Children's Mercy Kansas City
SHARE @ Children's Mercy

Manuscripts, Articles, Book Chapters and Other Papers

4-2023

Necrotizing enterocolitis in premature infants-A defect in the brakes? Evidence from clinical and animal studies.

Venkatesh Sampath
Children's Mercy Hospital

Maribel Martinez
Children's Mercy Kansas City

Michael Caplan

Mark A. Underwood

Alain Cuna
Children's Mercy Hospital

Let us know how access to this publication benefits you

Follow this and additional works at: https://scholarlyexchange.childrensmercy.org/papers

 Part of the Pediatrics Commons

Recommended Citation

Sampath V, Martinez M, Caplan M, Underwood MA, Cuna A. Necrotizing enterocolitis in premature infants-A defect in the brakes? Evidence from clinical and animal studies. Mucosal Immunol. 2023;16(2):208-220. doi:10.1016/j.mucimm.2023.02.002

This Article is brought to you for free and open access by SHARE @ Children's Mercy. It has been accepted for inclusion in Manuscripts, Articles, Book Chapters and Other Papers by an authorized administrator of SHARE @ Children's Mercy. For more information, please contact hlsteel@cmh.edu.

SOCIETY FOR MUCOSAL IMMUNOLOGY

REVIEW

Necrotizing enterocolitis in premature infants—A defect in the brakes? Evidence from clinical and animal studies

Venkatesh Sampath [1,2,✉], Maribel Martinez [1,2], Michael Caplan [3], Mark A Underwood [4] and Alain Cuna [1,2]

© 2023 The Author(s). Published by Elsevier Inc. on behalf of Society for Mucosal Immunology.
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

A key aspect of postnatal intestinal adaptation is the establishment of symbiotic relationships with co-evolved gut microbiota. Necrotizing enterocolitis (NEC) is the most severe disease arising from failure in postnatal gut adaptation in premature infants. Although pathological activation of intestinal Toll-like receptors (TLRs) is believed to underpin NEC pathogenesis, the mechanisms are incompletely understood. We postulate that unregulated aberrant TLR activation in NEC arises from a failure in intestinal-specific mechanisms that tamponade TLR signaling (the brakes). In this review, we discussed the human and animal studies that elucidate the developmental mechanisms inhibiting TLR signaling in the postnatal intestine (establishing the brakes). We then evaluate evidence from preclinical models and human studies that point to a defect in the inhibition of TLR signaling underlying NEC. Finally, we provided a framework for the assessment of NEC risk by screening for signatures of TLR signaling and for NEC prevention by TLR-targeted therapy in premature infants.

Mucosal Immunology (2023) 16:208–220; https://doi.org/10.1016/j.mucimm.2023.02.002

INTRODUCTION

Successful adaptation of the neonatal gut to the postnatal *milieu* is critical for survival. The gastrointestinal system undergoes dynamic biochemical, structural, and functional changes to accomplish its key roles in nutrition, immunity, self-renewal, and organ-organ crosstalk[1]. This well-orchestrated adaptation program in full-term neonates can represent a hazard to premature infants. The challenges faced by the immature intestine include acquiring the ability to digest and absorb nutrients to sustain somatic and brain growth and developing a symbiotic relationship with microbiota to prevent disease. A decreased absorptive surface, dysmature innate and adaptive intestinal immune responses, dysmotility, increased intestinal permeability, and increased exposure to pathogenic bacteria can program intestinal maladaptation and disease in premature infants[2–4]. Necrotizing enterocolitis (NEC), typified by inflammation, cell death, loss of barrier function, and in severe cases, systemic shock, is the most severe disease arising from a failure in postnatal gut adaptation[4,5]. Herein, we present evidence from clinical, experimental, and genetic studies to support the hypothesis that NEC is a phenotype for defects in the brakes inhibiting intestinal epithelial innate immune signaling. Because the pathogenesis of spontaneous intestinal perforation (SIP), transfusion-associated NEC, and NEC in term neonates differs from classical NEC in premature infants, these are not discussed here.

NEC PATHOGENESIS

NEC is diagnosed in 5%–12% of infants born at less than 33 weeks gestation, and surgical NEC has a mortality of 20%–35%[6]. Although several risk factors, including prematurity, formula feeding, gut ischemia, genetic predisposition, and intestinal dysbiosis, have been implicated in NEC, its exact pathogenesis remains unknown. In the last decade, research advances propelled using transgenic mouse models, gut microbiome studies, human genetics, and lactation science have enhanced our understanding of NEC. Collectively, these advances have provided compelling evidence for the importance of Toll-like receptor (TLR) signaling as a central mechanism in NEC development[7–11].

TLRs are innate immune receptors that recognize conserved structural motifs in bacteria and viruses called pathogen-associated molecular patterns (PAMPs) and host stress/danger molecules[12]. Collectively, TLRs recognize a diverse repertoire of PAMPs, including lipids, proteins, carbohydrates, and nucleic acids derived from bacteria, viruses, and fungi. The most studied TLR is TLR4, which is the pattern recognition receptor for lipopolysaccharide (LPS), a component of the gram-negative bacterial outer membrane that causes septic shock[4]. After the recognition of PAMPs, TLRs trigger innate immune defense mechanisms by activating transcriptional factors such as nuclear factor κ light chain enhancer of activated B cells (NFκB) and interferon regulatory factor 3[12]. Although TLRs are essential in the host's defense against invading pathogens, aberrant or pro-

[1]Division of Neonatology, Children's Mercy Kansas City, Kansas City, Missouri, USA. [2]School of Medicine, University of Missouri Kansas City, Kansas City, Missouri, USA. [3]Department of Pediatrics, North Shore University Health System, Evanston, Illinois, USA. [4]Department of Pediatrics, University of California Davis, Sacramento, California, USA. ✉ email: vsampath@cmh.edu

Received: 6 January 2023 Revised: 3 February 2023 Accepted 8 February 2023
Published online: 15 February 2023

longed activation can contribute to several immune and inflammatory diseases.

Several lines of evidence from mouse studies implicate TLR4 in the pathogenesis of NEC. Jilling et al.[7] demonstrated that the addition of bacteria to a formula + cold stress model of experimental NEC upregulated intestinal TLR4 expression and increased the incidence of NEC in mice. When C3HeJ mice with functional TLR4 deficiency were subjected to experimental NEC, the incidence of NEC was substantially diminished. Leaphart et al.[8] found similar findings of increased TLR4 expression and experimental NEC in wild-type mice and a reduction in NEC in mice with global or enterocyte-specific TLR4 deficiency. Subsequent studies provided additional mechanistic knowledge on how TLR4 activation can cause NEC-like injury. Leaphart et al.[8] showed that TLR4 activation increased injury through enterocyte apoptosis, whereas Yazji et al.[13] found that endothelial TLR4 activation impairs intestinal perfusion, thereby causing enterocyte injury through gut ischemia. In addition to directly inducing gut injury, TLR activation has also been shown to inhibit intestinal repair by impairing enterocyte proliferation and migration[14,15].

Studies in humans further support a critical role for TLR4 signaling in NEC. Ileal tissue samples obtained from human fetuses demonstrated an increased expression of TLR4 signaling compared with mature enterocytes, suggesting that susceptibility of preterm infants to NEC might be related to a state of TLR4 hyper-responsiveness in the immature intestinal tract[16]. Support for this hypothesis was further provided by the analysis of tissue samples from preterm infants who underwent intestinal resection for NEC and demonstrated significantly higher levels of TLR4 protein expression than those from controls[17]. Furthermore, stool microbiome studies in preterm infants have provided insight into what triggers aberrant TLR4 activation in human NEC. The landmark study by Warner et al.[9] elegantly demonstrated that a pathologic predominance of gram-negative bacteria, which can activate pro-inflammatory TLR4 signaling, preceded the development of NEC in premature infants. Subsequent studies have replicated the temporal relationship between enrichment of Gammaproteobacteria (the class of bacteria including the majority of pathogenic gram-negative bacteria) in the gut before NEC onset in preterm infants[18]. Finally, genetic studies have found that genetic mutations in TLR signaling pathway are associated with NEC vulnerability in preterm infants, suggesting a role for inherent genetic susceptibility in pathologic TLR4 activation[19–21]. These studies in mice and humans reveal that aberrant intestinal activation of the TLR family of innate immune receptors by microbiota in the setting of developmental dysmaturity underpins NEC pathogenesis in preterm infants. In the next section, we will review how TLR signaling in the newborn intestine is regulated to prevent pathologic TLR activation and maintain homeostasis.

ESTABLISHING THE BRAKES ON INTESTINAL TLR SIGNALING AFTER BIRTH—KEY TO SUCCESSFUL POSTNATAL INTESTINAL ADAPTATION

The preterm infant's intestine exhibits a state of heightened TLR sensitivity

Establishing the symbiotic host-microbiota relationships that sustain mucosal homeostasis and immune tolerance to microbiota while preventing bacterial invasion is key to intestinal adaptation. An important component of successful gut adaptation lies in preventing dysfunctional TLR activation in the neonatal intestine, which underpins NEC pathogenesis in premature infants[7,8]. The preterm infant's vulnerability to NEC arises partially from an imbalance in intestinal pro- versus anti-inflammatory signaling that favors deviant mucosal intestinal TLR activation[16,22,23]. Nanthakumar et al. showed that human intestinal epithelial expression of TLR4, TLR2, and its adapter MyD88 was increased in preterm neonates compared with term neonates, whereas the expression of the TLR inhibitors such as single immunoglobulin (Ig) interleukin (IL)-1-related receptor (SIGIRR) and A20 was decreased[16]. An analysis of preterm infant stool using proteomics and RNA sequencing also revealed a native "primed" state of TLR activation in preterm infants, characterized by enrichment of pathways facilitating increased TLR and inflammatory signaling[24–26]. Elegant studies on the evolving intestinal microbiota in preterm infants have revealed the proclivity of the preterm gut to be colonized with bacteria capable of triggering TLR activation at the expense of bacteria that inhibit TLR signaling[2,9,18,27,28]. The combination of a "primed" state of TLR sensitivity and intestinal dysbiosis confers vulnerability to unregulated TLR activation in the preterm infant. Therefore, the deployment of programs that tamponade dysfunctional intestinal TLR activation, i.e. "the brakes," is critical. In this section, we highlight the mechanisms from mouse and human studies that reveal how "the brakes on TLRs" are established (Fig. 1).

