytes, dendritic cells, neutrophils, and microglia. TLR expression is not only limited to immune cells but is also expressed on endothelial, epithelial cells, neuronal cells, neuronal progenitor cells, astrocytes, oligodendrocytes, satellite cells, myocytes, and platelets (Fig. 2) (70–72).

Toll-like Receptor Formation

The COOH-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 signaling through homotypic or heterotypic dimerization (73). The extracellular domains are responsible for binding PAMPs and each TLR subtype recognizes specific patterns of microbial components upon infection (Fig. 1) (74). X-ray crystallography supports the hypothesis that ligand-binding induces dimerization of the extracellular domains, which leads to juxtaposition that activates the intracellular TIR domain and induces the activation of downstream signaling cascades (73). TLR1-6, TLR1-9, and TLR1-13 are present as monomers in solution and dimerization take place upon ligand binding; conversely, TLR7 and TLR18 exist as preformed dimers that subsequently change conformation (16), though recent studies suggest that TLR1/2 and TLR2/6 are also present as preformed dimers (75, 76). Thus, the ligand recognition and activation mechanism upon ligand binding

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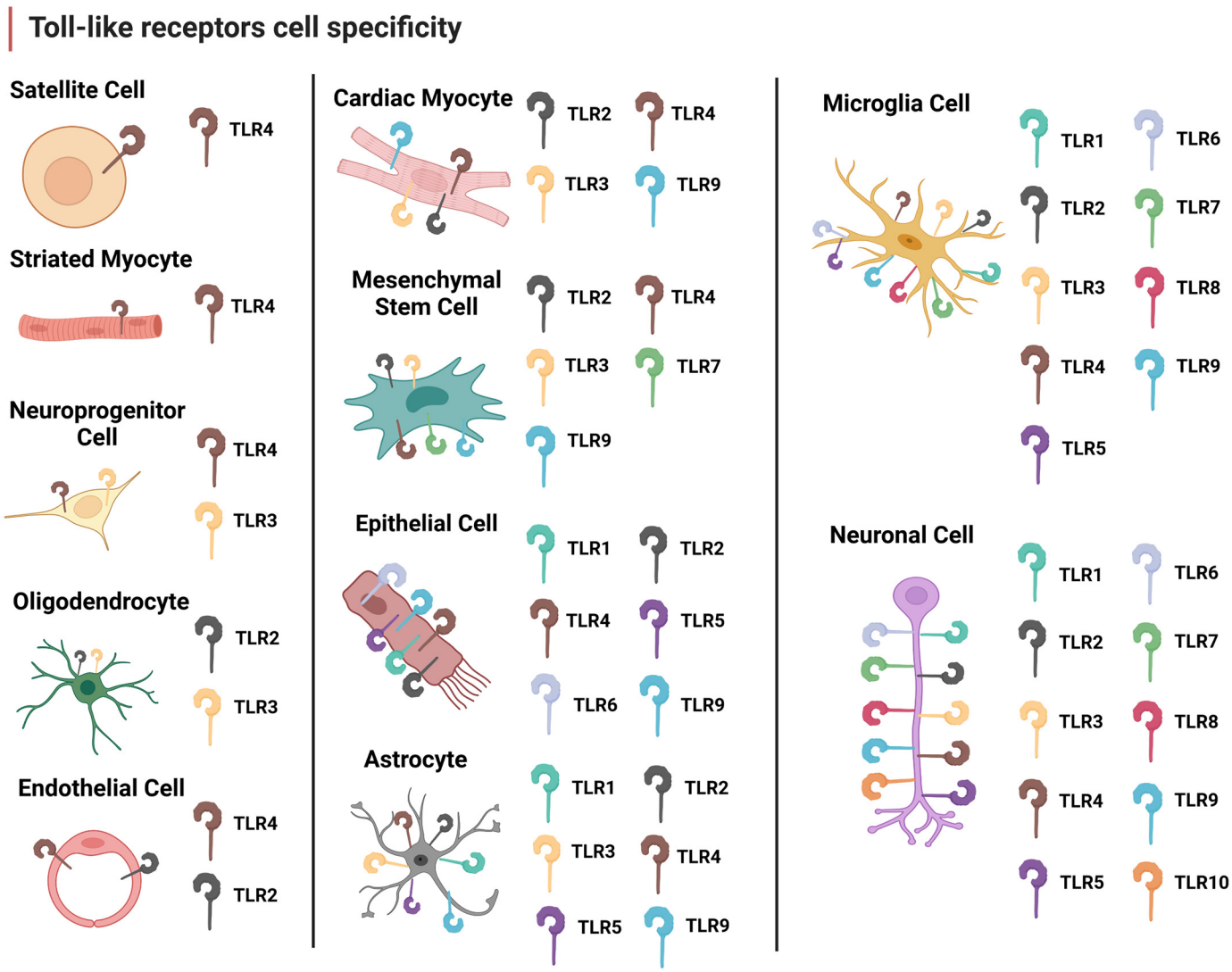


Figure 2. Toll-like receptors and their expression in multiple cell types. Activation of distinct signaling TLRs pathways is cell-type specific, ranging from neuroprogenitor to epithelial cells that are part of the central nervous system or respiratory mucosal immune system, respectively. For example, endothelial cells that constitute the mucosa barrier of the respiratory tract express both TLR4 and the coreceptor TLR2/6 that play a crucial role in defense 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. (165, 173, 188, 189, 192, 218, 224, 261–266). Created with BioRender.com.

are dependent on the structural rearrangement and availability of TLRs on the cell membrane. Toll-like receptor 4 recognizes GN bacterial protein LPS and has been the most extensively studied TLR subtype, whereas GP bacterial infection is primarily mediated through TLR2 by its recognition of lipotechoic acid (LTA) and peptidoglycan (PGN) (Fig. 1). Toll-like receptors 4 and TLR2 will be prominent in our discussion but we will also extend the discussion to other subtypes where appropriate.

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 recognizes LPS is then activated by formation of a cluster of differentiation 14 (CD14)/myeloid differentiation protein 2 (MD2)/TLR4 complex. CD14 is generally localized to lipid rafts in the plasma membrane. This complex is required for LPS-mediated signaling through TLR4 and is upregulated during neonatal bacterial sepsis (77). Common polymorphisms in the LBP gene expression are associated with an increased risk of sepsis from GN infection in adult males compared with females (78). Novel variants in LBP have also been identified in preterm infants with bronchopulmonary dysplasia (79).

