HHS Public Access

Author manuscript

Semin Fetal Neonatal Med. Author manuscript; available in PMC 2019 December 01.

Published in final edited form as:

Semin Fetal Neonatal Med. 2018 December ; 23(6): 394–399. doi:10.1016/j.siny.2018.08.002.

Innate and adaptive immunity in necrotizing enterocolitis

Madison A. Mara[a], **Misty Good**[a], and **Joern-Hendrik Weitkamp**[b,*]

[a]Department of Pediatrics, Division of Newborn Medicine, St Louis Children's Hospital, Washington University School of Medicine, St Louis, Missouri, USA

[b]Department of Pediatrics, Division of Neonatology, Monroe Carell Jr Children's Hospital at Vanderbilt, Vanderbilt University Medical Center, Nashville, Tennessee, USA

SUMMARY

Necrotizing enterocolitis (NEC) is the most frequent and devastating gastrointestinal disease of premature infants. Although the precise mechanisms are not fully understood, NEC is thought to develop following a combination of prematurity, formula feeding, and adverse microbial colonization. Within the last decade, studies increasingly support an important role of a heightened mucosal immune response initiating a pro-inflammatory signaling cascade, which can lead to the disruption of the intestinal epithelium and translocation of pathogenic species. In this review, we first describe the cellular composition of the intestinal epithelium and its critical role in maintaining epithelial integrity. We then discuss cell signaling during NEC, specifically, tolllike receptors and nucleotide oligomerization domain-like receptors. We further review cytokines and cellular components that characterize the innate and adaptive immune systems and how they interact to support or modulate NEC development.

Keywords

Necrotizing enterocolitis; Innate immunity; Adaptive immunity; Intestinal epithelium; Toll-like receptors; Prematurity

1. Introduction

The onset of necrotizing enterocolitis (NEC) is thought to be affected by three primary factors: prematurity, formula feeding, and unbalanced microbial colonization of the intestine [1,2]. However, a prevalent unifying hypothesis has proposed that the preterm gut environment is highly sensitive to postnatal colonization with potentially pathogenic bacteria, which elicits inappropriate immune responses supporting NEC development [2].

*Corresponding author. Address: Department of Pediatrics, Division of Neonatology, Monroe Carell Jr. Children's Hospital at Vanderbilt, Vanderbilt University Medical Center, Nashville, TN, USA 11114 Doctor's Office Tower 2200 Children's Way Nashville, TN 37232, USA. Tel.: +1 (615) 322-3476; fax: +1 (615) 343-6182. hendrik.weitkamp@vanderbilt.edu (J.-H. Weitkamp).

Conflict of interest statement

None declared.

Accordingly, abundant studies indicate that both innate and adaptive immune systems contribute to NEC pathology. By reviewing the functions of multiple components of the innate and adaptive immune systems in the neonatal intestine, we aim to provide a comprehensive understanding of the role of the immune system in NEC development.

2. Innate immune system

2.1. Physical barrier of the intestine

The intestinal epithelium is composed of a complex network with approximately seven different cell types working together to balance the multiple functions of the small intestine, including nutrient absorption, antigen recognition, maintenance of mucosal integrity, and protection from micro-organisms. These cell types are joined by tight junctions and form a cryptvillus structure characteristic of the small intestine [3]. The villus is covered with enterocytes, goblet, enteroendocrine, and tuft cells, whereas the crypts house Paneth cells, transit amplifying cells, and the progenitor stem cell that gives rise to all the intestinal cell types [4]. Together, these cells make up the intestinal epithelium and act as the first major barrier of the intestinal innate system.

2.1.1. Enterocytes—As absorption is a predominant role of the small intestine, enterocytes (also known as intestinal epithelial cells, IECs) are responsible for uptake of water, nutrients, and vitamins [5]. Enterocytes make up approximately 80% of the intestinal epithelium, are renewed every three to five days, and are connected by tight junctions, which together maintain intestinal integrity [4,6]. During digestion, enterocytes are exposed to antigens from food, native microflora, and foreign micro-organisms [6]. Therefore, enterocytes can sample and identify antigens passing through the intestinal lumen and relay the signal to the underlying intraepithelial lymphocytes via pattern-recognition receptors (PPRs) [5,7,8]. PPRs expressed by IECs, such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domains (NODs), recognize antigens on pathogenic bacteria and elicit an immune response against infection [5,7]. Studies demonstrate that PPRs have a direct linkage to NEC, which are discussed later in this review. In addition to PPRs, enterocytes are capable of presenting major histocompatibility class (MHC) I and II molecules and non-classical MHC molecules such as MHC class I polypeptide-related sequence A or B, both of which can signal directly with lymphocytes to initiate an immune response [6,7,9]. Taken together, enterocytes play an important role in overall function of the small intestine.

2.1.2. Goblet cells—Goblet cells are an important cell type that help maintain the gut barrier and are responsible for the production of the mucus layer between the epithelium and lumen. They make up approximately 4% of the epithelium and reside along the crypts up to the villus tip, allowing for mucus secretion [4]. The mucus secreted by goblet cells is composed of glycoproteins known as mucins, which are regulated by the *mucin* (MUC) gene. Mucins are responsible for facilitating interactions between host epithelium and commensal or pathogenic micro-organisms [9,10]. MUC2 is the primary mucin and important for goblet cell morphology, as demonstrated by *MUC2*-deficient mice that fail to develop distinguishable goblet cells [11,12]. Goblet cells have also been implicated in

immune tolerance to commensal bacteria and are capable of passaging antigens from the lumen to $CD103^+$ dendritic cells, thus promoting intestinal immune homeostasis [13].