Studies in mice and cell lines revealing the mechanisms by which TLR signaling is negatively regulated after birth

Developmental changes in intestinal TLR4 expression and localization

Expression studies of TLR4 in fetal and newborn mice indicate a pattern of increasing expression of intestinal TLR4 *in utero*, followed by a significant reduction after delivery at term gestation[17]. This reduction of intestinal TLR4 expression after birth is postulated as an important mechanism for downregulating the inflammatory TLR response to colonizing bacteria in the postpartum period[29]. The specific localization of TLR4 within intestinal epithelial cells (IECs) is another mechanism by which TLR activation is muted in the intestinal tract[30]. IECs are polarized cells with an apical surface membrane that faces the gut lumen and a basolateral membrane that is protected from exposure to luminal microbes. Studies using fetal enterocytes have demonstrated that excessive TLR4 receptors are found on the apical surface membrane of immature enterocytes. With maturity, however, the surface expression of TLR4 is decreased as TLR4 receptors are internalized. In addition to TLR4, immunohistochemistry studies indicate that TLR2 and TLR5 are also differentially expressed in the basolateral membrane of IECs[31–33]. The localization of TLRs in these relatively protected compartments has been demonstrated as early as 18–21 weeks of gestation in human fetal ileal samples[31]. These studies indicate that the developmental changes in TLR4 expression and localization contribute to the neonatal gut's ability to adapt to colonizing microbes without eliciting pro-inflammatory responses.

Postnatal repression of downstream effectors of intestinal TLR signaling

In addition to developmental changes in TLR4, postnatal repression of the downstream effectors of TLR signaling is another mechanism by which intestinal TLR4 inhibition is achieved postnatally. For example, the downregulation of IL-1 receptor-associated kinase 1 (IRAK1)—a kinase that is activated when LPS binds and activates the TLR4/MYD88 receptor adapter com-

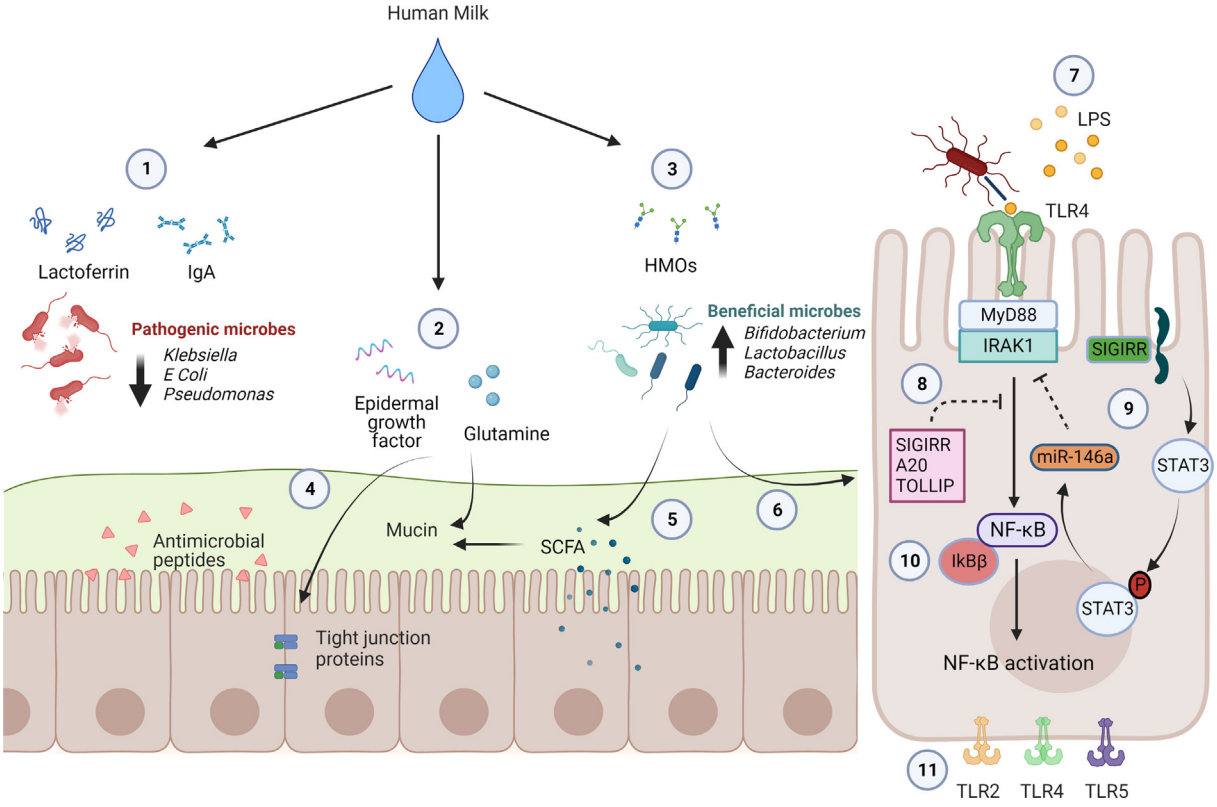

Fig. 1 Overview of mechanisms from mouse and human studies that reveal how "the brakes on TLRs" are established in the neonatal gut. (1) Human milk components such as lactoferrin and secretory IgA inhibit growth of pathogenic microbes that can activate TLR signaling. (2) Epidermal growth factor and glutamine in breastmilk enhance intestinal epithelial barrier by inducing mucin production and tight junction protein activity. (3) HMOs present in human milk promote growth of commensal microbes such as *Bifidobacteria* and *Lactobacillus*. (4) The intestinal epithelial barrier—which includes mucin, antimicrobial peptides, and tight junction proteins—acts as a physical barrier that separates pathogenic microbes from the developing gut. (5) SCFAs, produced in part from the fermentation of HMOs by commensal bacteria, induce mucosal mechanisms such as increased mucin production to help repress intestinal TLR sensitivity. (6) Commensals such as *Bifidobacterium* and *Lactobacillus*, whose growth was promoted in part by HMOs, induce negative regulators of TLR4 (SIGIRR, A20, TOLLIP) to suppress TLR4 activity in the gut. (7) Development of postnatal tolerance to LPS through reduced surface TLR4 expression and repression of IRAK1. (8) Postnatal induction of genes that inhibit intestinal TLR signaling (SIGIRR, A20, TOLLIP) helps prevent aberrant intestinal TLR activation to colonizing microbes. (9) Postnatal repression of IRAK1 through SIGIRR-STAT3-miR-146a pathway dampens TLR4 signaling and contributes to LPS tolerance. (10) Developmental increase in IκB sequesters NFκB in the cytoplasm preventing its transcriptional activation. (11) Compartmentalization of TLRs in the basolateral membrane of IECs decreases TLR activation from bacteria in the apical lumen. HMOs = human milk oligosaccharides; IEC = intestinal epithelial cell; Ig = immunoglobulin; LPS = lipopolysaccharide; NEC = necrotizing enterocolitis; SCFAs = Short-chain fatty acids; Th = T helper; TLR = toll-like receptor.

plex[12,34]—has been shown in mice by Lotz et al.[35] to dampen TLR4 signaling and contribute to the postnatal acquisition of intestinal endotoxin tolerance. Additional studies by this group have identified that microRNA-146a (miR-146a) mediates the translation repression of IRAK1 protein in the postnatal intestine[36]. How miR-146a-dependent IRAK1 repression is regulated was recently clarified by Yu et al.[22], who investigated the role of SIGIRR, a major inhibitor of TLR signaling, in postnatal intestinal adaptation[37]. Using SIGIRR transgenic mice that genocopy a SIGIRR stop mutation identified in human NEC, Yu et al.[22] showed that SIGIRR induces intestinal miR-146a expression through STAT3, thus uncovering a novel pathway for TLR inhibition through SIGIRR-STAT3-miR-146a inhibition of IRAK1.

Another example of postnatal repression of TLR4 signaling is the developmental increase in the expression of IκB, a protein that sequesters NFκB in the cytoplasm preventing its transcriptional activation[23]. Using human enterocyte cell lines and primary rat enterocytes, Claud et al.[23] demonstrated that an increased expression of IκB with intestinal maturation was associated with a decreased TLR activation. Unlike IRAK1, the mechanisms that regulate postnatal increase in IκB remain poorly understood. Nevertheless, these studies together demonstrate that postnatal repression of downstream TLR effectors (IRAK1 through SIGIRR-STAT3-miR-146a; NFκB through IκB) are key mechanisms that help prevent inappropriate TLR activation in the neonatal gut by bacteria.

Induction of genes that inhibit intestinal TLR signaling

Genes that negatively regulate TLR signaling are crucial for maintaining mucosal homeostasis and preventing inappropriate activation of TLR-mediated inflammation. Several negative regulators of TLR signaling in the intestine have been identified, including SIGIRR, Toll-interacting protein (TOLLIP), and A20. Fawley et al.[10] investigated the ontogeny of SIGIRR in rats and mice

and found that intestinal SIGIRR expression is induced postnatally, gradually increasing with maturation from postnatal day 1 to postnatal day 14. Using isolated ileal epithelium from immature and mature human tissue samples, Nanthakumar et al.[16] found a similar pattern of increasing expression of SIGIRR, as well as TOLLIP and A20, with intestinal maturation. Interestingly, supplementation with probiotics—such as *Bifidobacterium infantis*, *Lactobacillus acidophilus*, and *Lactobacillus rhamnosus GG*—also induces intestinal expression of SIGIRR, A20, and TOLLIP[38,39]. Intestinal antimicrobial peptides (AMPs) produced by Paneth cells upon gut maturation also help tamponade TLR signaling. The AMP cathelicidin induces TOLLIP that reduces apoptosis, thereby limiting intestinal permeability[40]. In pigs, β-defensin 114 induced by TLR-activated NFκB suppresses both inflammation and apoptosis[41]. Thus, the postnatal induction of TLR inhibitors, whether intrinsically with intestinal maturation or with probiotic supplementation, helps dampen inflammatory signaling as the newborn gut adapts to a microbe-rich environment.

In addition to genes that inhibit TLR signaling directly, other pattern recognition receptors can also play a role in curbing TLR4 signaling postnatally[21,42]. TLR9 is an endosomal TLR that recognizes bacterial DNA rich in unmethylated Cytosine-phosphate-Guanine (CpG) motifs. Using timed-pregnant mice, Gribar et al.[17] showed that TLR4 and TLR9 exhibited a pattern of reciprocal expression in the developing intestine, and that after birth, intestinal TLR9 expression increased in conjunction with a reduction in TLR4 expression. The authors further demonstrated with *in vivo* and *in vitro* experiments that the activation of TLR9 with bacterial DNA inhibits TLR4 signaling and protects the gut against NEC-like injury. Other studies demonstrated that TLR9 activation is required for the probiotic *Lactobacillus rhamnosus* to provide protection against experimental NEC in mice[43], and that fecal metagenomic signature of low levels of CpG DNA, suggestive of low gut TLR9 activity is associated with NEC in preterm infants[44]. These findings indicate that postnatal increase in intestinal TLR9 activity is another mechanism that helps inhibit TLR4 signaling in the developing intestinal tract.

Two other genes that may also be relevant to establishing brakes on TLR4 activation postnatally are peroxisome proliferator-activated receptor-γ (PPARγ) and nucleotide binding and oligomerization domain 2 (NOD2). PPARγ is a nuclear receptor that attenuates intestinal TLR inflammation through different mechanisms, such as the inactivation of p65 complex, induction of IκB, and interference with AP-1 activity[45]. NOD2 is a pattern recognition receptor that recognizes the muramyl dipeptide component of the bacterial cell wall and has been shown to inhibit TLR2 and TLR4 activity in the intestines[46]. In murine models of NEC, the treatment with agonists for PPARγ or NOD2 was effective in reducing the incidence and severity of experimental NEC[47–49]. Thus, although their developmental expression has not yet been fully elucidated, PPARγ and NOD2 are potent TLR inhibitors that can help maintain homeostasis in the naïve and immature newborn gut.