Cooperation of TLRs with other receptors has also been reported. TLR2 is crucial for the recognition of PGN and is known to form heterodimers with TLR1 and TLR6 that bind lipoproteins depending on whether they are triacylated or diacylated, respectively (80). However, the detection of GP bacteria relies on both TLR2 and the nucleotide oligomerization

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domain (NOD) leucine-rich repeat-containing family 2. NOD2 are mainly intracellular innate immune sensors involved in host defense with crucial roles in promoting specificity of protein recognition, enhancing antigen binding, and maintaining intracellular cross talk. Nucleotide oligomerization domain 2 (NOD2) has been reported to be an essential sensor for PGN through muropeptide detection (81). Both TLR2 and NOD2 cooperate to upregulate signaling pathways that promote inflammation and are nonredundant (82, 83). Many studies suggest that NOD2 plays a role in regulating inflammation in trophoblasts, astrocytes, and intestinal epithelial cells (84–87). NOD2 may also act downstream of TLR2, along the TLR2-NOD2-RIPK2 (receptor-interacting serine/threonine kinase 2) pathway, whereby NOD2 plays a key role in increasing IL-10 expression in response to *Streptococcus pneumonia* (88). In support of a role for NOD2 as a negative regulator of inflammation, Udden et al. (89) 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 suppresses colorectal tumorigenesis by downregulation of NF-κB and mitogen-activated protein kinase, blunting the proliferation of cancerous cells.

Cross talk 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 signaling, resulting in either synergistic stimulation of IL-12, a cytokine crucial for stimulating adaptive immunity when TLR4 signaling intensity is low, or in contrast inhibition of IL-12 synthesis when signaling intensifies maintaining intestinal mucosal homeostasis (90, 91). A recent study by Goethel et al. (92) in neonatal mice demonstrated that antibiotic treatment started 1 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 that NOD2 is a potent microbial sensor in the gut, is functionally protective against mucosal damage, and influences the resilience to perturbations. Also interesting is the fact that NOD2 activation has been demonstrated to inhibit serotonin transport through the RIPK2 intracellular pathway (93). Data from Layunta et al. (93) infers that NOD2 could modulate intestinal infection by interacting with TLRs and modulating the intestinal serotonergic system. As reviewed in this section, TLRs are dependent on accessory proteins and perform their functions in parallel with costimulatory receptor pathways such as NOD2. There are mixed reports with regard to the expression of NOD2 in preterm infants. Chen et al. (94) report a decrease in NOD2 expression as well as decreased release of proinflammatory mediators compared with adults using peripheral blood samples, whereas Granland et al. (95) report no differences but blood was sampled from both peripheral and cord blood. It is also reported that ≥2 NOD2 loss-of-function mutations are associated with an increased risk of NEC in preterm infants (94, 96). It is important for us to identify each of the players within the innate immune system of the newborn infant as loss of function in accessory proteins or costimulatory pathways will significantly impinge on neonatal outcomes.

Toll-like Receptor Signaling Cascades

The molecular mechanisms underlying TLR signaling cascades are still under investigation. However, one of the major signaling pathways of TLRs is the MyD88-dependent pathway, which utilizes 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 NF-κB, expression of inflammatory genes, and subsequent production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1β, and cyclooxygenase-2 (COX-2). LPS exposure activates TNF-α synthesis and COX-2 expression in a MyD88-dependent manner (97). 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 NF-κB (98). Later, in vitro rodent studies demonstrated that IRAK-1 participates in NF-κB activation of IL1R(TIR)-TLR dimerization (99) and was subsequently shown to be an essential regulator for activation of TLR7-signaling and TLR9-signaling pathways (32). 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 (100). 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 (101). Important research has demonstrated that polymorphisms or mutations in TLRs and downstream signaling molecules such as MyD88 and IRAK-4 are associated with increased risk of infection in children (102) and adults (103). In humans, the IRAK-1 haplotype is associated with poorer outcomes in sepsis and increase in NF-κB expression. However, animal studies have shown that heterozygous females for the wild-type IRAK-1 haplotypes show no adverse clinical effects (104). Regulation of host defense during exposure to LPS is also dependent on TLR4-driven induction of a signaling adapter p62, crucial in selective autophagy and ubiquitination of protein aggregates (105). 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 (106). The basal level of TLR expression and the activation of their downstream signaling cascades will influence the neonatal response to infection (36, 107).

Cooperation of Toll-like Receptors and Antioxidants

The neonatal transition to a relatively high oxygen environment can activate several transcription factors, including NF-κB that can promote inflammation but also nuclear factor erythroid 2-related factor 2 (Nrf2) that initiates the activation of endogenous antioxidant defense mechanisms (108).

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In human tracheal smooth muscle cells, ROS (reactive oxygen species) 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 (109). This cross talk between Toll-like receptor signaling and the Nrf2 pathway is important in the regulation of inflammation. The Nrf2 signaling pathway is a major regulator of antioxidant production and cooperates with TLRs to suppress chronic inflammation (110). 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, an Nrf2-inducer molecule (111). 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 (112).

REGULATION OF TOLL-LIKE RECEPTOR

Feedback Regulation

TLR expression is under feedback regulation (Fig. 3). Positive feedback control is mediated through the transcription factor NF-κB, a master regulator of inflammation and the immune response (113). Many studies demonstrate cooperativity between several transcription factors in the regulation of TLR transcription. For example, NF-κB 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 (114, 115). Moreover, TLR2 transcription has been reported to require TNF-α and androgen receptor activation function-1 cooperativity to promote activation of the TLR2 promoter and stimulate transcription (116). 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, namely, sTLR2 and sTRL4, are released into the circulation from peripheral blood mononuclear cells upon exposure to microbial ligands (117). In this study, sTLR2 correlated positively with TNF-α; however, it was previously reported that sTLR2 is capable of modulating TLR2 signaling in human plasma and breast milk by interacting with CD14 (membrane or soluble forms) and decreasing the bacterial response (118). Interestingly, sTLR4 did not activate production of cytokines (117). Furthermore, Iwami et al. (119) report that in mice,