Goblet cell differentiation is promoted by kruppel-like factor 4 (Klf4) and E47-like factor 3 (Elf3), but inhibited by the Notch signaling pathway [4,14]. Sodhi et al. demonstrated that tolllike receptor 4 (TLR4) activation is capable of upregulating Notch signaling, independent of microbial interactions [15]. They also found that TLR4 and Notch signaling are increased whereas goblet cells and *MUC2* expression are decreased in mice and premature infants with NEC [15]. Since MUC2 plays an important role in regulating gut barrier integrity, any decrease in MUC2 could potentially increase the risk for NEC. Other studies have also shown that MUC2 is significantly decreased in infants with NEC, which can be exacerbated by factors such as immature goblet cells due to prematurity, increased Notch signaling, or mutations leading to aberrant goblet cell development [14,16–18].

2.1.3. Paneth cells/antimicrobial peptides—Paneth cells are significant contributors to the integrity of the intestinal epithelium since they produce and secrete peptides that rapidly kill or inactivate micro-organisms [19–21]. On average, five to 12 Paneth cells reside near the bottom of the crypt and are renewed every two to three weeks, allowing them to produce enough antimicrobial peptides (AMPs) to maintain homeostasis [20,21]. AMPs include a-defensins (human) or cryptidins (mice), lysozyme C, secretory group IIA phospholipase A2 (sPLA2), C-type lectins (REG3a in humans, REG3g in mice), and angiogenin 4 (ANG4) in mice [19–21]. In the small intestine, Paneth cells secrete these peptides in response to pathogen-associated molecular patterns (PAMPs), or molecular motifs expressed by classes of micro-organisms [21].

Although Paneth cells produce AMPs to counteract enteric pathogens, genetic mutations can lead to the disruption of the intestinal epithelium and increase susceptibility of inflammatory bowel diseases (IBD). Crohn's disease (CD) results in ileal inflammation and is associated with mutations of NOD2, which is a PPR predominantly present in Paneth cells [20,22,23]. NOD2 is a receptor that recognizes muramyl dipeptide, which is found in both Gram-positive and Gram-negative bacteria. Studies have shown that NOD2-deficient mice have fewer cryptidins at both baseline and post-infection as compared to wild-type (WT) mice [24]. Additionally, NOD2deficient mice challenged with *Helicobacter hepaticus* develop granulomatous lesions in the ileum, which is common in patients with Crohn's disease, and they express pro-inflammatory T_H1-related genes, such as the cytokine interferon-γ (IFN-γ) and the transcription factor T-bet [20,25]. In human studies, the effect of NOD2 loss-of-function mutations has been evaluated to determine the risk of developing lethal gastrointestinal tract diseases, such as NEC and focal intestinal perforation [26]. Moreover, an unconventional mouse model has been described, in which Paneth cells are depleted with dithazone and the intestine is exposed to *Klebsiella pneumoniae* [27–29]. This model yields a phenotype similar to human NEC, yet, due to intentional damage of Paneth cells, conclusions regarding AMPs cannot be made [29]. Taken together, further research is needed to elucidate the role of Paneth cells and/or Paneth cell-derived AMPs in the prevention or the pathogenesis of NEC.

2.2. Pattern recognition receptors

Pattern recognition receptors (PRRs) are conserved receptors that have evolved to sense the presence of pathogenic and endogenous molecules released during infection and injury. There are currently four classes of PRR families [30], yet for the purpose of this review we focus on the two classes that have been linked to NEC: TLRs and nucleotide oligomerization domain-like receptors (NLRs).

2.2.1. Toll-like receptors—TLRs are well-characterized transmembrane receptors that primarily function to initiate immune responses against bacteria, viruses, and other pathogenic micro-organisms [30,31]. Presently, there have been 10 TLRs identified in humans and 13 in mice, with each TLR sensing for a distinct pathogenic or endogenous ligand [30,31]. Once the ligand binds to its associated TLR, the receptor dimerizes and recruits the myeloid differentiation factor 88 (MyD88) (or Toll/IL-1R domain containing adaptor inducing IFNβ (TRIF) with the exception of TLR3), which causes downstream signaling to the nuclear factor-κB (NF-κB) pathway [31–33]. NF-κB is a transcription factor that translocates to the nucleus and induces an inflammatory response, drawing effector cells to the location of the initial pathogen or injury [31]. This conserved response is tightly regulated, yet exacerbated TLR responses have been heavily implicated in the pathogenesis of NEC.

Within the last few decades, numerous studies have suggested that TLR4 activation and signaling is a significant contributor to NEC development [34–43]. Since prematurity is a major risk factor in developing NEC, several studies have investigated differences between the preterm and term TLR expression in the intestinal tract. Interestingly, these studies indicate that there are higher TLR4 expression levels in premature murine and human intestine as compared to full-term controls [31,44–46]. Investigators have demonstrated that TLR4 activation in this immature environment results in increased enterocyte apoptosis, reduced enterocyte proliferation and migration, and the eventual breakdown of the intestinal epithelium [34,36,37,39,40,47]. Once epithelial integrity is lost, pathogens are able to enter the circulatory system resulting in systemic inflammation. Moreover, TLR4 activation has been shown to reduce endothelial nitric oxide synthase (eNOS) in the intestinal endothelium, causing decreased blood flow and ischemia that may further support the development of NEC [43,48].