Human studies revealing the biological mechanisms by which intestinal TLR signaling is kept in check in neonates

Development of an effective intestinal epithelial barrier after birth

The intestinal epithelial barrier is a complex and dynamic system that prevents the translocation of potentially harmful microbes that can otherwise activate pro-inflammatory TLR signaling in the gut[50]. Although the intestinal epithelial barrier of infants is "leaky" immediately after birth, rapid maturation takes place over the first few days of life, especially in term infants[51]. In contrast, preterm infants possess a less well-developed intestinal epithelial barrier that can take up to 6 weeks after birth to mature to the level of term infants[26]. Components of the intestinal epithelial barrier include the mucus layer, AMPs, the single layer of epithelial cells, and the tight junction proteins between them[52]. The mucus layer, comprised chiefly of mucin produced by goblet cells, acts primarily by physical separation of potentially pathogenic gut microbiota from host tissues[53]. The mucus layer also contains intestinal alkaline phosphatase (IAP), an endogenous protein secreted by IECs that serves to inactivate the bacterial endotoxin LPS[54]. Intestinal AMPs, secreted by Paneth cells and other specialized immune cells, can eliminate pathogenic microbes that trigger TLR signaling through the direct disruption of bacterial membranes, inhibition of vital bacterial intracellular pathways, and recruitment of immune cells[55]. In the adult intestine, tight junction proteins, such as claudin and occludin, form a tight barrier between IECs that prevent paracellular passage of microbial antigens, such as LPS, but this mechanism might be deficient in the immature intestine[56–58]. Together, these individual components contribute to the intestinal epithelial barrier and work in concert to inhibit excessive TLR signaling in the developing intestine.

The developmental processes that govern the transition of the newborn gut mucosal barrier from "leaky" to "tight" remain incompletely understood. Emerging studies suggest that complex, bidirectional interactions between the developing infant's intestinal epithelium and the colonizing luminal microbes contribute to this developmental transition[53,55]. For example, although the newborn's intestinal tract can produce AMPs as early as 24 weeks gestation, AMP production and activity are substantially increased postnatally by the presence of colonizing microbes[59,60]. This postnatal increase in AMP activity, in turn, acts on colonizing microbes to help shape the developing gut microbiota[55]. Another example is the relationship between IAP production and gut microbes. Intestinal production of IAP is developmentally regulated, with lower levels in preterm infants that increase with gestational age[61,62]. Interestingly, low levels of IAP results in gut dysbiosis, whereas increased IAP activity promotes the growth of commensal organisms[63]. The interaction between the host and microbiota is also demonstrated by the relationship between the developing mucus layer and colonizing bacteria. Karav et al.[64] showed that infants colonized predominantly by *Bifidobacterium longum* have diminished degradation of colonic mucus compared with infants colonized predominantly by *Bacteroides*. Synergistic relationships between colonizing microbes and the host mucosa are crucial for the proper establishment of the intestinal epithelial barrier and TLR tolerance to colonizing bacteria[65]. Conversely, disruptions to this intricate process—such as with preterm birth, hospitalization, or antibiotic use—can lead to a leaky gut barrier and inappropriate TLR activation in the intestines[66].

Human milk oligosaccharides inhibit intestinal TLR signaling by altering the microbiome and regulating mucosal responses

Human milk contains a broad range of bioactive molecules, cellular components, and microbes that shape the developing infant intestinal microbiome and the innate immune system[67,68]. Human milk oligosaccharides (HMOs) are highly abundant in breast milk and yet not digestible by the human intestine, rais-

ing the question of the biological utility of these complex glycans that have no inherent nutritional value[68]. HMOs impact the gut microbiota by being digestible only by a small number of gut microbes, predominantly *Bifidobacterium* and *Bacteroides* species[69]. This ensures a selective expansion of *Bifidobacterium* and *Bacteroides* spp in the gut of breastfed infants, while decreasing the enrichment of NEC-associated Gammaproteobacteria and other gram-negative bacteria, which activate pro-inflammatory TLR4 signaling[69]. In clinical trials, neonatal supplementation with *Bifidobacterium* spp that can use HMOs results in the suppression of T helper (Th)2 and Th17 responses, augmented Th1 responses, decreased plasma levels of inflammatory cytokines, and reduced NEC rates[69,70]. In human tissue/cell-based *in vitro* models, *Bifidobacterium* spp have been shown to repress TLR4 and TLR2 mediated inflammatory responses by augmenting negative regulators, such as A20[71,72]. Probiotics containing *Bifidobacterium* spp have been noted to be the most efficacious for preventing NEC[73]. In addition to promoting the growth of commensals, such as bifidobacteria, HMOs in breast milk also directly inhibit mucosal TLR signaling. HMOs resemble intestinal epithelial cell surface glycans and as such act as anti-adhesive antimicrobials that bind gut pathogens and prevent mucosal adherence and invasion[74]. HMOs also decrease the epithelial expression of sialylated glycans used by pathogens, such as *E. coli*, to invade the mucosa, inhibit neutrophil activation, leukocyte adhesion and trafficking from blood, and alter T-cell polarization toward a Th1 response versus an allergenic Th2 response[75–78]. These studies indicate the importance of HMOs in regulating TLR signaling by altering the gut microbial composition and inducing mucosal mechanisms that repress intestinal TLR sensitivity.

Human milk components mitigate intestinal TLR signaling

In addition to HMOs, human milk also contains several metabolites and bioactive molecules that exhibit direct and indirect anti-TLR activity[79–86]. Human milk has soluble TLR2 and soluble CD14 (a TLR co-receptor) that specifically act as decoy receptors for lipopeptides and LPS from gram-positive and gram-negative bacteria, preventing mucosal TLR activation[79,80]. Human milk also has glycoproteins, such as lactoferrin and lactadherin, that inhibit NFκB activation or induce STAT3- and IL-10 signaling to suppress pro-inflammatory TLR signaling[11,81–83]. Human milk is also enriched in antibacterial peptides such as β-defensins, enzymes such as lysozyme, short-chain fatty acids, bacteriocins, and other bioactive molecules, which inhibit TLRs and the growth of pathogenic bacteria[87,88]. Secretory Igs are another key component of human milk that provide passive immunity against gut microbes. Although secretory IgG and secretory IgM are present in human milk, secretory IgA is the most abundant component[85,86]. Secretory IgA inhibits mucosal TLR activation by coating bacteria and preventing their invasion, facilitating luminal excretion, and instructing antigen-presenting cells to sample IgA-bound commensals marked for tolerance rather than pathogens[80,85–87]. Other components of human milk can mitigate intestinal TLR signaling indirectly by enhancing the intestinal barrier integrity. For example, epidermal growth factor increases mucin production by goblet cells and normalizes the expression of tight junction proteins occludin and claudin-3[89], whereas glutamine promotes enterocyte proliferation[90] and induces the expression and function of several tight junction proteins, including occludin, claudin-4, junction adhesion molecule-A, and zonula occludens[91]. It is important to note that pasteurization, although necessary to destroy microbes for banking of donor milk, denatures bioactive proteins and peptides, significantly decreasing the antibacterial activity of human milk; however, HMOs are unaffected by pasteurization[92]. In summary, human milk has a broad repertoire of bioactive factors that tamponade TLR activation in the neonatal gut facilitating adaptation to extrauterine life.

Human milk microbiota—another potential modulator of intestinal TLR signaling?

The human milk microbiota consists of microbes found in milk and on the mother's skin, which impact the establishment of the intestinal microbiota in the infant[93,94]. The predominant microbes in milk include *Staphylococcus* and *Streptococcus*, followed by smaller numbers of *Corynebacterium*, *Cutibacterium*, *Lactococcus*, *Lactobacillus*, *Bifidobacterium*, and *Enterobacter* spp[93,95]. Several factors, including maternal diet, genetics, hygiene, HMO composition, glucose tolerance, ethnicity, and geography, alter the microbial composition of breast milk[96,97]. Although it is well known that human milk protects against NEC compared with formula milk, the role of human milk microbiota in regulating TLR signaling is not well understood. Human milk microbiota are assumed to play a putative role in preventing colonization with more harmful bacteria, inhibiting gut inflammation, and aiding the maturation of the immune system[93–95,98,99]. Although outside of the scope of this review, several factors such as vaginal delivery and skin-to-skin care contribute to the establishment of gut microbiota less capable of activating intestinal TLR signaling[2,100,101].

NEC - A PHENOTYPE FOR A DEFECT IN THE BRAKES ON TLRS?

In the previous section, we outlined the molecular mechanisms that suppress dysfunctional intestinal TLR activation during postnatal adaptation. In this section and Table 1, we provide evidence from experimental models of NEC and human studies to support our hypothesis that defects in inhibition of intestinal TLR signaling (faulty brakes) underlie the pathogenesis of NEC[5,20,102].

TLR signaling in experimental models of NEC—Is TLR4 the culprit?

The evidence for a direct role in TLR activation in NEC comes from studies that reveal (a) a decreased NEC-like injury in transgenic mice lacking functional TLR4; (b) increased NEC vulnerability in transgenic mice deficient in proteins that inhibit TLR signaling; and (c) decreased NEC with bioactive molecules, prebiotics, and probiotics known to decrease bacteria-mediated intestinal TLR activation. Classical studies by Jilling et al.[7] and Leaphart et al.[8] revealed the obligate requirement for TLR4 in experimental NEC, as global deficiency of *Tlr4* or IEC-specific *Tlr4* deletion prevented NEC. Several animal studies have also shown an increased expression of TLR4 in NEC[3,42,103,104]. TLR4 stimulation activates intestinal inflammation, cell death, necroptosis, and microvascular injury while inhibiting mucosal regeneration through the MAPK, HMGB1, NFκB, and VEGF-dependent pathways[8,10,13,102,105–107]. These data are consistent with human studies showing that Gammaproteobacteria, which activate TLR4 signaling, are the most common bacteria implicated in preterm NEC[2,18]. Interestingly, flagellin from gram-negative bacteria can also stimulate TLR5/MyD88-dependent inflammation[34]. However, TLR5 is decreased in experimental models of NEC and studies in *Tlr5* –/– mice have not been reported[42,108]. Simi-

Table 1. Summary of studies in animal models and infants supporting the hypothesis that defects in inhibition of intestinal TLR signaling underlie the pathogenesis of NEC.