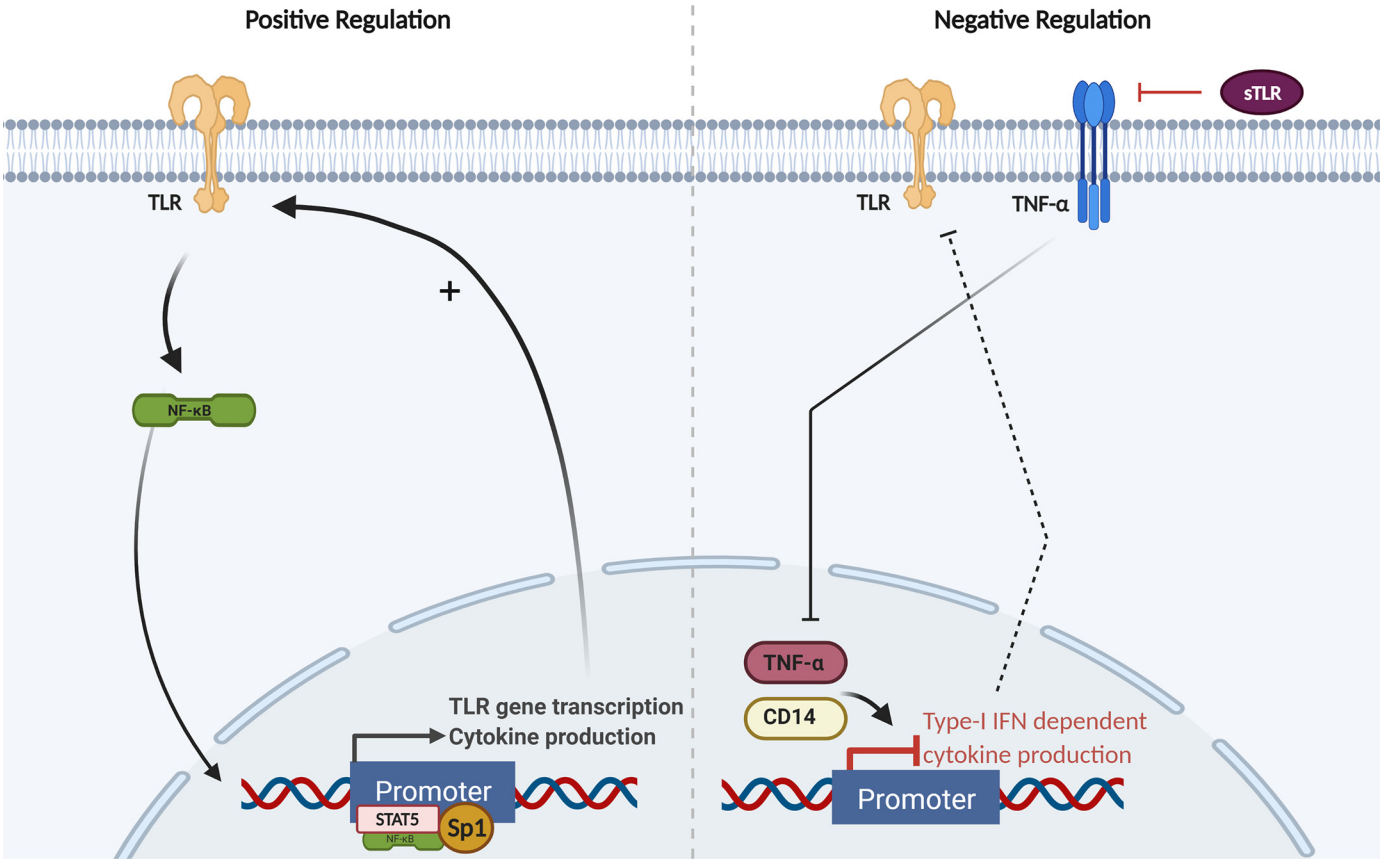


Figure 3. TLRs feedback regulation during sepsis. TLRs positive feedback control is mediated through the transcription factor NF-κB that interacts with Sp1 and STAT5 elements to upregulate mRNA and protein levels of TLRs. In contrast, soluble (s) receptors correlated positively with TNF-α and inhibit mediated TNF-α and CD14 signaling, modulating negatively TLRs expression. Sp1, stimulation factor 1; STAT5, signal transducer and activator of transcription 5; TLRs, Toll-like receptors. Created with BioRender.com.

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sTLR4 inhibits LPS-mediated TNF-α signaling, 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 (120). An in vitro study demonstrated that NF-κB 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 (97). 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 (121). Unraveling 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 that we optimize breast milk delivery to the preterm infant, whether that be mother's own milk or where there are challenges, we facilitate the use of donor milk for preterm infants.

Epigenetic and Chromatin Modifications

Human and murine TLR2 gene expression is controlled by distinct, nonconserved regulatory elements, with some regions located in a cytosine-phosphate-guanine (CpG) island that modulates TLR2 gene repression (Fig. 4). These sites contain putative binding sites for several transcription factors, including Sp1 (122). It is well established that hypermethylation of the gene promoter silences gene expression, whereas 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 (123, 124). 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 (125). Altered methylation of TLR genes in neonatal sepsis has not been identified in pilot studies, but other inflammatory genes have been identified (126, 127).

In a murine alveolar macrophage cell line, the TLR2 promoter is reported to undergo chromatin remodeling following infection with GP bacteria. This chromatin remodeling 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 (128). In addition, 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 NF-κB complex to the nucleus downstream of TLR2, thereby preventing the production of proinflammatory cytokines (124). A study on infants with confirmed prenatal chorioamnionitis revealed gain in histone tail modification H3K4me3 in monocytes distinct from noninfected infants, but it is clear that their contribution to neonatal sepsis risk will depend on the location of the epigenetic changes, be they at promoter, intron, exon, and intergenic regions (20). 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.