Studies have also indicated that TLR4 is required for NEC development, and is not simply an outcome of the disease. In one such study, TLR4-mutant mice (C3H/HeJ) were protected from developing experimental NEC, whereas wild-type littermates experienced disruption of the intestinal epithelium and severe disease [40]. Others utilized global and intestinal-specific TLR4 gene deletion that demonstrated preservation of the intestinal epithelium and downregulation of pro-inflammatory cytokines [15]. Likewise, it has been shown that, by inhibiting TLR4 signaling, mice were protected against experimental NEC [34,37,38,46,48]. For example, administration of amniotic fluid [37], breast milk [38], or human milk oligosaccharide 2′-fucosyllactose (2′FL) [48] attenuated TLR4 signaling, thus significantly decreasing the severity of experimental NEC in mice. Together, these studies suggest an important role that TLR4 plays in NEC pathogenesis.

Other TLRs have also been studied in the context of intestinal injury. TLR2, which is expressed in enterocytes, immune cells in the lamina propria, and in enteroendocrine cells, induces the production of anti-inflammatory cytokines, such as IL-10, to modulate the immune response [49]. However, imbalances in TLR2-mediated NF-κB activation can lead to proinflammatory cytokine production and induce intestinal injury. Furthermore, TLR2 also supports preservation of tight junctions between enterocytes, thus maintaining gut integrity [49]. Studies have also demonstrated protection against NEC by modulating the expression of TLR9 [36,39]. By administering CpG-DNA (ligand to TLR9), TLR4-mediated signaling is modulated and prevents enterocyte apoptosis and bacterial translocation, thus decreasing experimental NEC severity [39]. Although these studies yield a basic understanding of the interactions between TLRs and NEC pathogenesis, more studies are needed to discover additional roles of TLRs during NEC.

2.2.2. Nucleotide oligomerization domain-like receptors (NLRs)—NLRs are another class of PRRs that recognize PAMPs and DAMPs within the cytoplasm of cells. NOD1 and NOD2, two members of this class, have been suggested to modulate NEC development via TLR regulation [47,50–52]. NOD1, expressed by enterocytes, recognizes peptidoglycans found in the cell walls of Gram-negative bacteria and activates an immune response though the Peyer's patches [3,53]. Similarly, NOD2 recognizes intracellular muramyl dipeptide (MDP) that is found in most bacterial cell walls and is expressed by monocytes, dendritic cells, and Paneth cells [3,30,53]. Multiple studies have explored the roles played by NOD1 and NOD2 in NEC. In a study by Richardson et al., activation of NOD2 by MDP decreased TLR4 signaling and decreased NEC severity in mice [47]. When MDP was administered, the apoptosis regulatory protein SMAC-diablo was downregulated, thus inhibiting TLR4-mediated enterocyte apoptosis [47]. In another study, investigators found that NOD2 expression is required for colonization of commensal bacteria and suppression of pathogenic bacteria [52]. Using NOD2-deficient mice, *H. hepaticus* was gavaged to WT and NOD2-deficient mice, resulting in NOD2-deficient mice unable to clear *H. hepaticus* infection [52]. In relation to NEC, mutations in NOD2 may promote colonization of pathogenic microflora in the premature intestine and lead to NEC development [52]. Finally, both NOD1 and NOD2 activate the NFκB pathway, which, depending on the stimulus, could modulate TLR4 expression or act synergistically with TLRs [30,47,50].

2.3. Neutrophils

Neutrophils are major drivers of innate immune responses. They are the first cells at the site of injury, produce bactericidal compounds, and attract and influence other cell types, such as monocytes and dendritic cells [54]. Their implication in NEC remains to be fully elucidated, yet there are a few studies to note. One study used a *Cronobacter sakazakii* NEC mouse model to induce NEC in WT mice and mice depleted of neutrophils, finding an increase in proinflammatory cytokines, nitric oxide, and enterocyte apoptosis in neutrophil-depleted mice (72%) compared to WT mice (40%) [55]. Another study investigated the incidence of neutropenia (neutrophil counts of ≤1000/mL) in small for gestational age (SGA) human neonates, which suggested that these infants have a 4-fold increased risk to develop NEC [56]. These findings indicate that neutrophils may attenuate the immune response in NEC.

However, one study demonstrated that overexpression of the bactericidal oxidative species produced by neutrophils could contribute to the severity of NEC [57]. In rats with and without neutrophils (depleted with vinblastine), treatment with platelet activating factor (PAF) and LPS were used to induce NEC [57]. Rats with neutrophils experienced greater hypotension, reduced intestinal perfusion, and necrotic bowel compared to neutrophil-depleted rats [57]. This suggests that the reactive oxidative metabolites produced by neutrophils may promote intestinal injury and that the depletion of neutrophils may be protective in NEC. Nonetheless, the variation in neutrophil function may be confounded due to the diverse models used to induce NEC-like disease in animals and further studies are needed to confirm their role in NEC.

2.4. Innate lymphoid cells

Within the last decade, a new population of cells in the innate immune system, known as innate lymphoid cells (ILCs), have been increasingly investigated in order to determine their function in immunity. These cells are derived from a common lymphoid progenitor and embody the traditional lymphoid cell morphology, yet lack characteristic receptors and antigen specificity that are typical in adaptive lymphocytes [58]. ILCs are largely grouped into three different classes (subclasses exist among each group; see [59]), which are defined by cell-surface markers, transcriptional factors, and cytokine expression; however, each class elicits a distinct immune response [58]. Group 1 (ILC1s) produces cytokines INF-γ and TNF to protect the intestinal epithelium from invading viruses, bacteria, or other intracellular micro-organisms; Group 2 (ILC2s) produces type-2 cytokines (IL-4, IL-5, IL-9, IL-13) to facilitate the expulsion of parasites from the intestine; and ILC group 3 (ILC3s) produces lymphotoxin (LT), IL-17A, IL-22, and INF-γ, which help mediate responses to commensal and extracellular microbes [58].