	Author(PMID)	Experimental study design	Main findings
Animal model studies			
Experimental models of NEC implicating TLR4 as key mediator of NEC	Liu (19608731)	Neonatal rat NEC model	Intestinal expression of TLRs and cytokines precedes histologic injury in experimental NEC
	Jilling (16920968) Leaphart (17878380)	Neonatal mouse NEC model	TLR4 mutant mice protected from development of experimental NEC compared to wildtype mice
	Leaphart (17878380) Neal (23455503)Yazji (23650378)	Neonatal mouse NEC model	Mechanistic studies in mice with global and epithelial specific TLR4 deficiency demonstrating how TLR4 stimulation induces mucosal injury, impairs microvascular perfusion, and inhibits intestinal repair
Genetic ablation of TLR inhibitors results in NEC-like intestinal injury	Lee (11009421)	A20-deficient mice	A20-deficient mice develop severe intestinal inflammation, hypersensitivity to lipopolysaccharide, and die in the neonatal period.
	Fawley (28846670)	SIGIRR-deficient mice	SIGIRR-deficient mice have mild, spontaneous intestinal inflammation at baseline and more severe experimental NEC
	Gribar (19109197)	TLR9-deficient mice	Deficiency of TLR9, which is known to repress TLR4 signaling, exacerbate NEC severity.
	Richardson (20580721)	NOD2-deficient mice	Failure of NOD2 signaling leads to NEC through increased TLR4-mediated enterocyte apoptosis
Treatment with agents that inhibit TLR decreases severity of experimental NEC	Cho (33188181)	Neonatal mouse NEC model	Human recombinant IL-37 engages SIGIRR, represses TLR signaling, and decreases severity of experimental NEC
	Corsini (27973471)	Neonatal rat NEC model	Pioglitazone, an inhibitor of TLR signaling through peroxisome proliferator-activated receptor ⍰, decreases severity of experimental NEC
	Li (35316914)	Neonatal rat NEC model	miR-21 injection inhibits TLR signaling and decreases severity of experimental NEC in newborn mice
	Villamor-Martinez (33363060)Zani (23525603)	Neonatal rat NEC model	Stem cells or exosomes derived from stem cells exert their beneficial effect in NEC partly by inhibiting TLR and other inflammatory pathways
	Rentea (23331804, 22703783)Riggle (23158403)	Neonatal rat NEC model	Enteral and systemic supplementation with intestinal alkaline phosphatase (IAP) increases IAP activity and decreases NEC-related intestinal injury.
Bioactive molecules and peptides from breastmilk represses intestinal TLR signaling and help in NEC protection	Gopalakrishna (31209335)	Preterm infant fecal samples and neonatal mouse NEC model	Relative decrease of IgA-bound bacteria in preterm stool is associated with development of NEC, and mouse pups reared by IgA-deficient dams are susceptible to NEC.
	Jantscher-Krenn (22138535)	Neonatal rat NEC model	Supplementation with the human milk oligosaccharide disialyllacto-N-tetraose (DSLNT) reduces NEC in neonatal rats.
	Liu (31483289)	Neonatal mouse NEC model	Lactoferrin and lactoferrin-induced myeloid-derived suppressor cells inhibited NFκB activation and attenuated experimental NEC
	Lu (17515866)	Neonatal rat NEC model	Polyunsaturated fatty acids (PUFA) supplementation in rats suppresses pro-inflammatory platelet-activating factor receptor (PAFR) and TLR4 gene expression and reduces experimental NEC

(*continued on next page*)

Table 1 (**continued**)

	Author(PMID)	Experimental study design	Main findings
	Dvorak (11751169)Feng (16410124)Chen (34012839)	Neonatal rat NEC model	Epidermal growth factor (EGF) and heparin-binding EGF supplementation reduces the development of NEC.
Studies in infants			
Genetic signatures of deviant TLR signaling are associated with human NEC	Härtel (26752461)	Gene association study of a large cohort of preterm infants	Preterm infants with $\geq$ 2 NOD2 gene variants have an increased risk for NEC
	Sampath (27893720)	Gene association study of a large cohort of preterm infants	Hypomorphic variant of autophagy gene ATG16L1 is associated with NEC in preterm infants
	Sampath (25963006)	Exome sequencing of preterm infants with NEC	Loss of function mutations in SIGIRR are enriched in preterm infants with NEC
Gut microbiome studies reveal enrichment of NEC-associated *Gammaproteobacteria* and other Gram-negative microbes that activate TLR4 signaling	Warner (26969089) Pammi (28274256)	Prospective case-control studies of preterm infants	Increased relative abundance of *Gammaproteobacteria* precede NEC in preterm infants
RNA and miRNA sequencing analysis identify dysregulated TLR signaling pathways in human NEC samples	Chan (24368664)	Gene expression profile analysis of human NEC and SIP intestinal tissues	Dysregulation of TLR4 and NFκB pathways are observed in NEC samples compared to controls
	Nanthakumar (21445298)	Gene expression profile analysis of resected ileum from fetuses, NEC patients, and controls	Immature enterocytes from fetuses have increased expression of TLR4 and decreased expression of SIGIRR and A20 compared to mature enterocytes from controls. Enterocytes from NEC infants exhibit further imbalance with increased TLR and decreased SIGIRR/A20.
	Ng (26274503)	miRNA profile analysis of NEC, SIP, and control intestinal tissues in preterm infants	Dysregulated expression of miRNA/mRNA pairs that regulated TLR4 and NFκB signaling are identified in NEC infants
	Knight (24965658)	RNA sequencing of intact human epithelial cells in stool samples	Preterm infants have upregulated NFκB and IL-1B compared to term infants, indicating dysregulated TLR response in preterm infants vulnerable to NEC
Clinical intervention studies provide indirect evidence for a defect in the brakes on TLR signaling in NEC	Henrick (34143954)	Randomized clinical trial	Breastfed infants given *Bifidobacterium infantis* exhibit a reduction in intestinal Th2- and Th17-type responses
	Pammi (28658720)	Cochrane meta-analysis of randomized clinical trials	Lactoferrin, which inhibits TLR signaling by chelating iron required by bacteria and preventing their adherence to the intestinal mucosa, may decrease risk of NEC in small clinical trials
	Bernabe-Garcia (33671220)	Randomized clinical trial	NEC was lower in docosahexaenoic acid (DHA)-treated group compared to controls, suggesting that DHA supplementation may prevent NEC in preterm infants

DHA = docosahexaenoic acid; EGF = epidermal growth factor; IAP = intestinal alkaline phosphatase; Ig = immunoglobulin; IL = interleukin; NEC = necrotizing enterocolitis; NOD = nucleotide binding and oligomerization domain; SIGIRR = single immunoglobulin interleukin-1-related receptor; Th = T helper; TLR = Toll-like receptor.

larly, the role of TLR2, which senses lipopeptides from gram-positive bacteria, such as *Staphylococcus* spp, and can stimulate pro-inflammatory TLR signaling has not been evaluated[34]. TLR2 signaling in the neonatal gut might protect against NEC because the efficacy of *Lactobacillus reuteri* to prevent NEC is lost in *Tlr2* –/– mice[109]. Platelet-activating factor (PAF), which is elevated in human NEC, also induces TLR4 expression in cell lines and animal models of NEC, suggesting that TLR4 activation is central to neonatal intestinal injury induced by PAF[104,110,111]. Finally, studies using novel peptides that target functional domains of TLR4 have been shown to attenuate experimental NEC[112]. Collectively, these studies implicate TLR4 but not other TLRs in the causation of preterm NEC.

Deficiency of gut mucosal proteins that suppress TLR signaling results in exaggerated NEC vulnerability

Important mechanistic insights into the role of defective TLR regulation in NEC pathogenesis can be gleaned from studies in mice with genetic deficiency of various TLR inhibitors. A20 is a ubiquitin-editing enzyme that inhibits TLR and tumor necrosis factor-α-induced NFκB activation. The genetic ablation of A20 in mice results in spontaneous inflammation in multiple organs, including the intestine, which results in perinatal death[113,114]. SIGIRR is an orphan receptor that strongly represses TLR and IL-1R signaling[37]. We have shown that SIGIRR–/– mice have mild, spontaneous intestinal inflammation at baseline with more severe experimental NEC, in parallel with exaggerated activation of canonical TLR signaling[10]. Both NOD2 and TLR9 are also known to suppress TLR4 signaling in the gut[115,116]. Consistent with this, the deficiency of NOD2 and TLR9 exacerbates TLR4-mediated experimental NEC[17,48]. Interestingly, mutations in SIGIRR and NOD2 are implicated in human NEC and are further discussed in the subsequent sections. In conclusion, experimental studies clearly demonstrate that a loss in the brakes on intestinal TLR signaling exaggerates NEC vulnerability and severity in animal models.

Treatment with substances that augment TLR inhibition in the gut reduces NEC

Studies in mice that augmented TLR inhibition have been shown to decrease the severity of experimental NEC. IL-37 is a known ligand for human SIGIRR, with no known mouse homologue. Nevertheless, treatment with human recombinant IL-37 or transgenic IL-37 overexpression in mice inhibits TLR signaling and decreases the severity of experimental NEC[108]. IL-22 is critical for intestinal epithelial homeostasis because it regulates IEC proliferation, mucous secretion, and production of AMPs[117,118]. Although the expression of IL-22 or its receptor is low during the period of NEC vulnerability in mice and human neonates, treatment with recombinant IL-22 decreased inflammation and induced epithelial regeneration in a mouse model of TLR-dependent NEC[117]. IAP, an endogenous protein secreted by the intestinal epithelium and present in the mucus layer, is a potent inactivator of the TLR4 ligand LPS. Low IAP enzyme activity was associated with NEC in preterm infants[119] and in experimental rat models[120]. Exogenous supplementation of IAP, either enterally[121,122] or systemically[123], has been shown to increase IAP activity and protect rat pups subjected to experimental NEC[124]. PPARγ is a known inhibitor of intestinal TLR4 signaling, and treatment with the PPARγ agonist, pioglitazone, decreased the severity of experimental NEC in a rat model[47,125,126]. Interestingly, the lung surfactant protein A, a collection with antimicrobial properties, has been shown to attenuate TLR4-mediated NEC[127]. Several microRNAs, including miR-146, let-7, and miR-21, are also known to inhibit TLR signaling by translational repression of targeted mRNAs and intraperitoneal injections with miR-21 decreased severity of enteral LPS-induced experimental NEC in newborn rats[22,128–131]. Finally, stem cells or exosomes derived from stem cells exert their beneficial effect in NEC partly by inhibiting TLR and other inflammatory pathways, although the precise mechanisms remain unclear[132–134]. Together, these studies that mediate TLR inhibition in the gut provide additional evidence to support the loss of the brakes on TLR in NEC pathogenesis.