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 3 h, more specifically, TLR2 protein induction is reached within 1 h and subsequently decreases, suggesting the activation of proteolysis (129). In

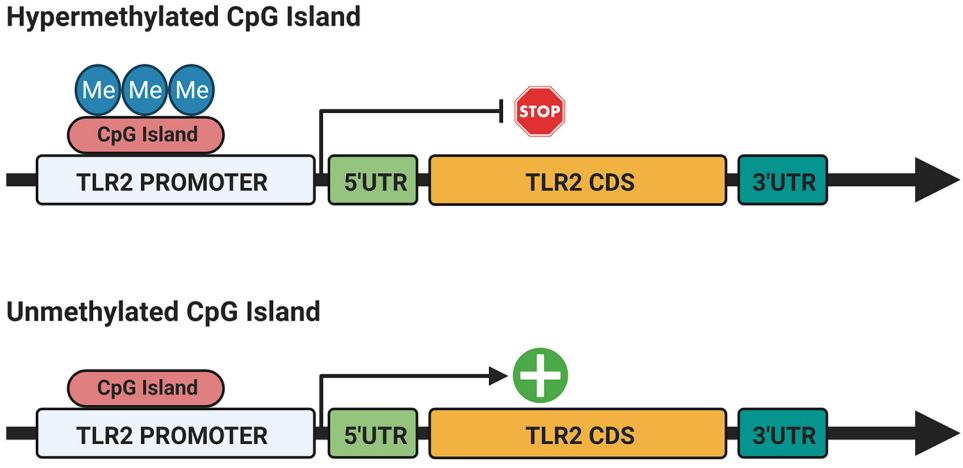


Figure 4. TLR2 transcriptional regulation is mediated through methylation of CpG islands. TLR2 is epigenetically regulated by hypermethylation of the promoter that silences gene expression, whereas hypomethylation activates transcription with aggravation of the proinflammatory response. CpG, cytosine-phosphate-guanine; TLR, Toll-like receptor. Created with BioRender.com.

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lung tissue, TLR4 has been shown to present a half-life between 2 and 3 h (130). TLR3 has a half-life of 3 to 12 h in human embryonic kidney cells (HEKs) 293 (131). However, the length of the TLR3 protein critically impacts its half-life: in the same cell cultures, TLR3 full-length protein has a half-life of 3 h, and the cleaved protein fragments present with half-lives of over 7 h (132).

Uncontrolled TLR expression and activation will contribute to the 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 (133). TLR2 is directly ubiquitinated by RING finger E3 ligase, PPP1R11 both in vitro and in vivo in white blood cell samples isolated from patients with Gram-positive infections and mouse lungs, respectively. This signaling cascade leads to degradation of TLR2, which greatly impacts Gram-positive bacterial clearance (134). In macrophages, a lysosome-associated small guanosine triphosphatase (GTPase) Rab7b has been reported to negatively regulate LPS-induced production of TNF-α 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 signaling, by establishing the translocation of TLR4 into lysosomes (135). 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 (136, 137). TLR9 response to inflammation in macrophages has been also demonstrated to be negatively regulated by Rab7b as it promotes TLR9 protein degradation (138). 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 an RNF216-dependent pathway in response to circulating microRNAs. Negative regulation of TLR expression and promotion of TLR degradation are important steps for regulation of 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.

IMPLICATIONS OF EARLY LIFE IMMUNE ACTIVATION

Sensitization

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 has 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. (139), TLR4 activation by the binding of its ligand LPS on mononuclear phagocytes is responsible for the production of proinflammatory cytokines (TNF-α, IL1, and IL-6), bioactive lipids (prostaglandins), and ROS. In the brain, TLR sensitization has been the focus of intense investigation. For example, LPS dramatically increases vulnerability to hypoxic-ischemic (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 (140). Vulnerability to HI has also been reported to increase with TLR3 activation in mice at *postnatal day 9*, which markedly increases both white and gray matter injury in a TLR3/TRIF-dependent pathway (141). The impact of TLRs in the central nervous system (CNS) has also been investigated 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 (142). At the cellular level, TLR ligands have been reported to sensitize B cell receptor signaling 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 signaling is present and allows discrimination between microbial and inert antigens by controlling the threshold for BCR activation (143). One study using *TLR3*$^{-/-}$ mice demonstrated that TLR3 enhances antigen-independent skin inflammation in allergic contact dermatitis with increased expression of TNF-α (144). Finally, in adults, the TLR5 ligand flagellin promotes asthma symptoms by sensitizing the allergic response to indoor allergens (145).

Tolerance

Previous exposure to or preconditioning with LPS is proposed to contribute to a state of hyporesponsiveness or endotoxin tolerance, which may limit inflammation-induced tissue injury (107). In preterm sheep exposed to repeated prenatal GN bacterial protein LPS, it was reported that TLR4 signaling exhibited decreased expression of the positive intracellular regulator IRAK-4 and increased expression of the 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 (146). In premature infants who developed NEC and GN bacterial infection, exposure of primary enterocytes to LPS after birth leads to TLR tolerance, such that NF-kB is not stabilized and it is thought that this hyporesponsiveness plays a role in host-immune stabilization of microbial colonization (147). In a study by Dhillon et al. (148), 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 negative controls, 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 inflamma-

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tion. A novel TLR2 and TLR4 antagonist, OxPAPC, administrated to rats 24 h before an LPS challenge has been reported to prevent the stress-induced potentiation of hippocampal proinflammatory response from isolated microglial (149). A study by Kumral et al. (29) performed in neonatal rats suggests that the number of apoptotic cell deaths and hypomyelination in the periventricular white matter region is significantly reduced with administration of LPS (preconditioning) 24 h before acute LPS exposure. In the latter report, analysis of antioxidant gene expression revealed an upregulation of superoxide dismutase (SOD) 1, 2, and 3 as well as TNF-α expression levels, suggesting that low-dose LPS given 1 day before administration of a potent dose protects against endotoxin-induced white matter injury in cooperation with antioxidant enzymes (29); however, no assessment of TLR expression was made in this study. Interestingly, neural anti-inflammatory pathways have been demonstrated to be reprogrammed 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 (150). In LPS-exposed rats, administration of dobutamine (a β1-adrenergic agonist), in hippocampal slices from 6-day-old mice, upregulates the expression of SOD3 and survival genes and decreases the inflammatory response by modifying TLR4 signaling pathways, including IFN-β and NF-κB (151). In addition, upregulation of SOD3 has been proposed to play a role in the activation of signaling cascades downstream from the β1-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 (152, 153). 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. In addition, 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.

EXPRESSION OF TLRs AND DEVELOPMENTAL PATTERNS

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 has 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 not only reflect their protective role but also point toward their functional role in neurodevelopment outcomes such as neurogenesis (154–156). 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 defense against pathogenic, nonpathogenic microorganisms, and endogenous DAMPs (157, 158).