As investigators have continued to study these cells, research has implicated ILCs in intestinal inflammation [60–63]. For example, some studies show that patients with CD have markedly increased infiltration of ILC1s [60,61]. In a study by Bernink et al., resected bowel from CD patients contained a significantly higher frequency of ILC1s as compared to control bowel, which corresponded with increased INF-γ in these patients [60]. Murine colitis studies further supported these findings and suggested that ILC1 infiltration is a response to CD and does not play a causative role [60,61]. ILC3s have also been suggested to contribute to intestinal inflammation during IBD [62,63]. Buonocore et al. demonstrated in $Rag2^{-/-}$ mice, which fail to generate B and T- cells, that ILC3s mount a pro-inflammatory response by producing IL-17A and IFN-γ in an IL-23-dependent manner [62]. Interestingly, another study utilizing the $Tbx21^{-/-}$ $Rag2^{-/-}$ ulcerative colitis (TRUC) model of IBD discovered that TNF-α from DCs acted synergistically with IL-23 to stimulate ILC3s to produce IL-17A, thus demonstrating a novel interaction between DCs and ILC3s [63]. Taken together, these studies support the role of ILC1s and ILC3s in the pathogenesis of intestinal inflammation.

3. Adaptive immune system

3.1. Macrophages

Macrophages are important effector cells that contribute to the maintenance of homeostasis as well as initiating an immune response during injury. Intestinal macrophages typically reside beneath the epithelial layer in the lamina propria, where they are activated upon exposure to LPS and IFN-γ and respond by releasing pro-inflammatory cytokines and nitric oxide [53]. However, macrophage activation is amplified in the immature intestine, but is downregulated by tumor growth factor-β (TGF-β) until the intestine is fully developed [64]. Studies related to NEC have observed high intestinal infiltration of macrophages and significantly lower levels of the TGF-β_2 isoform, suggesting that an exacerbated macrophage response in the premature intestine may play a role in NEC development [64,65].

In investigating the macrophage response during NEC, studies have discovered a pathway describing the relationship between TGF-β, mothers against decapentaplegic homolog 7 (Smad7), and NF-κB [66,67]. Along with low levels of TGF-β_2 in in-vitro and in-vivo models of NEC, MohanKumar et al. found an increase in expression of Smad7 in macrophages and an increased sensitivity to bacterial products, such as LPS [64,66,67]. Additionally, Smad7overexpressing macrophages activated the NF-κB pathway and induced a pro-inflammatory response [66]. Together, these studies suggest that an imbalance in this macrophage response pathway may influence the inflammatory state that occurs in the premature intestine during NEC.

3.2. Dendritic cells

Intestinal dendritic cells (DCs) are specialized antigen presenting cells (APCs) that play a critical role in establishing self-tolerance and maintaining homeostasis in the small intestine [68]. By sampling antigens that pass through the lumen of the gut, DCs can differentiate between antigens on pathogenic bacteria from those found on food and commensal microbiota. However, investigation of their role in NEC has been fairly limited. In one study, investigators used the opportunistic pathogen *Cronobacter sakazakii*, which may contaminate infant formula, to induce NEC in newborn mice [69]. When infected with *C. sakazakii,* a significant number of DCs were recruited to the lamina propria, which resulted in an increased release of cytokines IL-10 and TGF-β [69]. With higher levels of cytokines, specifically TGF-β, NEC-related symptoms such as enterocyte apoptosis and epithelial disruption were observed [69]. This study concluded that, in a *C. sakazakii*-induced model of NEC, DCs contribute to the breakdown of intestinal epithelial integrity [69]; however, additional studies are necessary to elucidate the role of DCs in premature infants with NEC.

3.3. T-cells

Historically, neonates were assumed to be deficient in T-cells and other adaptive immune cells [70]. However, in 1996, novel studies investigating the neonatal adaptive immune system demonstrated that neonatal T-cells were capable of adult-level responses [70–73]. Since then, continued research has shown that neonatal T-cell responses are increasingly variable and dependent on the timing and conditions surrounding antigen exposure [70]. As

such, evidence has emerged establishing a primary role of T-cells in the development of NEC. For the purpose of this review, we focus on T-cell subsets that have been directly implicated in the pathogenesis of NEC.

3.4. Intraepithelial lymphocytes

Intraepithelial lymphocytes (IELs) primarily consist of antigen-experienced T-cells that, upon exposure to familiar antigens, release cytokines to recruit effector cells to destroy invading pathogens and growth factors to stimulate epithelial repair [74]. In the gut, IELs are interspersed between epithelial cells of the intestinal epithelium and function to elicit and regulate both innate and adaptive responses during infection [74,75]. Thus, mucosal IELs act to preserve the epithelial barrier and maintain homeostasis [74,75]. Mucosal IELs are highly heterogeneous and are distinguished based on T-cell receptors (TCR) γδ or αβ, as well as on CD8 co-receptor expression [75]. γδ IELs are the first T-cell subset present in the intestine during embryogenesis [76,77]. In the study by Gibbons et al., neonatal γδ IELs were found to produce higher levels of cytokines, such as IFN-γ and IL-10, as compared to neonatal αβ IELs and adult γδ IELs, indicating enhanced activity of γδ IELs during early life [76]. Murine studies using the DSS model of colitis showed that γδ IELs are important in managing invasion of bacteria in the event of mucosal injury [78]. Microarray analysis exhibited a complex transcriptional response by γδ IELs, including transcriptional enrichment of cytoprotective properties (i.e. heat shock proteins), anti-inflammatory cytokines, and transcription factors that promote healing, all of which support epithelial regeneration [78]. Interestingly, this study also demonstrated that commensal bacteria provide immunomodulatory factors that promote the protective transcriptional response by γδ IELs during mucosal injury [78]. Furthermore, Weitkamp et al. discovered significantly lower $CD8^+$ γδ IELs in preterm infants with NEC compared to control infants, suggesting that γδ IELs depletion occurs during the development of NEC [77]. There were also decreases in gene expression for retinoic acid-related orphan nuclear hormone receptor C (RORC) and occludin, which promote early pathogen colonization resistence via IL-17 and γδ IELs migration into the epithelium, respectively [77]. Taken together, these data suggest that γδ IELs are critical for intestinal barrier integrity and that mucosal γδ IELs may be important in preventing NEC.