Treatment with human milk-derived bioactive molecules suppresses experimental NEC

As previously discussed, human milk contains several bioactive molecules and peptides that repress intestinal TLR signaling and help in NEC prevention[73,84,87,88,135]. In both rodent and piglet models of NEC, targeted treatment with specific human milk components—such as HMOs, secretory IgA, epidermal growth factor, and lactoferrin—have been shown to inhibit TLR signaling and decrease the incidence and severity of experimental NEC[72,85,136,137]. More recently, we showed that the short-chain fatty acid, butyrate, an anaerobic fermentation product of indigestible HMOs, protect against experimental NEC in mice by inducing the expression of SIGIRR and A20[138,139]. In an elegant study, Liu et al.[136] showed that both lactoferrin and lactoferrin-induced myeloid-derived suppressor cells inhibited NFκB activation and attenuated experimental NEC. Epidermal growth factor (EGF) is another bioactive component of human milk with important immunomodulatory properties[140]. EGF levels were found to be low in infants with NEC[141,142], whereas the supplementation of EGF reduced NEC in the experimental rat models[143–146]. The elegant mechanistic studies by Good et al.[147] and others[146] have shown that EGF in human milk protects against NEC by inhibiting LPS-mediated TLR4 activation and autophagy. Docosahexaenoic acid, an ω3 polyunsaturated fatty acid present in human milk, was also found to inhibit TLR4 expression and experimental NEC in neonatal rats[148,149]. Several other biologically active lipids, carbohydrates, or proteins with potential effects on TLR signaling have been shown to reduce experimental NEC[150].

Signatures of dysregulated TLR signaling are associated with human NEC

The role of dysregulated intestinal TLR signaling in NEC has been investigated using genetic/genomic studies, biomarker studies in stool and blood, and biochemical/molecular studies on intestinal samples obtained from infants with and without NEC. The genetic basis of NEC vulnerability has been probed to explain differences in NEC incidence among preterm infants with similar clinical risk factors and to identify biomarkers that can predict disease[151,152]. Single-nucleotide polymorphisms in TLR receptors, such as TLR4, TLR2, and TLR5, and downstream kinases, such as IRAK1, and co-receptors, such as CD14, have not been associated with increased or decreased NEC[21,151,153]. This is consistent with the hypothesis that decreased TLR signaling arising from hypomorphic variants does not contribute to NEC. Interestingly, a hypomorphic NFKB1 insertion/deletion polymorphism, which is more prevalent in African American infants known to have increased NEC rates, was associated with increased NEC[21]. Nuclear oligomerization domain (NOD) con-

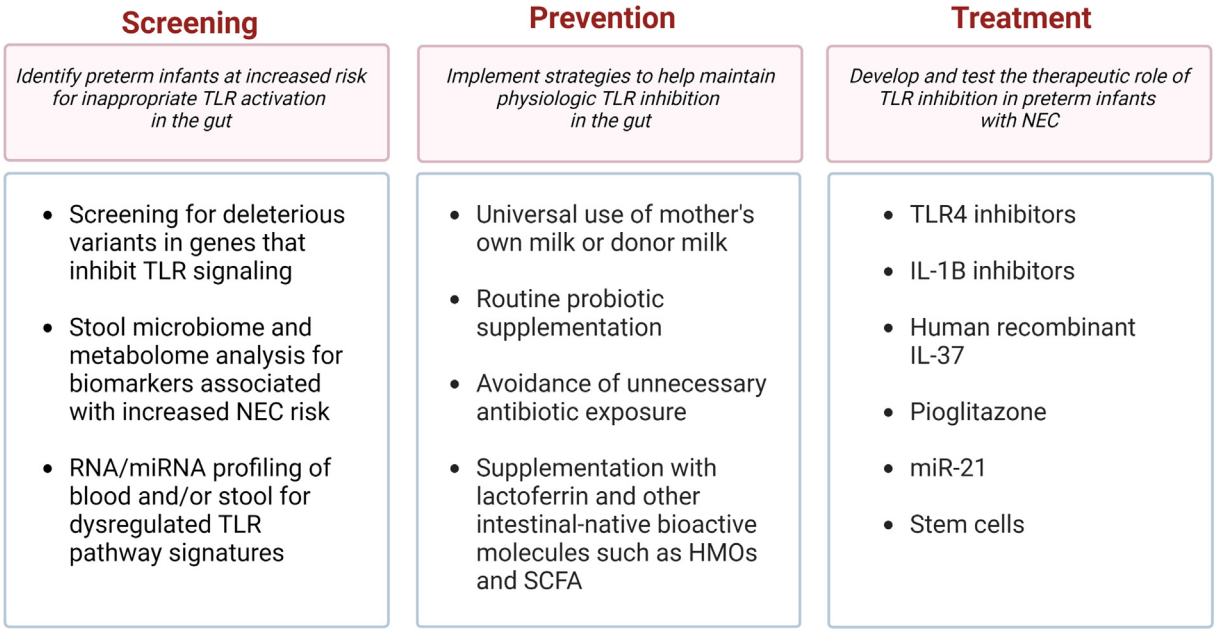

Fig. 2 Conceptual framework. Clinical applications for improving outcomes of NEC based on the concept that NEC as a defect in the brakes on TLR signaling. HMOs = human milk oligosaccharides; Ig = immunoglobulin; IL = interleukin; LPS = lipopolysaccharide; NEC = necrotizing enterocolitis; SCFAs = short-chain fatty acids; TLR = Toll-like receptor.

taining receptors are known regulators of TLR signaling, with NOD2 inhibiting intestinal TLR4 activation in NEC[48,154]. The presence of two or more hypomorphic mutations in NOD2 was associated with increased NEC in a large cohort of preterm infants, whereas each of these variants individually did not increase NEC risk[155,156]. Similarly, SIGIRR is a known inhibitor of TLR and IL-1R signaling, and the loss of SIGIRR in mice induces TLR hyper-responsiveness and exacerbates experimental NEC[10,22]. We used a sequencing-based approach to show that rare loss-of-function SIGIRR mutations were enriched in preterm infants with NEC in a small study[19]. Studies showing increased NEC risk with hypomorphic NOD2 and SIGIRR variants is consistent with the hypothesis that mutations in genes that inhibit TLR signaling can increase NEC vulnerability. In a large study, the loss-of-function ATG16L1 (T300A) variant was shown to protect against NEC[155]. Although interactions between TLR and autophagy signaling is complex, autophagy contributes to intestinal injury in TLR4-mediated experimental NEC[106]. These studies suggest that the genetic dysregulation of TLR signaling alters NEC susceptibility.

In addition to genetic studies, the direct investigation of the intestinal tissue samples obtained from surgical NEC and non-NEC controls have revealed clues to dysregulated TLR signaling. Nanthakumar et al. showed that enterocytes from fetuses had increased the RNA expression of TLR4, TLR2, MYD88, and cytokines but decreased the expression of SIGIRR and A20 compared with mature enterocytes, with further decreases in NEC infants[16]. Genome-wide RNA expression of intestinal tissue from NEC, SIP, and controls revealed the dysregulation of TLR4, NFκB1, and AP1 pathways in NEC selectively[157]. Similarly, microRNA profiling of intestinal tissue from NEC and control infants revealed dysregulated expression of microRNAs/RNA pairs that regulate TLR4 and NFκB signaling[131]. Although several studies have used proteomic profiling and enzyme-linked immunosorbent assay of plasma and urine samples to show increased cytokine expression in NEC, a definitive link to TLR activation is not established[24,158]. RNA sequencing of the intact human epithelial cells present in stool samples indicate increased NFκB and IL-1β in preterm versus term infants, consistent with a dysregulated TLR response in preterm infants prone to NEC[25].

Indirect evidence for a defect in the brakes on TLR signaling in NEC comes from clinical intervention studies

Several individual studies and meta-analyses have shown that probiotics that tamponade intestinal TLR signaling, notably, *Bifidobacterium* spp and *Lactobacillus* spp, decrease NEC incidence substantially[2,69,73]. Although it is believed that probiotics decrease NEC by altering the microbiome, this has been contested by studies showing that probiotics alter the intestinal epithelial transcriptome, inducing the TLR inhibitors SIGIRR and A20[38,39]. Disialyllacto-N-Tetraose, an HMO used by *Bifidobacterium* spp, is decreased in breast milk of preterm infants who subsequently develop NEC[137,159]. Lactoferrin, an iron-binding glycoprotein, inhibits TLR signaling by chelating iron required by bacteria, destabilizing bacterial membranes, and preventing their adherence to the mucosa[136,160]. In early clinical trials, lactoferrin used alone or when combined with probiotics decreased NEC in preterm infants[160]; however, subsequent larger studies have not confirmed the prevention of NEC with lactoferrin[161,162]. Although docosahexaenoic acid has been shown in independent studies to decrease NEC incidence in preterm infants, a meta-analysis of clinical trials did not show protection[148,150]. Although several biologically active molecules have been tested to prevent NEC, current evidence suggest that only probiotics can reduce TLR-mediated NEC in premature infants[5,73,150].

CONCLUSION, CONCEPTUAL FRAMEWORK, AND FUTURE DIRECTIONS

A remarkable feature of the human intestine is the ability to establish a symbiotic holobiont with 10–100 trillion bacteria con-

stituted of 300–500 bacterial species[163]. Although the full-term neonate adapts to postnatal life living in harmony with co-evolved gut microbiota, this is often perturbed in the preterm neonate[28,101,126,163]. Complex and temporally regulated developmental programs that attenuate intestinal epithelial TLR signaling after birth show dysmature responses in preterm infants, making them vulnerable to TLR activation and NEC[3–5,22]. The experimental data from animal models and human studies support the hypothesis that a defect in the inhibition of intestinal TLR signaling, in particular TLR4, is central to the pathogenesis of NEC. The vulnerability of the preterm infant to NEC arises partly because of the proclivity of the immature intestine to aberrant TLR activation and is exaggerated in the presence of genetic risk factors and other adverse environmental influences, such as diet, mucosal injury, and intestinal dysbiosis. A conceptual framework founded on a defect in the brakes on intestinal TLR signaling in NEC (Fig. 2) may allow us to screen, prevent, and treat NEC. The screening for the genetic, microbiota, and metabolic signatures of NEC vulnerability in blood and stool could be instituted right after birth. The infants at risk of NEC could be initiated on TLR-mitigation strategies, such as probiotics, lactoferrin, short-chain fatty acids, and HMOs, in addition to the mandatory use of human milk (Fig. 2). In addition, at the onset of NEC, TLR4-inhibitors/peptides, anti-inflammatory strategies (IL-1β blockade, IL-37 to stimulate SIGIRR), and exosome therapy could be instituted to decrease the morbidity and mortality of NEC (Fig. 2)[5,9,151]. A combination of these strategies is likely required to prevent NEC in the future.

AUTHOR CONTRIBUTIONS

VS conceptualized the review, wrote the first draft of the manuscript, and supervised all aspects of its production. AC co-wrote the first draft of the manuscript and prepared the tables and figure. MM, MC, and MAU contributed to the content and writing of the manuscript. All authors reviewed, provided critical feedback, and approved the final version of the manuscript as submitted.

DECLARATIONS OF COMPETING INTEREST

The authors have no competing interests to declare.

FUNDING

This study was supported by institutional funds from Children's Mercy Hospital (VS, AC), R01DK117296 (VS), and K08DK125735 (AC).