Intestinal tract.

After birth, the external environment of the gut lumen is quickly colonized and contains a significant repository of diverse microorganisms that contribute positively to physiological homeostasis (159). In preterm infants, aberrant gut microbiota and intestinal barrier disruption can develop as a result of cesarean delivery, parenteral feeding, antibiotic treatment, and hypoxic stress (160–162). A study by Zou et al. (161) 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 colonize the gut. In humans, it has been reported that probiotic supplementation results in a significantly lower incidence of LOS in preterm newborns exclusively fed breast milk with great benefit in the reduction of necrotizing enterocolitis (NEC) (163). Benefits of probiotic supplementation in a mouse model of NEC were shown to be dependent on TLR9, in animals aged 7–10 postnatal days (164). TLR expression along the gut has recently been described in mice between 6 and 12 wk of age (165). TLR9 expression was not observed in the gut epithelium in these mice however. Nonetheless, the expression of 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 were not detected in colonic organoids (165, 166). This suggests that there may be signaling 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 (167). In the latter report, human intestinal microvascular endothelial cells and human intestinal fibroblast were exposed to bacterial ligands for TLR2/6 and TLR4, 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 (168). Propyl hydroxylase enzymes (PHDs) are negative regulators of hypoxia-inducible factor 1α (HIF 1α), and the loss of PHDs under hypoxic conditions has been implicated in deleterious gut phenotypes (169). An ex vivo study in humans showed that a hypoxic insult of 1-h stabilized 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 (170).

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Respiratory tract.

In the lung, mechanisms that regulate mucosal inflammation impact lung development and depend on TLR signaling (171–173). 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 coordination have long been identified as complications of preterm birth (174–178). TLRs are evident along the length of the respiratory tract (Fig. 5). TLR9 is expressed in the nasal mucosa of healthy adult humans on mast cells, dendritic cells, and fibroblast-like cells (179). Sha et al. (180) demonstrated mRNA expression of all TLRs in the BEAS-2b cell line (human bronchial epithelium transformed by an adenovirus) and PBEC (human primary bronchial epithelial cells). Bronchial epithelial cells express functional TLR1–TLR6 and TLR9 in adults, although the responsiveness of TLR2 was shown to be relatively subdued (70, 181). TLR2 appears to have an apical surface location, whereas TLR4 is expressed more abundantly in the basolateral region of the epithelial cell (182). Expression of both TLR2 and TLR4 receptors is also evident in alveolar epithelium cells in human adults (183). 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 (171). In preterm lambs, both TLR4 and CLR-family PRRs in respiratory epithelia increased from late gestation to term birth (184). This was consistent with a study from Hillman et al. (185), 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, whereas TLR1 and TLR4 were significantly increased at 52 days suggesting a dynamic and developmental pattern of expression between members of the TLR family. In the guinea pig, all TLRs had significantly higher expression in lung during later gestational age compared with postnatal expression (186). A report by Awasthi et al. (187) performed in premature baboons shows 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

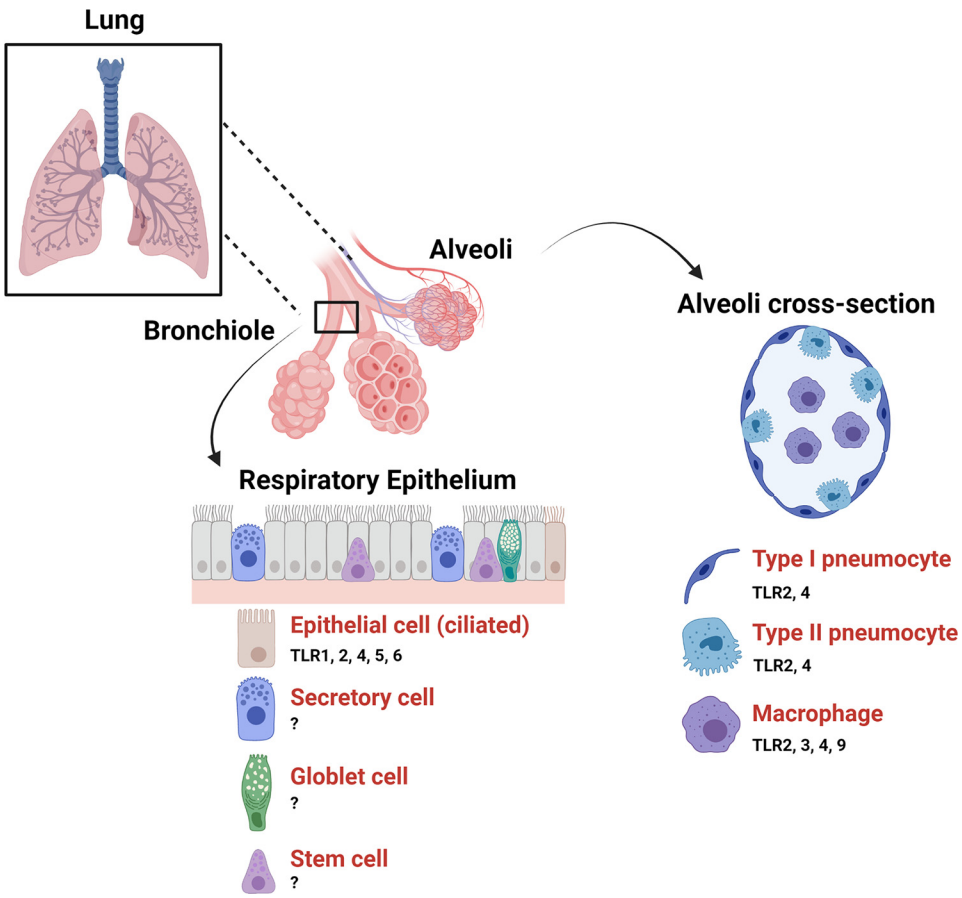


Figure 5. Toll-like receptors on the lung mucosa. Studies have reported the expression of TLRs in a variety of cells present in the lung mucosa and alveoli (Type II pneumocytes and macrophages). However, there is a paucity of investigation in specific cells present in the respiratory epithelium, such as the secretory, goblet, and stem cells. (183, 267, 268). Created with BioRender.com.