3.5. Regulatory T-cells

Regulatory T-cells (Tregs) are $CD4^+$ T-cells that act to suppress excessive immune responses as well as mediate immunological tolerance. Interestingly, Tregs that express forkhead box protein 3 (Foxp3) are present in the developing gut as early as 23 weeks of gestation, suggesting that they may participate in establishing intestinal homeostasis in neonates [79]. Therefore, murine and human studies were conducted to explore the immunomodulatory role of Tregs in NEC. In one such study, Egan et al. induced NEC in WT neonatal mice and observed significant mucosal barrier damage, increased pro-inflammatory cytokine levels, and a reduction in $CD4^+$ $Foxp3^+$ Tregs [34]. They also discovered that the neonatal intestine is rich in signal transducer and activator of transcription 3 (STAT3), which directs T-cell differentiation away from the Treg phenotype [34]. By treating neonatal mice with a STAT3 inhibitor, Tregs were restored and NEC severity decreased [34]. In another study, small intestine was resected from NEC and non-NEC infants and T-cell populations were analyzed

[80]. They found that intestinal resections from NEC patients had a significant decrease in Treg proportions compared to other Tcell populations [80]. Additionally, investigators discovered a pro-inflammatory cytokine profile in NEC tissue samples, which correspond to cytokines that have been shown to suppress Tregs [80]. Finally, patients with resolved NEC displayed restoration of the Treg cell population, suggesting that Treg depletion is a response to the hostile microenvironment induced by NEC [80]. Together, these studies support that Tregs serve a protective role in the fetal and neonatal intestine.

3.6. T-helper 17 cells

T-helper cells (T_H) are $CD4^+$ T-cells, which, once stimulated by APCs, actively participate in cell-mediated immune responses. T_H-cells can be further divided into subtypes based on the cytokines secreted and the stimuli they respond to; however, we now focus on T_H17 cells and evaluate their role in NEC. Several studies have demonstrated that T_H17-derived cytokines can cause intestinal inflammation during experimental NEC. For example, Egan et al. had demonstrated a decrease in Tregs in NEC-induced neonatal mice and they found a significant increase in the $CD4^+$ T_H17 cells, which primarily produce the inflammatory cytokine IL-17A [34]. IL-17A was determined to be a significant contributor to NEC pathogenesis, as demonstrated by the loss of tight junctions between intestinal epithelial cells, reduced enterocyte proliferation, and an increase in crypt apoptosis after administration to neonatal mice [34]. Additionally, they discovered that the microenvironment of the premature human intestine expresses high levels of IL-6, IL-22, and STAT3, all which drive T_H17 differentiation [34]. An additional study demonstrated that mice subjected to an experimental NEC model observed severe damage to the mucosal epithelium, primarily due to apoptosis of the $Lgr5^+$ intestinal stem cells residing at the bottom of intestinal crypts [81]. These intestinal stem cells are critical for the regeneration of other epithelial cell types; therefore, loss of these cells is detrimental to the integrity of the mucosal barrier [4,81]. Importantly, when mice subjected to experimental NEC were treated with all-trans retinoic acid (ATRA), which has been previously shown to induce Treg populations while limiting T_H17 differentiation, NEC severity was significantly reduced [34,81]. These studies demonstrate the negative effects of increased $CD4^+$ T_H17 cells on the neonatal small intestine during NEC, and highlight the use of ATRA as a potential preventative strategy or therapeutic for NEC.

4. Conclusions

The complex pathogenesis of NEC has yet to be completely elucidated, and, at least regarding the role of adaptive immunity in NEC, more studies evaluating the differences between NEC in animal models and human infants are needed. However, recent discoveries have resulted in obtaining a more comprehensive understanding of intestinal immunology in the premature gut, such as the delicate interplay between the intestinal epithelium, commensal bacteria, molecular signaling, and innate and adaptive immune cells. Importantly, these findings have led to testing of novel approaches in NEC prevention such as promoting an effective gut barrier, fostering immune surveillance without injury, and supporting a balanced intestinal microbiome during pregnancy and after birth. The latter can be achieved either through avoidance of antibiotics, supplementation with milk-derived

factors or probiotics. Although we have gained valuable insight, continued research efforts are necessary to define the pathogenesis of NEC and to develop more effective strategies to alleviate this disease.

Acknowledgment

We apologize to the authors whose work we were not able to include due to space limitations.

Funding sources

M.G. is supported by grants K08DK101608 and R03DK111473 from the National Institutes of Health, March of Dimes Foundation Grant No. 5-FY17–79, and the Children's Discovery Institute of Washington University and St Louis Children's Hospital.