REFERENCES

1. Chin, A. M., Hill, D. R., Aurora, M. & Spence, J. R. Morphogenesis and maturation of the embryonic and postnatal intestine. *Semin. Cell Dev. Biol.* **66**, 81–93 (2017).
2. Cuna, A., Morowitz, M. J., Ahmed, I., Umar, S. & Sampath, V. Dynamics of the preterm gut microbiome in health and disease. *Am. J. Physiol. Gastrointest. Liver Physiol.* **320**, G411–G419 (2021).
3. Afrazi, A. et al. New insights into the pathogenesis and treatment of necrotizing enterocolitis: toll-like receptors and beyond. *Pediatr. Res.* **69**, 183–188 (2011).
4. Hackam, D. J. & Sodhi, C. P. Bench to bedside - new insights into the pathogenesis of necrotizing enterocolitis. *Nat. Rev. Gastroenterol. Hepatol.* **19**, 468–479 (2022).
5. Caplan, M.S., et al. Necrotizing enterocolitis: using regulatory science and drug development to improve outcomes. *J. Pediatr.* **212**, 208–215.e1 (2019).
6. Alganabi, M., Lee, C., Bindi, E., Li, B. & Pierro, A. Recent advances in understanding necrotizing enterocolitis. *F1000Research* **8**, F1000 Faculty Rev-107 (2019).
7. Jilling, T. et al. The roles of bacteria and TLR4 in rat and murine models of necrotizing enterocolitis. *J. Immunol.* **177**, 3273–3282 (2006).
8. Leaphart, C. L. et al. A critical role for TLR4 in the pathogenesis of necrotizing enterocolitis by modulating intestinal injury and repair. *J. Immunol.* **179**, 4808–4820 (2007).
9. Warner, B. B. et al. Gut bacteria dysbiosis and necrotising enterocolitis in very low birthweight infants: a prospective case-control study. *Lancet* **387**, 1928–1936 (2016).
10. Fawley, J. et al. Single-Immunoglobulin interleukin-1-Related Receptor regulates vulnerability to TLR4-mediated necrotizing enterocolitis in a mouse model. *Pediatr. Res.* **83**, 164–174 (2018).
11. Chatterton, D. E. W., Nguyen, D. N., Bering, S. B. & Sangild, P. T. Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. *Int. J. Biochem. Cell Biol.* **45**, 1730–1747 (2013).
12. Kawai, T. & Akira, S. The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. *Nat. Immunol.* **11**, 373–384 (2010).
13. Yazji, I. et al. Endothelial TLR4 activation impairs intestinal microcirculatory perfusion in necrotizing enterocolitis via eNOS-NO-nitrite signaling. *Proc. Natl Acad. Sci. U. S. A.* **110**, 9451–9456 (2013).
14. Neal, M. D. et al. Toll-like receptor 4 is expressed on intestinal stem cells and regulates their proliferation and apoptosis via the p53 up-regulated modulator of apoptosis. *J. Biol. Chem.* **287**, 37296–37308 (2012).
15. Sodhi, C. P. et al. Toll-like receptor-4 inhibits enterocyte proliferation via impaired beta-catenin signaling in necrotizing enterocolitis. *Gastroenterology* **138**, 185–196 (2010).
16. Nanthakumar, N. et al. The mechanism of excessive intestinal inflammation in necrotizing enterocolitis: an immature innate immune response. *PLoS One* **6**: e17776.
17. Gribar, S. C. et al. Reciprocal expression and signaling of TLR4 and TLR9 in the pathogenesis and treatment of necrotizing enterocolitis. *J. Immunol.* **182**, 636–646 (2009).
18. Pammi, M. et al. Intestinal dysbiosis in preterm infants preceding necrotizing enterocolitis: a systematic review and meta-analysis. *Microbiome* **5**, 31 (2017).
19. Sampath, V. et al. SIGIRR genetic variants in premature infants with necrotizing enterocolitis. *Pediatrics* **135**, e1530–e1534 (2015).
20. Cuna, A. & Sampath, V. Genetic alterations in necrotizing enterocolitis. *Semin. Perinatol.* **41**, 61–69 (2017).
21. Sampath, V. et al. The NFkB1 (g.-24519delATTG) variant is associated with necrotizing enterocolitis (NEC) in premature infants. *J. Surg. Res.* **169**, e51–e57 (2011).
22. Yu, W. et al. SIGIRR mutation in human necrotizing enterocolitis (NEC) disrupts STAT3-dependent microRNA expression in neonatal gut. *Cell. Mol. Gastroenterol. Hepatol.* **13**, 425–440 (2022).
23. Claud, E. C. et al. Developmentally regulated IkappaB expression in intestinal epithelium and susceptibility to flagellin-induced inflammation. *Proc. Natl Acad. Sci. U. S. A.* **101**, 7404–7408 (2004).
24. Agakidou, E., Agakidis, C., Gika, H. & Sarafidis, K. Emerging biomarkers for prediction and early diagnosis of necrotizing enterocolitis in the era of metabolomics and proteomics. *Front. Pediatr.* **8**:602255.
25. Knight, J. M. et al. Non-invasive analysis of intestinal development in preterm and term infants using RNA-Sequencing. *Sci. Rep.* **4**, 5453 (2014).
26. Henderickx, J. G. E. et al. Maturation of the preterm gastrointestinal tract can be defined by host and microbial markers for digestion and barrier defense. *Sci. Rep.* **11**, 12808 (2021).
27. Baranowski, J. R. & Claud, E. C. Necrotizing enterocolitis and the preterm infant microbiome. *Adv. Exp. Med. Biol.* **1125**, 25–36 (2019).
28. Thomas, H. Paediatrics: gut microbiota dysbiosis precedes NEC. *Nat. Rev. Gastroenterol. Hepatol.* **13**, 252 (2016).
29. Lu, P., Sodhi, C. P. & Hackam, D. J. Toll-like receptor regulation of intestinal development and inflammation in the pathogenesis of necrotizing enterocolitis. *Pathophysiology* **21**, 81–93 (2014).
30. Yu, S. & Gao, N. Compartmentalizing intestinal epithelial cell toll-like receptors for immune surveillance. *Cell. Mol. Life Sci.* **72**, 3343–3353 (2015).
31. Fusunyan, R. D., Nanthakumar, N. N., Baldeon, M. E. & Walker, W. A. Evidence for an innate immune response in the immature human intestine: toll-like receptors on fetal enterocytes. *Pediatr. Res.* **49**, 589–593 (2001).
32. Rhee, S. H. et al. Pathophysiological role of toll-like receptor 5 engagement by bacterial flagellin in colonic inflammation. *Proc. Natl Acad. Sci. U. S. A.* **102**, 13610–13615 (2005).
33. Ortega-Cava, C. F. et al. Epithelial toll-like receptor 5 is constitutively localized in the mouse cecum and exhibits distinctive down-regulation during experimental colitis. *Clin. Vaccine Immunol.* **13**, 132–138 (2006).
34. Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **124**, 783–801 (2006).
35. Lotz, M. et al. Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells. *J. Exp. Med.* **203**, 973–984 (2006).