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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. Hillman et al. (185) 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 intraamniotic 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.

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 outcomes. 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 TLR1, TLR7, and TLR9 increased more than 10-fold by *postnatal day 12*. In the same study TLR2, TLR4, and TLR6 mRNA expression were higher in the postnatal period compared with the embryonic phase; however, MyD88 expression did not follow the same trend over this period (188). The expression of TLRs is evident on multiple cells expressed in the central nervous system neuroprogenitor cells, neurons, astrocytes, and microglia (189, 190).

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 (191). A study by Scheffel et al. (192) in mice, demonstrated differential protein inductions and functional outcomes upon TLR activation in postnatal microglia compared with adults suggesting specialization 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 suppressed by TLR activation in a MyD88-dependent pathway (193). Poor myelination is associated with cognitive and motor deficits. The previous study by Scheffel et al. (192) also 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.

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 normalized proinflammatory gene expression (194). Adenosine system is a potential regulator of this response in microglia. These neuronal cells are highly sensitive to subtle changes of adenosine triphosphate (ATP) that belong to the purinergic signaling system. Receptors that recognize purines such as P2X, P2Y, and P1 receptors mediate the generation of intracellular adenosine by degradation of ATP contributing to the microglial anti- and proinflammatory shift (195). Adenosine signals the downstream increase of cAMP by binding G protein-coupled adenosine receptors in the cytoplasm impairing the TLR-mediated signaling system (196). 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 (196). A large body of evidence suggests that the purinergic system might constitute a fundamental signaling network that establishes microglial response to infection and inflammation as well as mediating nerve repair by promoting myelination. Caffeine therapy used 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 nonselective antagonist of A1 receptors (197). Apnea of prematurity are recurrent clinical events that are accompanied by intermittent hypoxia and bradycardia, suggested to present worse outcomes in the male sex (198). Benefits of caffeine may extend beyond stabilizing breathing and may play a role in immune modulation and neuroprotection (199).

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 (165, 200). Cardiorespiratory dysfunction is one of the primary symptoms of infection with a requirement for increased ventilatory support and vasopressor administration in the days before LOS-induced death (201). The role of TLRs in cardiorespiratory control is poorly understood. Cardiorespiratory dysfunction is likely mediated by the local release of inflammatory cytokines and oxidative stress in addition to the action of cytokines on distant targets involved in homeostatic control.

Toll-like Receptors and Cardiorespiratory Control

The autonomic nervous system is composed 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 *Neonatal Pathogenic and Sterile Inflammation*, neonatal infection impinges on cardiorespiratory function destabilizing 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 defenses and are extremely sensitive to a wide range of proinflammatory molecules, such as IL-1, IL-6, and TNF-α (202). During infection, the release of

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such molecules stimulates vagal afferent signaling, which can be traced to crucial regions that regulate breathing such as the nucleus of the solitary tract (NTS) (203). TLR4 mRNA and protein are expressed in the rat nodose ganglion, another potential site of inflammatory-induced afferent vagal nerve stimulation (204). 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 (205). TLR4 has also been identified in the carotid body in adult human, rat, and mouse, and the rat petrosal ganglia, which signals directly to the NTS (206, 207).

Although 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 blood-brain barrier (BBB) by a saturable transport mechanism such as RAGE, the interaction of peripheral cytokines with the circumventricular organs that lack the BBB and peripheral immune-mediated activation of afferent neurons within the vagus nerve (208, 209). 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 cardiorespiratory control. In the paraventricular nucleus of the hypothalamus, regulation of TLR1 and TLR2 in neonatal mice brains after hypoxia has been proposed to play a crucial role in neonatal brain injury (210). 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.

Toll-like Receptors and Inflammatory Lung Disease

Alterations in the TLR pathway have 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 (211). Furthermore, a study performed in children aged 2–4 yr 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 (212). 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 with term babies. Conversely, preterm and term infants suffering from RSV had diminished TLR4 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 (213), though other TLRs likely play a role. Preconditioning in mice preexposed to CpG oligodeoxynucleotides (TLR9 ligand) was associated with increased circulating APCs, which protected the animal against subsequent neonatal RSV infection (214). 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 humans, nonsmall cell lung carcinoma, higher mRNA expression of TLR1–TLR3 and TLR5–TLR8 were significantly associated with increased cell survival and thus improved disease outcomes (215). TLR4 is negatively associated with COPD outcomes 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 (216). Both the upregulation of TLRs in the lungs under septic states, together with the suggested protective function of TLRs during inflammation, reveal the complexity of these receptor signaling 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.

Toll-Like Receptors and Cardiovascular Dysfunction

There is evidence that TLR signaling is implicated in cardiovascular dysfunction in neonates 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 h of exposure, by activation of TLR2 and TLR4 on cardiomyocytes (217). This study implicates cardiomyocyte TLR2 and TLR4 dysregulation in 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 (218). Remodeling of cardiomyocytes has been associated with an upregulation of PRRs that trigger host inflammation through inflammasome activation (219). A study by Li et al. (220) utilized a model of cardiac hypertrophy, neonatal mouse cardiomyocytes stimulated with phenylephrine (α1 adrenoceptor agonist), and 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 remodeling and might have a role as a therapeutic target for heart failure. 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-dependent 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 overexpression of proinflammatory endothelial TLR2 protein expression (221). 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 (30). 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 (222).

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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 signaling enhancing complexes (223). 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 (224). Nonetheless, it is known that alarmins released in a hyperoxic environment modulate TLR4 signaling. In a recent study by Mian et al. (225) the authors demonstrated that administration of a TLR4 antagonist to rats exposed to hyperoxia (80% oxygen from *postnatal days 3* to *10*) prevented neonatal hyperoxia-induced left ventricular hypertrophy and dysfunction. 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 stimulates 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 signaling network (226). 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.