References

[1]. Papillon S, Castle SL, Gayer CP, Ford HR. Necrotizing enterocolitis: contemporary management and outcomes. Adv Pediatr 2013;60:263–79. [PubMed: 24007848]

[2]. Nino DF, Sodhi CP, Hackam DJ. Necrotizing enterocolitis: new insights into pathogenesis and mechanisms. Nature Rev Gastroenterol Hepatol 2016;13:590–600. [PubMed: 27534694]

[3]. Santaolalla R, Abreu MT. Innate immunity in the small intestine. Curr Opin Gastroenterol 2012;28:124–9. [PubMed: 22241076]

[4]. van der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol 2009;71:241–60. [PubMed: 18808327]

[5]. Miron N, Cristea V. Enterocytes: active cells in tolerance to food and microbial antigens in the gut. Clin Exp Immunol 2012;167:405–12. [PubMed: 22288583]

[6]. Snoeck V, Goddeeris B, Cox E. The role of enterocytes in the intestinal barrier function and antigen uptake. Microbes Infect 2005;7:997–1004. [PubMed: 15925533]

[7]. Vitale S, Picascia S, Gianfrani C. The cross-talk between enterocytes and intraepithelial lymphocytes. Mol Cell Pediatr 2016;3:20. [PubMed: 27251606]

[8]. Hackam DJ, Good M, Sodhi CP. Mechanisms of gut barrier failure in the pathogenesis of necrotizing enterocolitis: Toll-like receptors throw the switch. Semin Pediatr Surg 2013;22:76–82. [PubMed: 23611610]

[9]. Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. Nat Rev Immunol 2003;3:331–41. [PubMed: 12669023]

[10]. Deplancke B, Gaskins HR. Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. Am J Clin Nutr 2001;73:1131S–41S. [PubMed: 11393191]

[11]. Johansson ME, Larsson JM, Hansson GC. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. Proc Natl Acad Sci USA 2011;108 Suppl 1:4659–65. [PubMed: 20615996]

[12]. Van der Sluis M, De Koning BA, De Bruijn AC, et al. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 2006;131:117–29. [PubMed: 16831596]

[13]. McDole JR, Wheeler LW, McDonald KG, et al. Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. Nature 2012;483:345–9. [PubMed: 22422267]

[14]. van Es JH, van Gijn ME, Riccio O, et al. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature 2005;435:959–63. [PubMed: 15959515]

[15]. Sodhi CP, Neal MD, Siggers R, et al. Intestinal epithelial Toll-like receptor 4 regulates goblet cell development and is required for necrotizing enterocolitis in mice. Gastroenterology 2012;143:708–18 e1–5. [PubMed: 22796522]

[16]. Hackam DJ, Upperman JS, Grishin A, Ford HR. Disordered enterocyte signaling and intestinal barrier dysfunction in the pathogenesis of necrotizing enterocolitis. Semin Pediatr Surg 2005;14:49–57. [PubMed: 15770588]

[17]. Clark JA, Doelle SM, Halpern MD, Saunders TA, Holubec H, Dvorak K, et al. Intestinal barrier failure during experimental necrotizing enterocolitis: protective effect of EGF treatment. Am J Physiol 2006;291:G938–49.

[18]. Martin NA, Mount Patrick SK, Estrada TE, et al. Active transport of bile acids decreases mucin 2 in neonatal ileum: implications for development of necrotizing enterocolitis. PloS One 2011;6:e27191. [PubMed: 22162748]

[19]. Gallo RL, Hooper LV. Epithelial antimicrobial defence of the skin and intestine. Nat Rev Immunol 2012;12:503–16. [PubMed: 22728527]

[20]. Bevins CL, Salzman NH. Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. Nat Rev Microbiol 2011;9:356–68. [PubMed: 21423246]

[21]. Elphick DA, Mahida YR. Paneth cells: their role in innate immunity and inflammatory disease. Gut 2005;54:1802–9. [PubMed: 16284290]

[22]. Hugot JP, Chamaillard M, Zouali H, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 2001;411:599–603. [PubMed: 11385576]

[23]. Ogura Y, Bonen DK, Inohara N, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 2001;411:603–6. [PubMed: 11385577]

[24]. Kobayashi KS, Chamaillard M, Ogura Y, et al. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. Science 2005;307:731–4. [PubMed: 15692051]

[25]. Biswas A, Liu YJ, Hao L, et al. Induction and rescue of Nod2-dependent Th1-driven granulomatous inflammation of the ileum. Proc Natl Acad Sci USA 2010;107:14739–44. [PubMed: 20679225]

[26]. Hartel C, Hartz A, Pagel J, 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 2016;22:249–56. [PubMed: 26752461]

[27]. Zhang C, Sherman MP, Prince LS, et al. Paneth cell ablation in the presence of Klebsiella pneumoniae induces necrotizing enterocolitis (NEC)-like injury in the small intestine of immature mice. Dis Models Mechs 2012;5:522–32.

[28]. McElroy SJ, Underwood MA, Sherman MP. Paneth cells and necrotizing enterocolitis: a novel hypothesis for disease pathogenesis. Neonatology 2013;103:10–20. [PubMed: 23006982]

[29]. Underwood MA. Paneth cells and necrotizing enterocolitis. Gut Microbes 2012;3:562–5. [PubMed: 22895084]

[30]. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell 2010;140:805–20. [PubMed: 20303872]

[31]. Lu P, Sodhi CP, Hackam DJ. Toll-like receptor regulation of intestinal development and inflammation in the pathogenesis of necrotizing enterocolitis. Pathophysiology 2014;21:81–93. [PubMed: 24365655]

[32]. Takeda K, Akira S. Roles of Toll-like receptors in innate immune responses. Genes Cells 2001;6:733–42. [PubMed: 11554921]

[33]. Barton GM, Kagan JC. A cell biological view of Toll-like receptor function: regulation through compartmentalization. Nat Rev Immunol 2009;9:535–42. [PubMed: 19556980]

[34]. Egan CE, Sodhi CP, Good M, et al. Toll-like receptor 4-mediated lymphocyte influx induces neonatal necrotizing enterocolitis. J Clin Invest 2016;126:495–508. [PubMed: 26690704]