36. Chassin, C. et al. miR-146a mediates protective innate immune tolerance in the neonate intestine. *Cell Host Microbe* **8**, 358–368 (2010).
37. Wald, D. et al. SIGIRR, a negative regulator of toll-like receptor-interleukin 1 receptor signaling. *Nat. Immunol.* **4**, 920–927 (2003).
38. Ganguli, K. et al. Probiotics prevent necrotizing enterocolitis by modulating enterocyte genes that regulate innate immune-mediated inflammation. *Am. J. Physiol. Gastrointest. Liver Physiol.* **304**, G132–G141 (2013).
39. Cuna, A. et al. NEC-like intestinal injury is ameliorated by Lactobacillus rhamnosus GG in parallel with SIGIRR and A20 induction in neonatal mice. *Pediatr. Res.* **88**, 546–555 (2020).
40. Holani, R. et al. Cathelicidins induce toll-interacting protein synthesis to prevent apoptosis in colonic epithelium. *J. Innate Immun.* **15**, 204–221 (2022).
41. Su, G., Luo, Y., Chen, D., Yu, B. & He, J. NF-κB-dependent induction of porcine β-defensin 114 regulates intestinal epithelium homeostasis. *Int. J. Biol. Macromol.* **192**, 241–249 (2021).
42. Liu, Y. et al. Changes in intestinal toll-like receptors and cytokines precede histological injury in a rat model of necrotizing enterocolitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* **297**, G442–G450 (2009).
43. Good, M. et al. 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.* **306**, G1021–G1032 (2014).
44. Shaw, A. G. et al. Premature neonatal gut microbial community patterns supporting an epithelial TLR-mediated pathway for necrotizing enterocolitis. *BMC Microbiol.* **21**, 225 (2021).
45. Delerive, P., Fruchart, J. C. & Staels, B. Peroxisome proliferator-activated receptors in inflammation control. *J. Endocrinol.* **169**, 453–459 (2001).
46. Watanabe, T., Kitani, A., Murray, P. J. & Strober, W. NOD2 is a negative regulator of toll-like receptor 2-mediated T helper type 1 responses. *Nat. Immunol.* **5**, 800–808 (2004).
47. Corsini, I. et al. Peroxisome proliferator-activated receptor-γ agonist pioglitazone reduces the development of necrotizing enterocolitis in a neonatal preterm rat model. *Pediatr. Res.* **81**, 364–368 (2017).
48. Richardson, W.M., et al. Nucleotide-binding oligomerization domain-2 inhibits toll-like receptor-4 signaling in the intestinal epithelium. *Gastroenterology* **139**, 904–917, 917.e1–6 (2010).
49. Good, M. et al. Amniotic fluid inhibits toll-like receptor 4 signaling in the fetal and neonatal intestinal epithelium. *Proc. Natl Acad. Sci. U. S. A.* **109**, 11330–11335 (2012).
50. Weström, B., Arévalo Sureda, E., Pierzynowska, K., Pierzynowski, S. G. & Pérez-Cano, F. J. The immature gut barrier and its importance in establishing immunity in newborn mammals. *Front. Immunol.* **11**, 1153 (2020).
51. van Elburg, R. M., Fetter, W. P. F., Bunkers, C. M. & Heymans, H. S. A. Intestinal permeability in relation to birth weight and gestational and postnatal age. *Arch. Dis. Child. Fetal Neonatal Ed.* **88**, F52–F55 (2003).
52. Managlia, E., Yan, X. & De Plaen, I. G. Intestinal epithelial barrier function and necrotizing enterocolitis. *Newborn (Clarksville)* **1**, 32–43 (2022).
53. Paone, P. & Cani, P. D. Mucus barrier, mucins and gut microbiota: the expected slimy partners? *Gut* **69**, 2232–2243 (2020).
54. Lallès, J. P. Intestinal alkaline phosphatase: novel functions and protective effects. *Nutr. Rev.* **72**, 82–94 (2014).
55. Ostaff, M. J., Stange, E. F. & Wehkamp, J. Antimicrobial peptides and gut microbiota in homeostasis and pathology. *EMBO Mol. Med.* **5**, 1465–1483 (2013).
56. Castoldi, A., Favero de Aguiar, C., Moraes-Vieira, P.M. & Olsen Saraiva Câmara, N. They must hold tight: junction proteins, microbiota and immunity in intestinal mucosa. *Curr. Protein Pept. Sci.* **16**, 655–671 (2015).
57. Ghosh, S. S., Wang, J., Yannie, P. J. & Ghosh, S. Intestinal barrier dysfunction, LPS translocation, and disease development. *J. Endocr. Soc.* **4**, bvz039 (2020).
58. Tulkens, J. et al. Increased levels of systemic LPS-positive bacterial extracellular vesicles in patients with intestinal barrier dysfunction. *Gut* **69**, 191–193 (2020).
59. Salzman, N. H. et al. Enteric defensin expression in necrotizing enterocolitis. *Pediatr. Res.* **44**, 20–26 (1998).
60. Kai-Larsen, Y. et al. Antimicrobial components of the neonatal gut affected upon colonization. *Pediatr. Res.* **61**, 530–536 (2007).
61. Yang, Y. et al. Ontogeny of alkaline phosphatase activity in infant intestines and breast milk. *BMC Pediatr.* **19**, 2 (2019).
62. Rentea, R. M., Rentea, M. J., Biesterveld, B., Liedel, J. L. & Gourlay, D. M. Factors known to influence the development of necrotizing enterocolitis to modify expression and activity of intestinal alkaline phosphatase in a newborn neonatal rat model. *Eur. J. Pediatr. Surg.* **29**, 290–297 (2019).
63. Malo, M. S. et al. Intestinal alkaline phosphatase promotes gut bacterial growth by reducing the concentration of luminal nucleotide triphosphates. *Am. J. Physiol. Gastrointest. Liver Physiol.* **306**, G826–G838 (2014).
64. Karav, S., Casaburi, G. & Frese, S. A. Reduced colonic mucin degradation in breastfed infants colonized by Bifidobacterium longum subsp. infantis EVC001. *FEBS Open Bio* **8**, 1649–1657 (2018).
65. Magalhaes, J. G., Tattoli, I. & Girardin, S. E. The intestinal epithelial barrier: how to distinguish between the microbial flora and pathogens. *Semin. Immunol.* **19**, 106–115 (2007).
66. Burgueño, J. F. & Abreu, M. T. Epithelial toll-like receptors and their role in gut homeostasis and disease. *Nat. Rev. Gastroenterol. Hepatol.* **17**, 263–278 (2020).
67. Patel, A. L. & Kim, J. H. Human milk and necrotizing enterocolitis. *Semin. Pediatr. Surg.* **27**, 34–38 (2018).
68. Nolan, L. S., Rimer, J. M. & Good, M. The role of human milk oligosaccharides and probiotics on the neonatal microbiome and risk of necrotizing enterocolitis: a narrative review. *Nutrients* **12**, 3052 (2020).
69. Henrick, B.M., et al. Bifidobacteria-mediated immune system imprinting early in life. *Cell* **184**, 3884–3898.e11 (2021).
70. Lin, C. et al. Intestinal 'infant-type' bifidobacteria mediate immune system development in the first 1000 days of life. *Nutrients* **14**, 1498 (2022).
71. O'Hara, A. M. et al. Functional modulation of human intestinal epithelial cell responses by Bifidobacterium infantis and Lactobacillus salivarius. *Immunology* **118**, 202–215 (2006).
72. Tomosada, Y. et al. Immunoregulatory effect of bifidobacteria strains in porcine intestinal epithelial cells through modulation of ubiquitin-editing enzyme A20 expression. *PLoS One* **8**:e59259.
73. Underwood, M. A. Probiotics and the prevention of necrotizing enterocolitis. *J. Pediatr. Surg.* **54**, 405–412 (2019).
74. Morrow, A. L. et al. Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants. *J. Pediatr.* **145**, 297–303 (2004).
75. Eiwegger, T. et al. Human milk–derived oligosaccharides and plant-derived oligosaccharides stimulate cytokine production of cord blood T-cells in vitro. *Pediatr. Res.* **56**, 536–540 (2004).
76. Eiwegger, T. et al. Prebiotic oligosaccharides: in vitro evidence for gastrointestinal epithelial transfer and immunomodulatory properties. *Pediatr. Allergy Immunol.* **21**, 1179–1188 (2010).
77. Bode, L., Rudloff, S., Kunz, C., Strobel, S. & Klein, N. Human milk oligosaccharides reduce platelet-neutrophil complex formation leading to a decrease in neutrophil beta 2 integrin expression. *J. Leukoc. Biol.* **76**, 820–826 (2004).
78. Bode, L. et al. Inhibition of monocyte, lymphocyte, and neutrophil adhesion to endothelial cells by human milk oligosaccharides. *Thromb. Haemost.* **92**, 1402–1410 (2004).
79. LeBouder, E. et al. 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 (2003).
80. Labéta, M. O. et al. Innate recognition of bacteria in human milk is mediated by a milk-derived highly expressed pattern recognition receptor, soluble CD14. *J. Exp. Med.* **191**, 1807–1812 (2000).
81. Ando, K. et al. Human lactoferrin activates NF-kappaB through the toll-like receptor 4 pathway while it interferes with the lipopolysaccharide-stimulated TLR4 signaling. *FEBS Journal* **277**, 2051–2066 (2010).
82. Aziz, M., Jacob, A., Matsuda, A. & Wang, P. Review: milk fat globule-EGF factor 8 expression, function and plausible signal transduction in resolving inflammation. *Apoptosis* **16**, 1077–1086 (2011).
83. Håversen, L., Ohlsson, B. G., Hahn-Zoric, M., Hanson, L. A. & Mattsby-Baltzer, I. Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-kappa B. *Cell. Immunol.* **220**, 83–95 (2002).
84. Cacho, N. T. & Lawrence, R. M. Innate immunity and breast milk. *Front. Immunol.* **8**, 584 (2017).
85. Gopalakrishna, K. P. et al. Maternal IgA protects against the development of necrotizing enterocolitis in preterm infants. *Nat. Med.* **25**, 1110–1115 (2019).
86. Atyeo, C. & Alter, G. The multifaceted roles of breast milk antibodies. *Cell* **184**, 1486–1499 (2021).
87. He, Y., Lawlor, N. T. & Newburg, D. S. Human milk components modulate toll-like receptor-mediated inflammation. *Adv. Nutr.* **7**, 102–111 (2016).
88. Newburg, D. S. & Walker, W. A. Protection of the neonate by the innate immune system of developing gut and of human milk. *Pediatr. Res.* **61**, 2–8 (2007).
89. Clark, J. A. et al. Intestinal barrier failure during experimental necrotizing enterocolitis: protective effect of EGF treatment. *Am. J. Physiol. Gastrointest. Liver Physiol.* **291**, G938–G949 (2006).
90. Wang, B. et al. Glutamine and intestinal barrier function. *Amino Acids* **47**, 2143–2154 (2015).
91. Wang, B. et al. L-glutamine enhances tight junction integrity by activating CaMK kinase 2-amp-activated protein kinase signaling in intestinal porcine epithelial cells. *J. Nutr.* **146**, 501–508 (2016).