TRANSLATIONAL OPPORTUNITIES IN NEONATAL IMMUNOLOGY

The mechanisms by which the innate immune system recognizes and responds to infection have been the focus of an extensive body of research. It is now clear that many PAMPs activate TLRs, which through intracellular signaling and effective cross talk, induce immune responses and rapid feedback control to fight invading pathogens and facilitate repair and regeneration in a variety of tissues (Fig. 6). 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

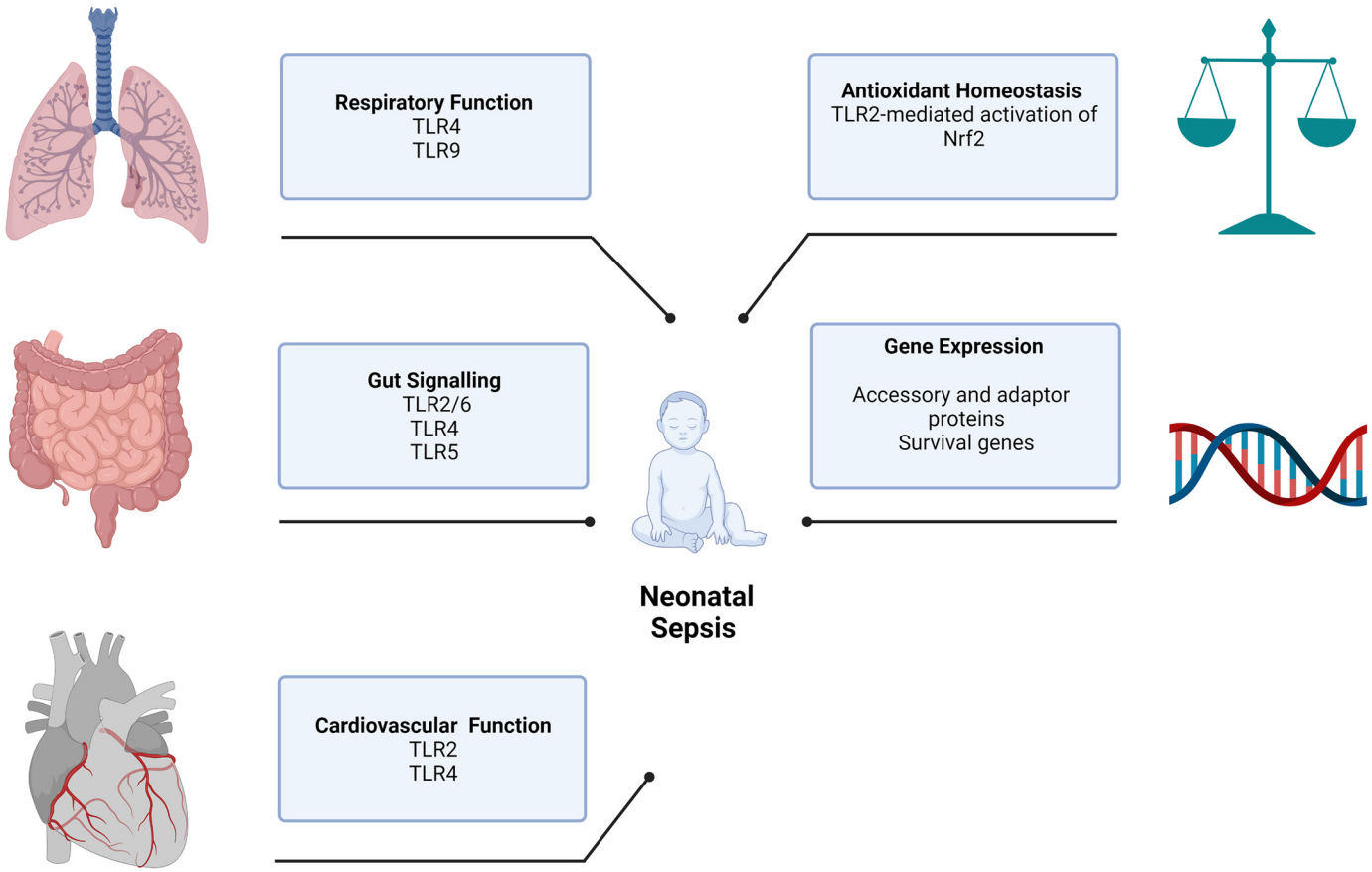


Figure 6. Overview of the development of neonatal sepsis and TLRs cooperation in infants. Effective mediated cross talk by TLRs is responsible for priming the innate immune response in many tissues upon infection. It is now clear that many PAMPs activate TLRs that actively modulate crucial body functions in neonates, such as respiratory, intestinal, and cardiovascular through effective regulation of the antioxidant response and gene expression. PAMPs, Pathogen or microbe-associated molecular patterns; TLRs, Toll-like receptors; Nrf, nuclear factor erythroid 2-related factor 2. Created with BioRender.com.

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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 differ between newborn infants and adults (227). Simultaneous increases in both proinflammatory 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 proinflammatory cytokines and promoting resolution of the inflammatory response (228). However, overproduction and overactivation of immune cells result in exaggerated cytokine response or "cytokine storm" and increased mortality in humans. Although cytokine storms are often associated with viral infections, very high cytokine concentrations in serum and cerebrospinal fluid samples after the development of bacterial-induced sepsis with *S. aureus* and *Staphylococcal* endotoxins was reported to be associated with sudden infant death (229). In neonates, the reduction of excessive inflammation could minimize mortality and decrease the frequency and intensity of devastating postsepsis damage, including chronic lung disease (230) and white matter damage (231), which may enhance longer-term outcomes. 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.

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 (232). This immunotherapy is known to increase allergen-specific IgG antibody levels and dampen the IgE response during allergen exposure (233). 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 with those that did not receive the vaccine (234); unfortunately, some developed countries have now excluded BCG's from their vaccination schemes, which likely imparts nonspecific immune benefits.

Benefits of administration of immune adjuvants that stimulate specific TLR (e.g., resiquimod and LPS) have been demonstrated in a neonatal mouse model predisposed to poor sepsis outcomes (235). A recent study by Yang et al. (236) has reported that monophosphoryl lipid A (MPLA), a TLR9 neutrophil agonist, protects against fatal bacterial sepsis in mice through the process of inflammatory preconditioning, which occurred in the setting of a low-dose bacterial challenge by enabling TLR9 membrane translocation and signaling. Moreover, this study suggested that depletion of TLR9 in $tlr9^{-/-}$ mice largely eliminated the neutrophil-mediated inflammatory preconditioning effect in this sepsis model. VTX-294, a novel TLR8 agonist, was demonstrated to induce the expression of TNF-α and IL-1β by leukocytes by acting in combination with MPLA (237). Morris and Surendran have advocated the need to develop novel adjuvants and the key may be to stimulate multiple TLR pathways, particularly TLR7 and TLR8, which are active in neonatal infants (238).