[35]. Neal MD, Leaphart C, Levy R, et al. Enterocyte TLR4 mediates phagocytosis and translocation of bacteria across the intestinal barrier. J Immunol 2006;176:3070–9. [PubMed: 16493066]

[36]. Afrazi A, Branca MF, Sodhi CP, et al. Toll-like receptor 4-mediated endoplasmic reticulum stress in intestinal crypts induces necrotizing enterocolitis. J Biol Chem 2014;289:9584–99. [PubMed: 24519940]

[37]. Good M, Siggers RH, Sodhi CP, et al. Amniotic fluid inhibits Toll-like receptor 4 signaling in the fetal and neonatal intestinal epithelium. Proc Natl Acad Sci USA 2012;109:11330–5. [PubMed: 22733781]

[38]. Good M, Sodhi CP, Egan CE, 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 2015;8:1166–79. [PubMed: 25899687]

[39]. Gribar SC, Sodhi CP, Richardson WM, et al. Reciprocal expression and signaling of TLR4 and TLR9 in the pathogenesis and treatment of necrotizing enterocolitis. J Immunol 2009;182:636–46. [PubMed: 19109197]

[40]. Leaphart CL, Cavallo J, Gribar SC, et al. A critical role for TLR4 in the pathogenesis of necrotizing enterocolitis by modulating intestinal injury and repair. J Immunol 2007;179:4808–20. [PubMed: 17878380]

[41]. Neal MD, Sodhi CP, Jia H, 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 2012;287:37296–308. [PubMed: 22955282]

[42]. Sodhi CP, Shi XH, Richardson WM, et al. Toll-like receptor-4 inhibits enterocyte proliferation via impaired beta-catenin signaling in necrotizing enterocolitis. Gastroenterology 2010;138:185–96. [PubMed: 19786028]

[43]. Yazji I, Sodhi CP, Lee EK, et al. Endothelial TLR4 activation impairs intestinal microcirculatory perfusion in necrotizing enterocolitis via eNOS-NO-nitrite signaling. Proc Natl Acad Sci USA 2013;110:9451–6. [PubMed: 23650378]

[44]. Nanthakumar N, Meng D, Goldstein AM, et al. The mechanism of excessive intestinal inflammation in necrotizing enterocolitis: an immature innate immune response. PloS One 2011;6:e17776. [PubMed: 21445298]

[45]. Fusunyan RD, Nanthakumar NN, Baldeon ME, Walker WA. Evidence for an innate immune response in the immature human intestine: toll-like receptors on fetal enterocytes. Pediatr Res 2001;49:589–93. [PubMed: 11264445]

[46]. Neal MD, Sodhi CP, Dyer M, et al. A critical role for TLR4 induction of autophagy in the regulation of enterocyte migration and the pathogenesis of necrotizing enterocolitis. J Immunol 2013;190:3541–51. [PubMed: 23455503]

[47]. Richardson WM, Sodhi CP, Russo A, et al. Nucleotide-binding oligomerization domain-2 inhibits toll-like receptor-4 signaling in the intestinal epithelium. Gastroenterology 2010;139:904–17, 17 e1–6. [PubMed: 20580721]

[48]. Good M, Sodhi CP, Yamaguchi Y, et al. The human milk oligosaccharide 2′-fucosyllactose attenuates the severity of experimental necrotising enterocolitis by enhancing mesenteric perfusion in the neonatal intestine. Br J Nutr 2016;116:1175–87. [PubMed: 27609061]

[49]. Cario E Barrier-protective function of intestinal epithelial Toll-like receptor 2. Mucosal Immunol 2008;1 Suppl 1:S62–6. [PubMed: 19079234]

[50]. Strober W, Murray PJ, Kitani A, Watanabe T. Signalling pathways and molecular interactions of NOD1 and NOD2. Nat Rev Immunol 2006;6:9–20. [PubMed: 16493424]

[51]. Le Mandat Schultz A, Bonnard A, Barreau F, et al. Expression of TLR-2, TLR-4, NOD2 and pNF-kappaB in a neonatal rat model of necrotizing enterocolitis. PloS One 2007;2:e1102. [PubMed: 17971865]

[52]. Petnicki-Ocwieja T, Hrncir T, Liu YJ, et al. Nod2 is required for the regulation of commensal microbiota in the intestine. Proc Natl Acad Sci USA 2009;106:15813–18. [PubMed: 19805227]

[53]. McElroy SJ, Weitkamp JH. Innate immunity in the small intestine of the preterm infant. Neoreviews 2011;12:e517–e26. [PubMed: 22639551]

[54]. Nathan C Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol 2006;6:173–82. [PubMed: 16498448]

[55]. Emami CN, Mittal R, Wang L, Ford HR, Prasadarao NV. Role of neutrophils and macrophages in the pathogenesis of necrotizing enterocolitis caused by Cronobacter sakazakii. J Surg Res 2012;172:18–28. [PubMed: 21601887]

[56]. Christensen RD, Yoder BA, Baer VL, Snow GL, Butler A. Early-onset neutropenia in small-for-gestational-age infants. Pediatrics 2015;136:e1259–67. [PubMed: 26459642]

[57]. Musemeche C, Caplan M, Hsueh W, Sun X, Kelly A. Experimental necrotizing enterocolitis: the role of polymorphonuclear neutrophils. J Pediatr Surg 1991;26:1047–9; discussion 9–50. [PubMed: 1941482]

[58]. Artis D, Spits H. The biology of innate lymphoid cells. Nature 2015;517:293–301. [PubMed: 25592534]

[59]. Gury-BenAri M, Thaiss CA, Serafini N, et al. The spectrum and regulatory landscape of intestinal innate lymphoid cells are shaped by the microbiome. Cell 2016;166:1231–46 e13. [PubMed: 27545347]