92. Colaizy, T. T. Effects of milk banking procedures on nutritional and bioactive components of donor human milk. *Semin. Perinatol.* **45**:151382.
93. Moubareck, C. A. Human milk microbiota and oligosaccharides: a glimpse into benefits, diversity, and correlations. *Nutrients* **13**, 1123 (2021).
94. Moossavi, S., et al. Composition and variation of the human milk microbiota are influenced by maternal and early-life factors. *Cell Host Microbe* **25**, 324–335.e4 (2019).
95. Fernández, L., Pannaraj, P. S., Rautava, S. & Rodríguez, J. M. The microbiota of the human mammary ecosystem. *Front. Cell. Infect. Microbiol.* **10**:586667.
96. LeMay-Nedjelski, L. et al. Examining the relationship between maternal body size, gestational glucose tolerance status, mode of delivery and ethnicity on human milk microbiota at three months post-partum. *BMC Microbiol.* **20**, 219 (2020).
97. LeMay-Nedjelski, L. et al. Maternal diet and infant feeding practices are associated with variation in the human milk microbiota at 3 months postpartum in a cohort of women with high rates of gestational glucose intolerance. *J. Nutr.* **151**, 320–329 (2021).
98. Lucas, A. & Cole, T. J. Breast milk and neonatal necrotising enterocolitis. *Lancet* **336**, 1519–1523 (1990).
99. Sullivan, S., et al. An exclusively human milk-based diet is associated with a lower rate of necrotizing enterocolitis than a diet of human milk and bovine milk-based products. *J. Pediatr.* **156**, 562–7.e1 (2010).
100. Neu, J. Developmental aspects of maternal-fetal, and infant gut microbiota and implications for long-term health. *Matern. Health Neonatol. Perinatol.* **1**, 6 (2015).
101. Milani, C., et al. The first microbial colonizers of the human gut: composition, activities, and health implications of the infant gut microbiota. *Microbiol. Mol. Biol. Rev.* **81**, e00036-17 (2017).
102. Pammi, M., De Plaen, I. G. & Maheshwari, A. Recent advances in necrotizing enterocolitis research: strategies for implementation in clinical practice. *Clin. Perinatol.* **47**, 383–397 (2020).
103. Nolan, L. S., Wynn, J. L. & Good, M. Exploring clinically-relevant experimental models of neonatal shock and necrotizing enterocolitis. *Shock* **53**, 596–604 (2020).
104. Ewer, A. K. et al. The role of platelet activating factor in a neonatal piglet model of necrotising enterocolitis. *Gut* **53**, 207–213 (2004).
105. Yu, R. et al. Inhibition of HMGB1 improves necrotizing enterocolitis by inhibiting NLRP3 via TLR4 and NF-κB signaling pathways. *J. Cell. Physiol.* **234**, 13431–13438 (2019).
106. Neal, M. D. et al. A critical role for TLR4 induction of autophagy in the regulation of enterocyte migration and the pathogenesis of necrotizing enterocolitis. *J. Immunol.* **190**, 3541–3551 (2013).
107. Yan, X. et al. Lack of VEGFR2 signaling causes maldevelopment of the intestinal microvasculature and facilitates necrotizing enterocolitis in neonatal mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* **310**, G716–G725 (2016).
108. Cho, S. X. et al. Characterization of the pathoimmunology of necrotizing enterocolitis reveals novel therapeutic opportunities. *Nat. Commun.* **11**, 5794 (2020).
109. Hoang, T. K. et al. Protective effect of Lactobacillus reuteri DSM 17938 against experimental necrotizing enterocolitis is mediated by toll-like receptor 2. *Am. J. Physiol. Gastrointest. Liver Physiol.* **315**, G231–G240 (2018).
110. Caplan, M. S., Simon, D. & Jilling, T. The role of PAF, TLR, and the inflammatory response in neonatal necrotizing enterocolitis. *Semin. Pediatr. Surg.* **14**, 145–151 (2005).
111. Soliman, A. et al. Platelet-activating factor induces TLR4 expression in intestinal epithelial cells: implication for the pathogenesis of necrotizing enterocolitis. *PLoS One* **5**:e15044.
112. Neal, M. D. et al. Discovery and validation of a new class of small molecule toll-like receptor 4 (TLR4) inhibitors. *PLoS One* **8**:e65779.
113. Martens, A. & van Loo, G. A20 at the crossroads of cell death, inflammation, and autoimmunity. *Cold Spring Harb. Perspect. Biol.* **12**:a036418.
114. Lee, E. G. et al. Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. *Science* **289**, 2350–2354 (2000).
115. Caruso, R., Warner, N., Inohara, N. & Núñez, G. NOD1 and NOD2: signaling, host defense, and inflammatory disease. *Immunity* **41**, 898–908 (2014).
116. Lee, J., Rachmilewitz, D. & Raz, E. Homeostatic effects of TLR9 signaling in experimental colitis. *Ann. N. Y. Acad. Sci.* **1072**, 351–355 (2006).
117. Mihi, B. et al. Interleukin-22 signaling attenuates necrotizing enterocolitis by promoting epithelial cell regeneration. *Cell Rep. Med.* **2**:100320.
118. Keir, M., Yi, Y., Lu, T. & Ghilardi, N. The role of IL-22 in intestinal health and disease. *J. Exp. Med.* **217**:e20192195.
119. Heath, M. et al. Association of intestinal alkaline phosphatase with necrotizing enterocolitis among premature infants. *JAMA Netw. Open* **2**:e1914996.
120. Biesterveld, B. E. et al. Intestinal alkaline phosphatase to treat necrotizing enterocolitis. *J. Surg. Res.* **196**, 235–240 (2015).
121. Rentea, R. M. et al. Enteral intestinal alkaline phosphatase administration in newborns decreases iNOS expression in a neonatal necrotizing enterocolitis rat model. *J. Pediatr. Surg.* **48**, 124–128 (2013).
122. Rentea, R. M. et al. Intestinal alkaline phosphatase administration in newborns is protective of gut barrier function in a neonatal necrotizing enterocolitis rat model. *J. Pediatr. Surg.* **47**, 1135–1142 (2012).
123. Riggle, K. M. et al. Intestinal alkaline phosphatase prevents the systemic inflammatory response associated with necrotizing enterocolitis. *J. Surg. Res.* **180**, 21–26 (2013).
124. Whitehouse, J. S. et al. The protective role of intestinal alkaline phosphatase in necrotizing enterocolitis. *J. Surg. Res.* **163**, 79–85 (2010).
125. Reddy, A. T. et al. Endothelial cell peroxisome proliferator-activated receptor γ reduces endotoxemic pulmonary inflammation and injury. *J. Immunol.* **189**, 5411–5420 (2012).
126. Shibolet, O. & Podolsky, D.K. TLRs in the gut. IV. Negative regulation of toll-like receptors and intestinal homeostasis: addition by subtraction. *Am. J. Physiol. Gastrointest. Liver Physiol.* **292**, G1469–G1473 (2007).
127. Liu, L. et al. Surfactant protein A reduces TLR4 and inflammatory cytokine mRNA levels in neonatal mouse ileum. *Sci. Rep.* **11**, 2593 (2021).
128. Donda, K., Bose, T., Dame, C. & Maheshwari, A. The impact of microRNAs in neonatal necrotizing enterocolitis and other inflammatory conditions of intestine: a review. *Curr. Pediatr. Rev.* **19**, 5–14 (2022).
129. Rupaimoole, R. & Slack, F. J. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat. Rev. Drug Discov.* **16**, 203–222 (2017).
130. Li, W., Huang, X. & Bi, D. miRNA-21 plays an important role in necrotizing enterocolitis. *Arch. Med. Sci.* **18**, 406–412 (2022).
131. Ng, P. C. et al. Comparative MiRNA expressional profiles and molecular networks in human small bowel tissues of necrotizing enterocolitis and spontaneous intestinal perforation. *PLoS One* **10**:e0135737.
132. Villamor-Martinez, E., Hundscheid, T., Kramer, B. W., Hooijmans, C. R. & Villamor, E. Stem cells as therapy for necrotizing enterocolitis: a systematic review and meta-analysis of preclinical studies. *Front. Pediatr.* **8**:578984.
133. Zani, A. et al. Amniotic fluid stem cells improve survival and enhance repair of damaged intestine in necrotising enterocolitis via a COX-2 dependent mechanism. *Gut.* **63**, 300–309 (2014).
134. Drucker, N. A. et al. Stem cell therapy in necrotizing enterocolitis: current state and future directions. *Semin. Pediatr. Surg.* **27**, 57–64 (2018).
135. Bode, L. Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology* **22**, 1147–1162 (2012).
136. Liu, Y. et al. Lactoferrin-induced myeloid-derived suppressor cell therapy attenuates pathologic inflammatory conditions in newborn mice. *J. Clin. Invest.* **129**, 4261–4275 (2019).
137. Jantscher-Krenn, E. et al. The human milk oligosaccharide disialyllacto-N-tetraose prevents necrotising enterocolitis in neonatal rats. *Gut* **61**, 1417–1425 (2012).
138. Ríos-Covián, D. et al. Intestinal short chain fatty acids and their link with diet and human health. *Front. Microbiol.* **7**, 185 (2016).
139. Yu, W. et al. Short chain fatty acids ameliorate necrotizing enterocolitis-like intestinal injury through enhancing notch1-mediated single immunoglobulin interleukin-1-related receptor, toll-interacting protein, and A20 induction. *Am. J. Physiol. Gastrointest. Liver Physiol.* **324**, G24–G37 (2023).
140. Dvorak, B., Fituch, C. C., Williams, C. S., Hurst, N. M. & Schanler, R. J. Increased epidermal growth factor levels in human milk of mothers with extremely premature infants. *Pediatr. Res.* **54**, 15–19 (2003).
141. Shin, C.E., Falcone, R.A., Stuart, L., Erwin, C.R. & Warner, B.W. Diminished epidermal growth factor levels in infants with necrotizing enterocolitis. *J. Pediatr. Surg.* **35**, 173–176; discussion 177 (2000).
142. Ahmed, H. M. & Kamel, N. M. The relation between serum levels of epidermal growth factor and necrotizing enterocolitis in preterm neonates. *Korean J. Pediatr.* **62**, 307–311 (2019).
143. Dvorak, B. et al. Epidermal growth factor reduces the development of necrotizing enterocolitis in a neonatal rat model. *Am. J. Physiol. Gastrointest. Liver Physiol.* **282**, G156–G164 (2002).
144. Feng, J., El-Assal, O.N. & Besner, G.E. Heparin-binding epidermal growth factor-like growth factor decreases the incidence of necrotizing enterocolitis in neonatal rats. *J. Pediatr. Surg.* **41**, 144–149; discussion 144–149 (2006).
145. Chen, W., Yang, C., Xue, H. & Huang, Q. The protective effect and mechanism of epidermal growth factor on necrotizing enterocolitis in a neonatal rat model. *Transl. Pediatr.* **10**, 900–913 (2021).
146. Maynard, A. A. et al. Epidermal growth factor reduces autophagy in intestinal epithelium and in the rat model of necrotizing enterocolitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* **299**, G614–G622 (2010).
147. Good, M. et al. Breast milk protects against the development of necrotizing enterocolitis through inhibition of toll-like receptor 4 in the intestinal

epithelium via activation of the epidermal growth factor receptor. *Mucosal Immunol.* **8**, 1166–1179 (2015).
148. Bernabe-García, M. et al. Efficacy of docosahexaenoic acid for the prevention of necrotizing enterocolitis in preterm infants: a randomized clinical trial. *Nutrients* **13**, 648 (2021).
149. Lu, J., Jilling, T., Li, D. & Caplan, M. S. Polyunsaturated fatty acid supplementation alters proinflammatory gene expression and reduces the incidence of necrotizing enterocolitis in a neonatal rat model. *Pediatr. Res.* **61**, 427–432 (2007).
150. de Lange, I. H. et al. Enteral feeding interventions in the prevention of necrotizing enterocolitis: a systematic review of experimental and clinical studies. *Nutrients* **13**, 1726 (2021).
151. Cuna, A., George, L. & Sampath, V. Genetic predisposition to necrotizing enterocolitis in premature infants: current knowledge, challenges, and future directions. *Semin. Fetal Neonatal Med.* **23**, 387–393 (2018).
152. Donda, K. T., Torres, B. A., Khashu, M. & Maheshwari, A. Single nucleotide polymorphisms in neonatal necrotizing enterocolitis. *Curr. Pediatr. Rev.* **18**, 197–209 (2022).
153. Szebeni, B. et al. Genetic polymorphisms of CD14, toll-like receptor 4, and caspase-recruitment domain 15 are not associated with necrotizing enterocolitis in very low birth weight infants. *J. Pediatr. Gastroenterol. Nutr.* **42**, 27–31 (2006).
154. Carneiro, L. A. M. & Travassos, L. H. The interplay between NLRs and autophagy in immunity and inflammation. *Front. Immunol.* **4**, 361 (2013).
155. Sampath, V. et al. A functional ATG16L1 (T300A) variant is associated with necrotizing enterocolitis in premature infants. *Pediatr. Res.* **81**, 582–588 (2017).
156. Härtel, C. et al. 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 (2016).
157. Chan, K. Y. Y. et al. Genome-wide expression profiles of necrotizing enterocolitis versus spontaneous intestinal perforation in human intestinal tissues: dysregulation of functional pathways. *Ann. Surg.* **260**, 1128–1137 (2014).
158. Benkoe, T. et al. Comprehensive evaluation of 11 cytokines in premature infants with surgical necrotizing enterocolitis. *PLoS One* **8**:e58720.
159. Masi, A. C. et al. Human milk oligosaccharide DSLNT and gut microbiome in preterm infants predicts necrotising enterocolitis. *Gut* **70**, 2273–2282 (2021).
160. Pammi, M. & Suresh, G. Enteral lactoferrin supplementation for prevention of sepsis and necrotizing enterocolitis in preterm infants. *Cochrane Database Syst. Rev.* **6**, CD007137 (2017).
161. Tarnow-Mordi, W. O. et al. The effect of lactoferrin supplementation on death or major morbidity in very low birthweight infants (LIFT): a multicentre, double-blind, randomised controlled trial. *Lancet Child Adolesc. Health* **4**, 444–454 (2020).
162. Pammi, M. & Suresh, G. Enteral lactoferrin supplementation for prevention of sepsis and necrotizing enterocolitis in preterm infants. *Cochrane Database Syst. Rev.* **3**, CD007137 (2020).
163. Postler, T. S. & Ghosh, S. Understanding the holobiont: how microbial metabolites affect human health and shape the immune system. *Cell Metab.* **26**, 110–130 (2017).