Although stimulation of TLR9 has been shown to act via effective preconditioning, stimulating TLR2 and TLR4 do not work in a similar manner. Injection of PAM (a TLR2 agonist) or LPS (a TLR4 agonist) alone results in neuroinflammatory alterations that have been reported to sensitize the immature brain-to-brain HI injury when injected systemically (231). Teasing apart the different mechanisms and environments in which tolerance versus sensitization occurs will be important to advance TLR-based therapies. Interestingly, in the study by Falck et al. (239), hypothermia treatment was highly neuroprotective after PAM-induced brain injury, potentially highlighting the usefulness of neonatal therapeutic hypothermia but this therapy is currently limited to term infants only with moderate-to-severe hypoxic-ischemic encephalopathy.

A clinical trial using a single dosage of 180 mg of Ticagrelor (a $P2Y_{12}$ receptor antagonist) in healthy individuals, reported reduced platelet-monocyte complex formation associated with an increase in proinflammatory cytokines in blood exposed to a TLR2 agonist, but a decrease in these cytokines in blood exposed to LPS, TLR4 agonist (240). These findings suggest that it is important to examine the therapeutic strategies to a variety of PAMPS as they may reveal opposing effects depending on the stimulus.

Biopharmaceutical research should consider immune development status during TLRs-based drug discovery. Variations likely exist in both the nature and intensity of simulated infection in different populations. For novel drug therapies, it will be important to demonstrate both improved survivals in preclinical models of acute neonatal sepsis and positive long-term outcomes.

Antioxidants

Neonatal response to infection is influenced by an altered redox environment. Diminished production of antioxidants during neonatal development in the presence of infection may lack the inflammatory suppression necessary to keep the system in check. Supplemental antioxidants have been studied as a means of preventing the accumulation of ROS in premature babies. In the brain, oxidative stress is a primary risk factor for neuronal damage in premature newborns and, therefore, of high clinical relevance (241).

A study by Sevastiadou et al. (242) showed that glutamine, an amino acid with immunomodulatory properties acts to mediate inflammation by decreasing the expression of oxidative stress-related genes and has suggested beneficial effects on intestinal integrity and the overall incidence of NEC and septicemia in preterm infants. Several additional studies have reported the benefits of glutamine supplementation in preterm babies, such as early improvement in liver function and increase in head circumference growth during

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the first year of life (243, 244). To determine if there is an effect of glutamine on TLRs, a study performed in adult patients in critical care demonstrated that daily glutamine supplementation lasting 5 days did not increase the expression of TLR2 and TLR4 in peripheral blood monocytes (245). However, glutamine mediates a decrease in proinflammatory cytokines (246) and an increase in the levels of glutathione and the oxidative capacity (247). However, studies still cannot infer if this modulation involves the TLR pathway.

Vitamin D is a potent modulator of both innate and adaptive immune systems by activation of monocytes/macrophages and is involved in the clearance of prooxidants. A study conducted in healthy adults shows that regulation of vitamin D receptor (VDR)-mediated TLR2/1 signaling is multifactorial, involving epigenetic modulation by the methylation of VDR key regulatory sites and genetic variation by the regulated translation of TIRAP proteins (248). In a review, Pludowshi et al. (249) discuss the emergence of vitamin D deficiency in recent years and the establishment of dietary supplementation in various countries such as Ireland, Canada, United Kingdom, and United States. Moreover, 5 μg of vitamin D is the recommended daily dose for newborns during the first 12 mo; however, premature infants may require higher doses. There is a dearth of studies focusing on the cooperation between TLRs and antioxidants during early life and this area should be explored in future studies.

Breast Milk

Breast milk has been championed in the last 20 years for its extensive benefits in disease prevention. Studies by LeBouder illustrate the bioactive components of breastmilk on immune modulation in the first few days after birth (118, 250). 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 noncoding RNA (ncRNA), such as miRNA and long noncoding RNA (lncRNA) (251). These miRNAs contained within porcine milk have been demonstrated to attenuate TLR4 response to LPS (252). 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 (238).

Probiotic Supplementation

Colonization 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 (253). Examining probiotic supplementation from *pregnancy wk 36*, Forsberg and colleagues collected postnatal mononuclear cells at birth, 2, 12, and 24 mo and examined TLR2 signaling. mRNA expression of TLR2 was unaltered in the probiotic supplementation cohort; at 12 mo, 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 (254). This strategy of maternal probiotic administration has not yet been explored in humans (255). 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 eight strains of bacteria in sepsis prevention in neonates is currently recruiting participants (256). Another new trial is assessing the benefits of multistrain probiotic products in the prevention of multidrug-resistant bacterial infection. Moreover, previous trials using synbiotics (mixture of probiotics and prebiotics) report significant beneficial effects in the treatment and the prevention of neonatal sepsis (257).

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 utilized 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 whether preconditioning lessens mortality and whether 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) (258, 259). 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 (260).

Perspectives and Significance

The TLR superfamily recognizes conserved molecular patterns expressed by bacteria and amplifies the downstream inflammatory and defensive response of the host to aid the elimination of the pathogen. Evidence shows that TLRs are constitutively expressed on many cell types across tissues such as the brain, lung, heart, and gut, in addition to circulating immune cells. TLR signaling cascades include accessory, adaptor proteins, and second messengers that promote intracellular stabilization of NF-κB, 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 newborns, TLR2 and TLR4 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 newborns 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

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underpinning the development of neonatal sepsis and neonatal cellular damage and delineating signaling networks. In addition, we should focus not only on the development of novel therapeutic strategies but also on the identification of early biomarkers to facilitate protection with consideration of personalized sex-specific approaches.

GRANTS

This review was funded by Science Foundation Ireland 18/SIRG/5483.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.L.D. and F.B.M. conceived and designed research; M.L.D. interpreted results of experiments; M.L.D. prepared figures; M.L.D. and F.B.M. drafted manuscript; M.L.D., K.M.O., E.M.D., K.D.O., and F.B.M. edited and revised manuscript; M.L.D., K.M.O., E.M.D., K.D.O., and F.B.M. approved final version of manuscript.

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