[60]. Bernink JH, Peters CP, Munneke M, et al. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. Nat Immunol 2013;14:221–9. [PubMed: 23334791]

[61]. Fuchs A, Vermi W, Lee JS, et al. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-gamma-producing cells. Immunity 2013;38:769–81. [PubMed: 23453631]

[62]. Buonocore S, Ahern PP, Uhlig HH, et al. Innate lymphoid cells drive interleukin-23dependent innate intestinal pathology. Nature 2010;464:1371–5. [PubMed: 20393462]

[63]. Powell N, Walker AW, Stolarczyk E, et al. The transcription factor T-bet regulates intestinal inflammation mediated by interleukin-7 receptor+ innate lymphoid cells. Immunity 2012;37:674–84. [PubMed: 23063332]

[64]. Maheshwari A, Kelly DR, Nicola T, et al. TGF-beta2 suppresses macrophage cytokine production and mucosal inflammatory responses in the developing intestine. Gastroenterology 2011;140:242–53. [PubMed: 20875417]

[65]. MohanKumar K, Kaza N, Jagadeeswaran R, et al. Gut mucosal injury in neonates is marked by macrophage infiltration in contrast to pleomorphic infiltrates in adult: evidence from an animal model. Am J Physiol 2012;303:G93–102.

[66]. MohanKumar K, Namachivayam K, Chapalamadugu KC, et al. Smad7 interrupts TGF-beta signaling in intestinal macrophages and promotes inflammatory activation of these cells during necrotizing enterocolitis. Pediatr Res 2016;79:951–61. [PubMed: 26859364]

[67]. Namachivayam K, Blanco CL, MohanKumar K, et al. Smad7 inhibits autocrine expression of TGF-beta2 in intestinal epithelial cells in baboon necrotizing enterocolitis. Am J Physiol 2013;304:G167–80.

[68]. Steimle A, Frick JS. Molecular mechanisms of induction of tolerant and tolerogenic intestinal dendritic cells in mice. J Immunol Res 2016;2016:1958650. [PubMed: 26981546]

[69]. Emami CN, Mittal R, Wang L, Ford HR, Prasadarao NV. Recruitment of dendritic cells is responsible for intestinal epithelial damage in the pathogenesis of necrotizing enterocolitis by Cronobacter sakazakii. J Immunol 2011;186:7067–79. [PubMed: 21551359]

[70]. Adkins B, Leclerc C, Marshall-Clarke S. Neonatal adaptive immunity comes of age. Nat Rev Immunol 2004;4:553–64. [PubMed: 15229474]

[71]. Sarzotti M, Robbins DS, Hoffman PM. Induction of protective CTL responses in newborn mice by a murine retrovirus. Science 1996;271:1726–8. [PubMed: 8596933]

[72]. Ridge JP, Fuchs EJ, Matzinger P. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. Science 1996;271:1723–6. [PubMed: 8596932]

[73]. Forsthuber T, Yip HC, Lehmann PV. Induction of TH1 and TH2 immunity in neonatal mice. Science 1996;271:1728–30. [PubMed: 8596934]

[74]. Cheroutre H, Lambolez F, Mucida D. The light and dark sides of intestinal intraepithelial lymphocytes. Nat Rev Immunol 2011;11:445–56. [PubMed: 21681197]

[75]. Sheridan BS, Lefrancois L. Intraepithelial lymphocytes: to serve and protect. Curr Gastroenterol Rep 2010;12:513–21. [PubMed: 20890736]

[76]. Gibbons DL, Haque SF, Silberzahn T, et al. Neonates harbour highly active gammadelta T cells with selective impairments in preterm infants. Eur J Immunol 2009;39:1794–806. [PubMed: 19544311]

[77]. Weitkamp JH, Rosen MJ, Zhao Z, et al. Small intestinal intraepithelial TCRgammadelta+ T lymphocytes are present in the premature intestine but selectively reduced in surgical necrotizing enterocolitis. PloS One 2014;9:e99042. [PubMed: 24905458]

[78]. Ismail AS, Behrendt CL, Hooper LV. Reciprocal interactions between commensal bacteria and gamma delta intraepithelial lymphocytes during mucosal injury. J Immunol 2009;182:3047–54. [PubMed: 19234201]

[79]. Weitkamp JH, Rudzinski E, Koyama T, et al. Ontogeny of FOXP3(+) regulatory T cells in the postnatal human small intestinal and large intestinal lamina propria. Pediatr Dev Pathol 2009;12:443–9. [PubMed: 19203136]

[80]. Weitkamp JH, Koyama T, Rock MT, et al. Necrotising enterocolitis is characterised by disrupted immune regulation and diminished mucosal regulatory (FOXP3)/effector (CD4, CD8) T cell ratios. Gut 2013;62:73–82. [PubMed: 22267598]

[81]. Nino DF, Sodhi CP, Egan CE, et al. Retinoic acid improves incidence and severity of necrotizing enterocolitis by lymphocyte balance restitution and repopulation of LGR5+ intestinal stem cells. Shock 2017;47:22–32. [PubMed: 27488085]

Practice points

- NEC is characterized by excessive inflammation likely resulting from a complex interplay between a host, microbes and nutritionally derived antigens.
- Currently, human milk and a diverse microbiome are the best-studied measures to prevent NEC by contributing to a balanced mucosal immune system.

Research directions

- Whereas animal models have been and will remain critical for further investigation of the pathogenesis of NEC, the unique feto-maternal environment with more advanced adaptive immunity at birth make correlation with human cells and tissues necessary.
- Given the important role of the immune system in the pathophysiology of NEC, clinical trials with molecules designed to support intestinal maturation and provide protective immune function or inhibit inflammatory processes are needed.